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Protective effects of a selective L-type voltage-sensitive calcium channel blocker, *S*-312-*d*, on neuronal cell death

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Abstract

Amyloid β protein (Aβ)- and human group IIA secretory phospholipase A_2 ($sPLA_2$-IIA)-induced neuronal cell death have been established as *in vitro* models for Alzheimer's disease (AD) and stroke. Both $sPLA_2$-IIA and Aβ causes neuronal apoptosis by increasing the influx of Ca^{2+} through L-type voltage-sensitive Ca^{2+} channel (L-VSCC). In the present study, we evaluated effects of a selective L-VSCC blocker, *S*-(+)-methyl 4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitro-phenyl)thieno[2,3-*b*]pyridine-5-carboxylate (*S*-312-*d*), on Aβ- and $sPLA_2$-IIA-induced neuronal apoptosis in primary cultures of rat cortical neurons. *S*-312-*d* significantly rescued cortical neurons from Aβ- and $sPLA_2$-IIA-induced cell death. Both cell death stimuli caused the appearance of apoptotic features such as plasma membrane blebs, chromatin condensation, and DNA fragmentation. *S*-312-*d* completely suppressed these apoptotic features. Before apoptosis, the two death ligands markedly enhanced an influx of Ca^{2+} into neurons. *S*-312-*d* significantly prevented neurons from $sPLA_2$-IIA- and Aβ-induced Ca^{2+} influx. Furthermore, the neuroprotective effect of *S*-312-*d* was more potent than that of another L-VSCC blocker, nimodipine. On the other hand, blockers of other VSCCs such as the N-type and P/Q-type calcium channels had no effect on the neuronal cell death, apoptotic features and Ca^{2+} influx. In conclusion, we demonstrated that *S*-312-*d* rescues cortical neurons from Aβ- and $sPLA_2$-IIA-induced apoptosis.

Keywords: *S*-312-*d*; L-type voltage-sensitive Ca^{2+} channel; Amyloid β protein; Secretory phospholipase A_2; Apoptosis; Cortical neurons

1. Introduction

Many neuronal processes are regulated by calcium influx through VSCC, including protein phosphorylation, gene expression, neurotransmitter release, action potential firing pattern [1]. On the basis of their pharmacological or electrophysiological properties, at least six distinct types of VSCCs have been identified and are designated L, N, P, Q, R, and T [2]. L-type VSCCs (L-VSCCs) mediate long-lasting Ca^{2+} currents in response to depolarization in excitable cells. Methyl 4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl)thieno[2,3-*b*]pyridine-5-carboxylate, *S*-312-*d*, is an L-type VSCC (L-VSCC) blocker [3]. *S*-312-*d* has displayed favorable effects against the occurrence of stroke and in significantly increasing the life span of stroke-prone spontaneously hypertensive rats [4,5]. *S*-312-*d* has been thought to indirectly protect neurons from ischemia by the relaxation of cerebral microvessels [4,5].

Brain L-VSCCs consist of five subunits: α_1, α_2, β, γ and δ [6]. The α_1 subunits form the ion-conducting pore of the channel and contain the binding sites for the dihydropyridine class of L-VSCC antagonists [7]. L-VSCCs are expressed in neurons [8] and astrocytes [9], and up-regulated in forebrain [10], hippocampus and cerebral cortex [11] after ischemia. Moreover, L-VSCC currents are elevated in CA1 neurons of the hippocampus in aged rats and

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Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; Aβ, amyloid β protein; $[Ca^{2+}]_i$, concentration of intracellular Ca^{2+}; IC_{50}, concentration giving 50% inhibition; L-VSCC, L-type voltage-sensitive calcium channels; MCA, middle cerebral artery; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye; PBS, phosphate-buffered saline; PG, prostaglandin; PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $sPLA_2$-IB, group IB $sPLA_2$; $sPLA_2$-IIA, group IIA $sPLA_2$; *S*-312-*d*, *S*-(+)-methyl 4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitro-phenyl)thieno[2,3-*b*]pyridine-5-carboxylate; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; ω-Aga-GVIA, ω-agatoxin GVIA; ω-CgTX-GVIA, ω-conotoxin GVIA; ω-CgTX-MVIIC, ω-conotoxin MVIIC.

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rabbits, due to increases in the density of L-VSCCs in neuronal cell membranes [12,13]. Elevated postsynaptic $[Ca^{2+}]_i$ and L-VSCC activity contributes to impaired synaptic plasticity [14] and working memory in aged hippocampal neurons [15]. Furthermore, L-VSCCs are upregulated in the hippocampus of AD patients, despite a significant decrease of cell density [16].

AD and stroke are two leading causes of age-associated dementia. AD is characterized by amyloid plaques, neurofibrillary tangles and neuronal loss [17,18]. Aggregated deposits of Aβ are generally assumed to have a causative role in neurodegeneration and development of AD. In AD brains [19] and in cultures of neurons exposed to Aβ [20,21], the dying neurons display the characteristics of apoptosis, such as formation of cell surface blebs, chromatin condensation, and DNA fragmentation. Aβ potentiates Ca^{2+} influx through L-VSCCs [21], elevates $[Ca^{2+}]_i$ and causes collapse of Ca^{2+} homeostasis [22].

Stroke is caused by a critical alteration of blood flow to a region of the brain. An acute obstruction of an artery results in ischemia i.e. insufficient blood flow to the tissue [23]. At the beginning of the stroke, there is a definite gradation of injury—a central area or core, with low blood flow already showing signs of massive cell death, and an outer area, the penumbra, that is still alive, but will malfunction for several days afterward. A rat with the MCA occluded has been established as an animal model for stroke [24]. MCA occlusion causes irreversible necrosis and infarction in the core [25]. On the other hand, cell death is induced not only via necrosis, but also via apoptosis, and cells remain viable for several hours in the penumbra [26]. Cortical $sPLA_2$-IIA is induced after MCA occlusion [27,28]. $sPLA_2$-IIA causes neuronal cell death via apoptosis [28]. Ca^{2+} influx through L-VSCC contributes to $sPLA_2$-IIA-induced neuronal apoptosis [29].

In the present study, we evaluated effects of *S*-312-*d* on Aβ- and $sPLA_2$-IIA-induced apoptosis in primary cultures of rat cortical neurons. Here, we provide the first evidence that *S*-312-*d* possessed the direct neuroprotective effects.

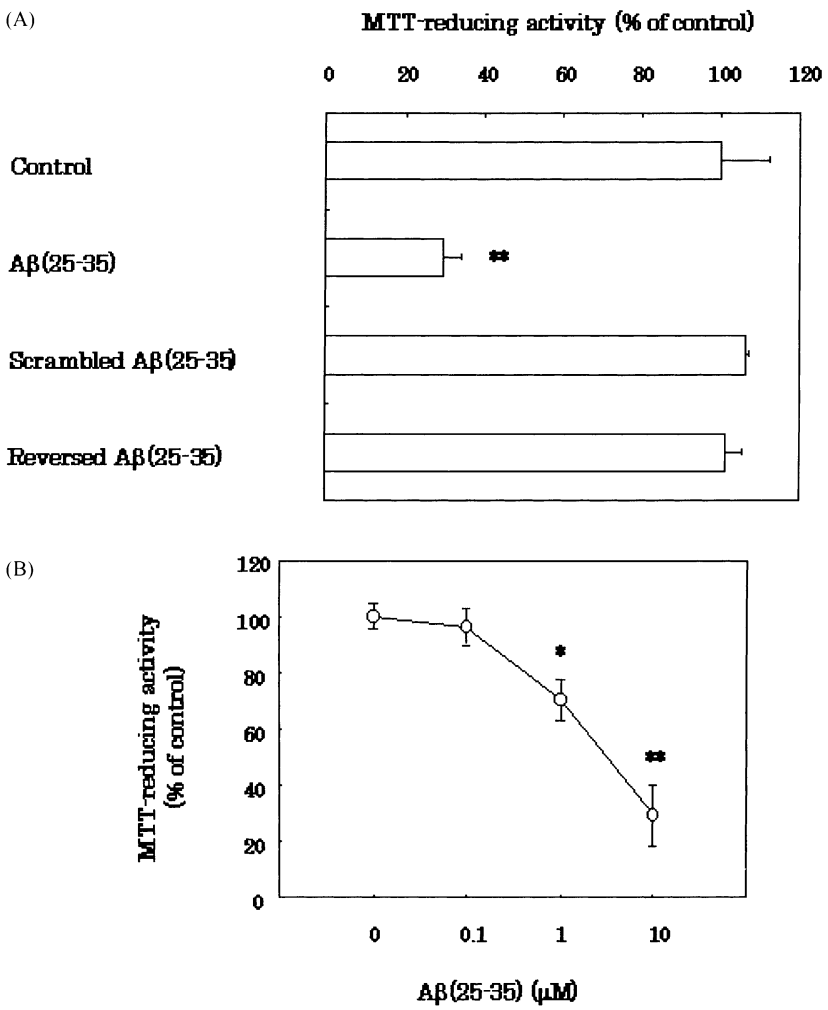

Fig. 1. Effects of Aβ on neuronal cell survival. (A) Aβ related peptides: rat cortical neurons were treated with various amyloid-related peptides. (B) Aβ(25–35): cortical neurons were treated with the indicated concentrations of Aβ(25–35). MTT reducing activity was determined 48 hr later. Data are expressed as means ± SEM values (N = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with controls by ANOVA followed by Dunnett's test.

2. Materials and methods

2.1. Materials

S-312-*d* and nimodipine were synthesized at the Shionogi Research Laboratories [3]. Human $sPLA_2$-IIA was prepared as described previously [30]. Aβ(25–35) was purchased from Bachem AG (Bubendorf, Switzerland). The scrambled and reversed forms of Aβ(25–35) were obtained from Takara. A stock solution of Aβ was prepared by solution of the peptide at 1 mM in deionized water and was incubated at 37° for 2–5 days to aggregate the peptide. ω-CgTX-GVIA, ω-Aga-GVIA and ω-CgTX-MVIIC were purchased from the Peptide Institute. [^{45}Ca]calcium chloride was purchased from Amersham International plc. Arabinosylcytosine C was purchased from Sigma. Dulbecco's modified Eagle's medium, Leibovitz's L-15 medium, trypsin, deoxyribonuclease I, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco. Hoechst 33258 fluorescent dye was purchased from Molecular Probes.

2.2. Animals

Experimental procedures were approved by the Institutional Animal Care and Use Committee at the Discovery Research Laboratories of Shionogi & Co, Ltd., and all efforts were made to minimize the number of animals used and their suffering. Pregnant Sprague–Dawley rats were used. The rats were individually housed in macrolon cages with free access to food and water and maintained on a 12-hr light/dark cycle, at 25° room temperature. All experiments were carried out according to the guidelines of the European Community's Council for Animal Experiments.

2.3. Tissue cultures

Neuronal cell cultures were prepared from cerebral cortices of day-19 Sprague–Dawley rat embryos as previously reported [31]. Cells were plated at a density of 2.5×10^5 cells/cm^2 on poly-L-lysine-coated dishes in

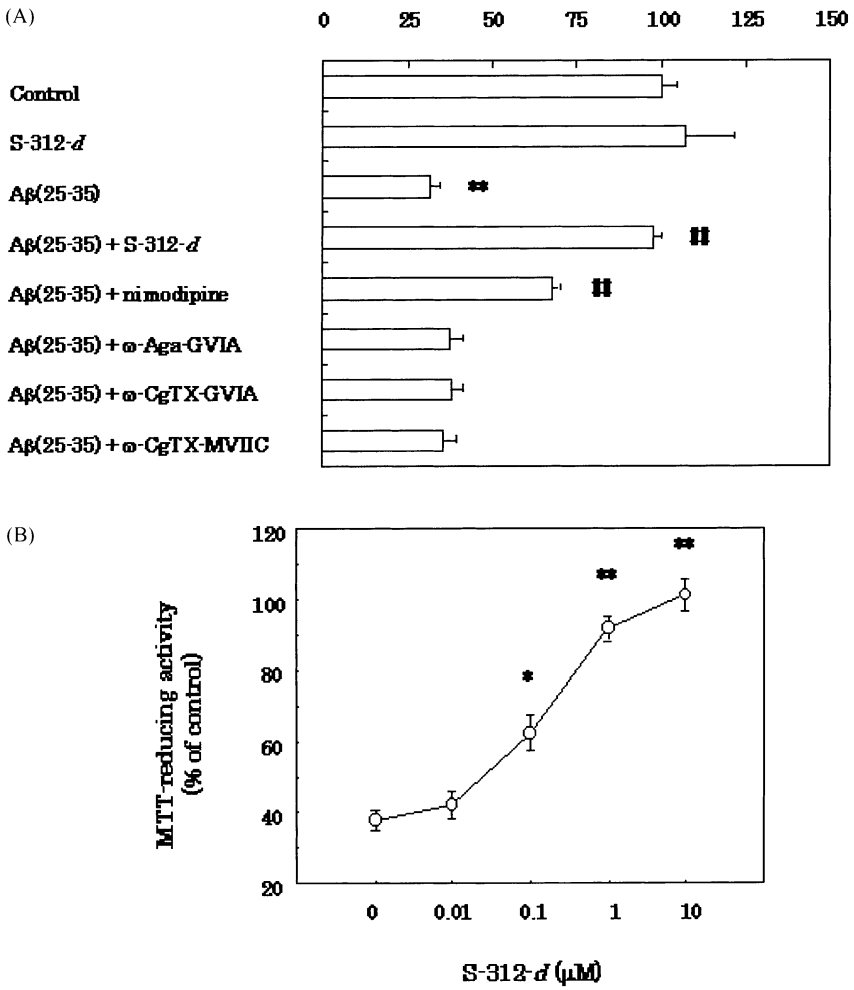

Fig. 2. Effects of *S*-312-*d* on Aβ(25–35)-induced neuronal cell death. (A) VSCC blockers: cortical neurons were treated with 10 μM Aβ(25–35) in the absence or presence of 3 μM *S*-312-*d* or 3 μM VSCC blockers. (B) *S*-312-*d*: cortical neurons were treated with *S*-312-*d* at the indicated concentrations in the presence of 10 μM Aβ(25–35). MTT-reducing activity was measured 48 hr later. Data are expressed as means ± SEM (N = 4). Data are expressed as means ± SEM (N = 4). $^{*}P < 0.05$ $^{**}P < 0.01$, compared with control, $^{\#\#}P < 0.01$, compared with Aβ(25–35) alone by ANOVA followed by Dunnett's test.

conditioning medium and Leibovitz's L-15 medium supplemented with 5% fetal bovine serum and 5% horse serum at 37°. Cultures were treated with 0.1 μM arabinosylcytosine C on day 1 and used for experiments on day 2 after plating. Most of the cells (more than 95%) were neurons, whereas there were a few astrocytes (less than 4%) and microglial cells (less than 3%). Thus, the present culture contained primarily neurons and few non-neuronal cells.

2.4. Analysis of neuronal survival

Neurons (2.5×10^5 cells/cm^2) were treated with 10 μM Aβ(25–35) or 1 μM sPLA$_2$-IIA in the presence or absence of *S*-312-*d* at 37° for 48 hr. Two different methods were employed for assessment of neurotoxicity of Aβ and sPLA$_2$-IIA, as previously reported [20]. First, the MTT reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed [32]. Second, residual cells were counted according to morphologic criteria; neurons with intact neurites and a smooth, round soma were considered viable, whereas those with degenerated neurites and an irregular soma were considered nonviable [33].

2.5. Fluoromicroscopic analysis

Assessment of condensation of chromatin was performed as previously described [34]. Neurons (2.5×10^5 cells/cm^2) were treated with 1 μM sPLA$_2$-IIA or 10 μM Aβ(25–35) in the presence or absence of *S*-312-*d* at 37° for 48 hr. Culture medium was exchanged with PBS containing 1 mM Hoechst 33258 fluorescent dye (Molecular Probes). Cells were incubated for 10 min at 37° in the dark and washed with PBS. Stained nuclei were categorized as follows: (i) normal nuclei, homogeneously stained chromatin; (ii) intact nuclei with condensed chromatin, crescent-shaped areas of condensed chromatin often located near the

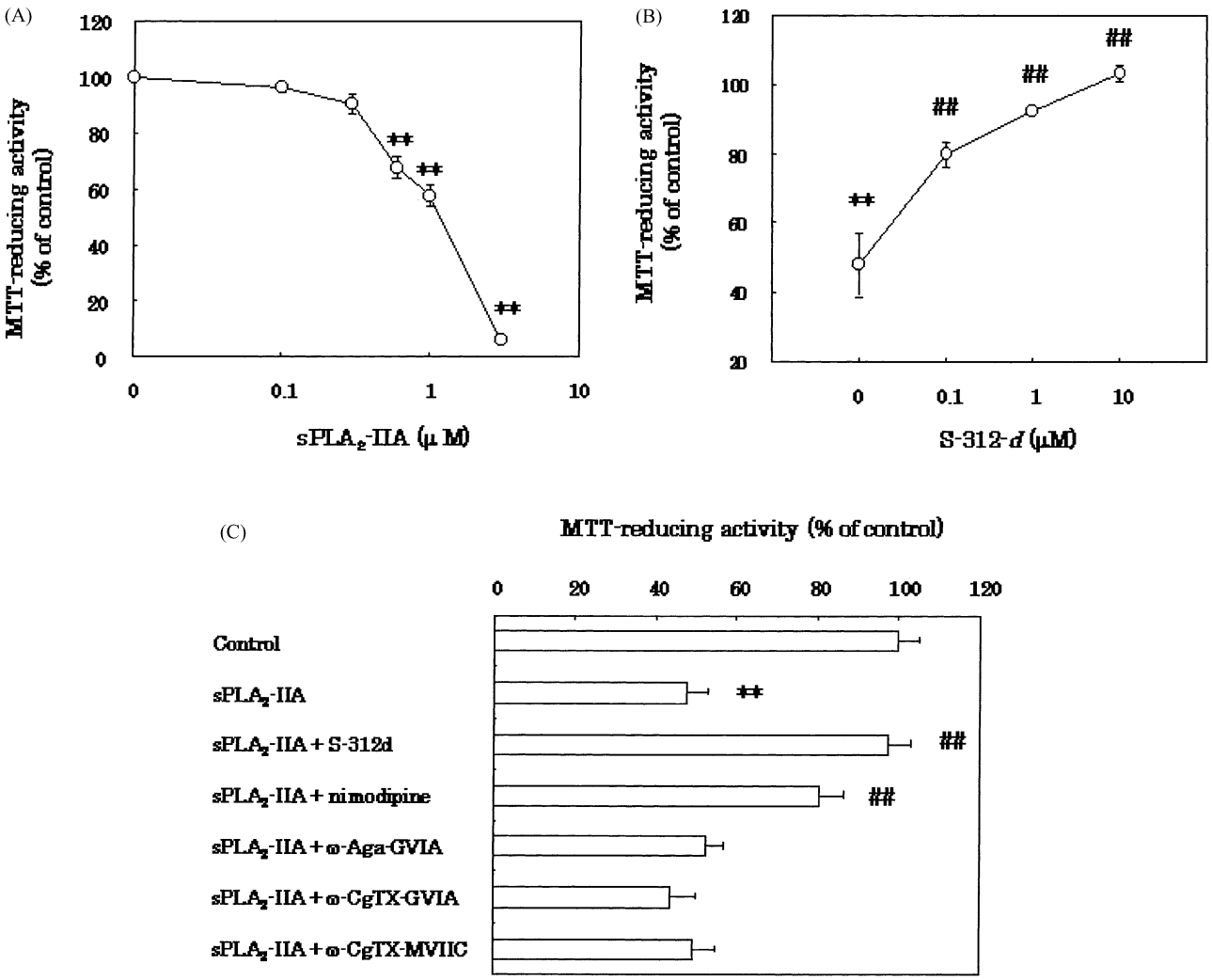

Fig. 3. Effects of *S*-312-*d* on sPLA$_2$-IIA-induced neuronal cell death. (A) sPLA$_2$-IIA: cortical neurons were treated with the indicated concentrations of sPLA$_2$-IIA. MTT reducing activity was determined 48 hr later. (B) *S*-312-*d*: cortical neurons were treated with *S*-312-*d* at the indicated concentrations in the presence of 1 μM sPLA$_2$-IIA. (C) VSCC blockers: cortical neurons were treated with 1 μM sPLA$_2$-IIA in the absence or presence of 3 μM *S*-312-*d* or 3 μM VSCC blockers. MTT-reducing activity was measured 48 hr later. Data are expressed as means ± SEM (N = 4). Data are expressed as means ± SEM (N = 4). $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with sPLA$_2$-IIA alone by ANOVA followed by Dunnett's test.

periphery of the nucleus; and (iii) fragmented nuclei, more than two condensed micronuclei within the area of a neuron.

2.6. In situ labeling of nuclear DNA fragments

Neurons (2.5×10^5 cells/cm^2) were treated with 1 μM sPLA$_2$-IIA or 10 μM Aβ(25–35) in the presence or absence of *S*-312-*d* at 37° for 48 hr. Cortical cell cultures were stained by the TUNEL (TdT-mediated dUTP-biotin nick end-labeling) technique, as described [35]. Apoptotic cells could be distinguished morphologically from necrotic cells by the presence of condensed brown nuclei.

2.7. Measurement of Ca^{2+} uptake

Neurons (2.5×10^5 cells/cm^2) were treated with 10 μM Aβ(25–35) or 1 μM sPLA$_2$-IIA in the presence or absence of *S*-312-*d* at 37° for 16 or 18 hr, respectively. Ca^{2+} uptake into cultured cells was measured as previously described [36]. Cortical cells were preincubated for 5 min at 37° with basal saline containing 145 mM NaCl, 10 mM Tris–HCl (pH 7.4), 0.4 mM KH_2PO_4, 1.2 mM $MgCl_2$, 3.1 mM KCl, 10 mM glucose, and 0.5 mM $CaCl_2$. The cells were then exposed to basal saline containing $^{45}CaCl_2$ (200 kBq/mL). Ca^{2+} uptake was terminated after 10 s of incubation by washing twice with basal saline without glucose. The cells

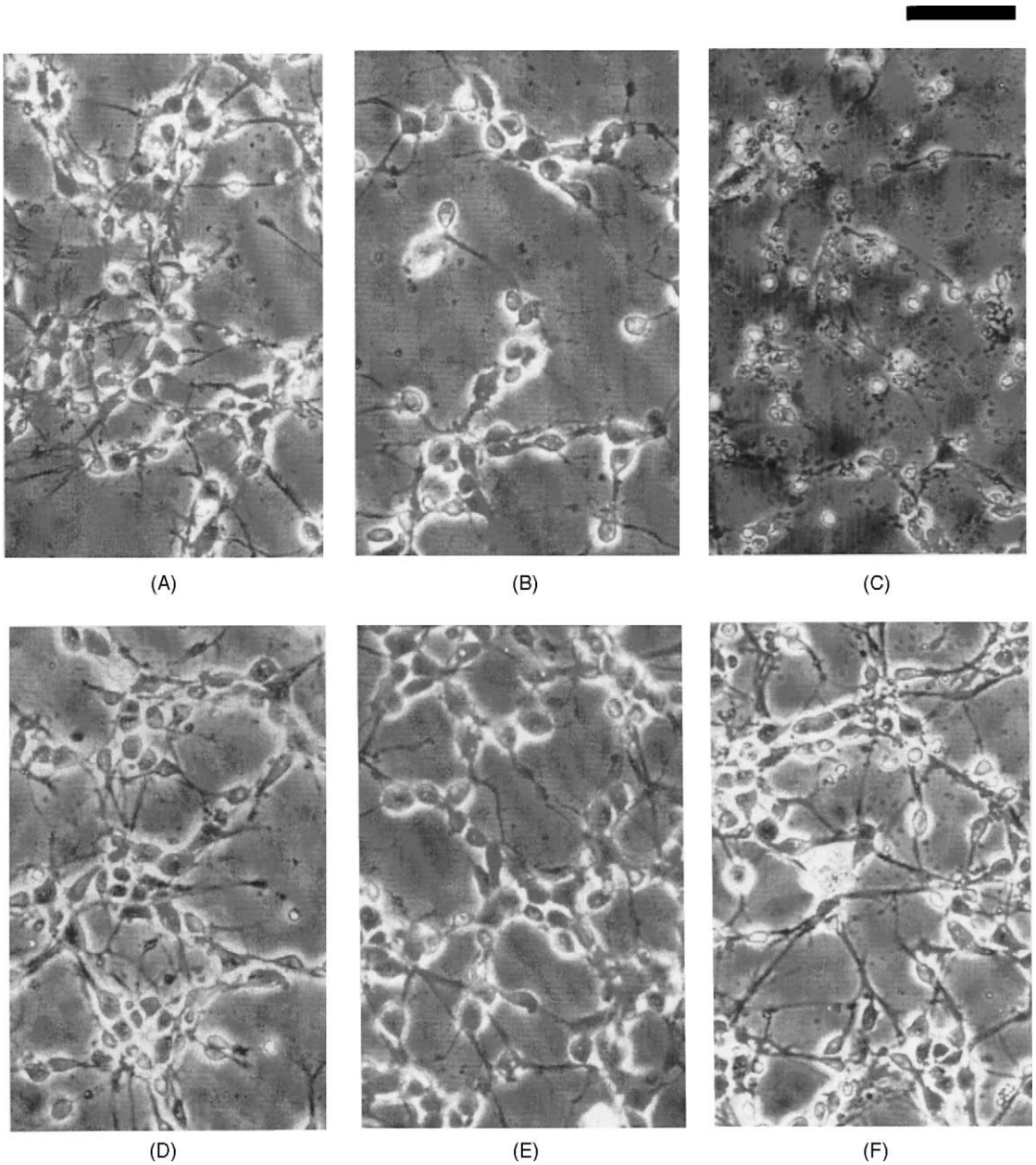

Fig. 4. Effects of *S*-312-*d* on Aβ(25–35)- and sPLA$_2$-IIA-induced morphological changes. Cortical neurons were treated with control (A), 1 μM sPLA$_2$-IIA (B), 10 μM Aβ(25–35) (C), 1 μM *S*-312-*d* (D), 1 μM sPLA$_2$-IIA + 1 μM *S*-312-*d* (E), or 10 μM Aβ(25–35) + 1 μM *S*-312-*d* (F). Neurons were examined by light microscopy 48 hr later. Scale bar = 100 μm.

were solubilized with 1 mL sodium dodecyl sulfate (0.5%) and deoxycholate (0.05%). Samples were mixed with scintillation fluid, Picofluor 40 (Perkin Elmer Life Science Products) and quantified by a liquid scintillation counter. Data are given as percentages of control.

2.8. Statistical analysis

Data are given as means ± SEM (N = numbers of observations). We performed at least two experiments on different days and confirmed their reproducibility. Data were analyzed statistically with Student's non-paired *t* test for comparison with the control group, and data on various inhibitors and blocker groups were analyzed statistically by two-way ANOVA followed by Dunnett's test for comparison with the Aβ- or the $sPLA_2$-IIA-treated group [37]. Concentration giving 50% inhibition (IC_{50}) values were calculated by Microsoft Excel Fit as previously reported [38].

3. Results

3.1. Effects of Aβ on neuronal cell survival

Primary cultures of dissociated cortical neurons were exposed to Aβ-related peptides for 48 hr, and their toxicity was quantified by the MTT reducing activity (Fig. 1). Naturally occurring cell death was not detected during experimental days. As shown in Fig. 1A, 10 μM Aβ(25–35), the toxic fragment of Aβ [31], caused neuronal cell death in a time-dependent manner after 24 hr and killed 70% of neurons at 48 hr. Aβ(25–35) showed neurotoxicity in a concentration-dependent manner (LD_{50} = 3.4 μM) (Fig. 1B). On the other hand, no neurotoxicity was observed by either scrambled Aβ(25–35) or Aβ(35–25), the reversed sequence of Aβ (25–35) (Fig. 1A).

3.2. Effects of S-312-d on Aβ(25–35)-induced neuronal cell death

S-312-*d* significantly attenuated neuronal cell death in the Aβ(25–35)-treated culture, whereas it alone did not influence the neuronal cell survival in the control culture (Fig. 2A). On the other hand, neither an N type VSCC blocker (ω-CgTX-GVIA) nor P/Q type VSCC blocker (ω-CgTX-MVIIC and ω-Aga-IVA) affected Aβ(25–35)-induced neuronal cell death (Fig. 2A). *S*-312-*d* prevented neurons from Aβ(25–35)-induced cell death in a concentration-dependent manner (Fig. 2B). Another L-VSCC blocker, nimodipine, significantly attenuated the neurotoxicity of Aβ(25–35) (Fig. 2A). The IC_{50} value of *S*-312-*d* (0.2 μM) was lower than that of nimodipine (1.7 μM). Thus, *S*-312-*d* significantly suppressed neurons from undergoing Aβ(25–35)-induced cell death.

3.3. Effects of S-312-d on $sPLA_2$-IIA-induced neuronal cell death

$sPLA_2$-IIA triggered neuronal cell death in a concentration-dependent manner (Fig. 3A). We examined effects of *S*-312-*d* on the $sPLA_2$-IIA-induced neuronal cell death. *S*-312-*d* protected neurons from $sPLA_2$-IIA-induced cell death in a concentration-dependent fashion (Fig. 3B). Another L-VSCC blocker, nimodipine, significantly reduced the neurotoxicity of $sPLA_2$-IIA (Fig. 3C). The IC_{50} value of *S*-312-*d* (0.04 μM) was lower than that of nimodipine (0.3 μM). On the other hand, neither an N type

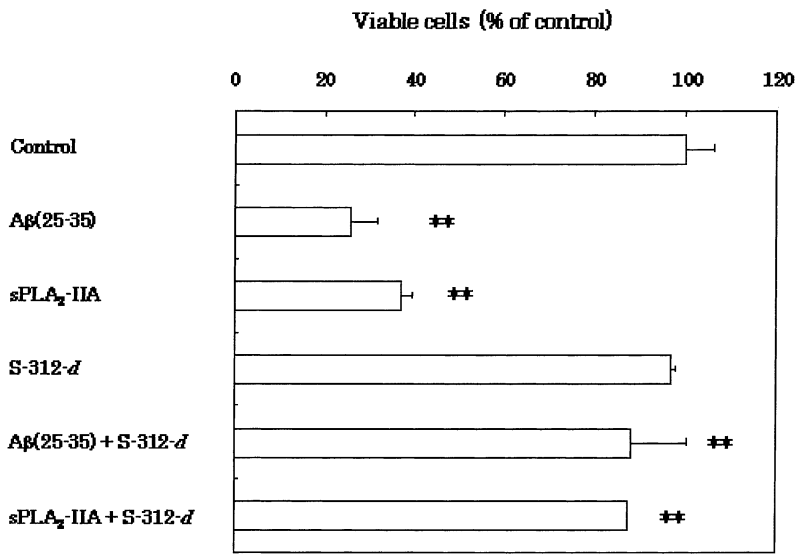

Fig. 5. Protective effects of *S*-312-*d* on Aβ(25–35)- and $sPLA_2$-IIA-induced neuronal cell death. Cortical neurons were treated with control, 10 μM Aβ(25–35), 1 μM $sPLA_2$-IIA, 1 μM *S*-312-*d*, 10 μM Aβ(25–35) + 1 μM *S*-312-*d* or 1 μM $sPLA_2$-IIA + 1 μM *S*-312-*d*. Cortical neurons were examined by light microscopy 48 hr later. Data are expressed as means ± SEM (N = 4). $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with Aβ(25–35) or $sPLA_2$-IIA alone, by ANOVA followed by Dunnett's test.

VSCC blocker (ω-CgTX-GVIA) nor P/Q type VSCC blocker (ω-CgTX-MVIIC and ω-Aga-IVA) affected sPLA$_2$-IIA-induced neuronal cell death (Fig. 3C). Thus, *S*-312-*d* significantly prevented neurons from undergoing sPLA$_2$-IIA-induced cell death.

3.4. Effects of S-312-d on Aβ(25–35)- and sPLA$_2$-IIA-induced light microscopic changes

We have reported that there is a close correlation between MTT-reducing activity and morphologic criteria [28]. Therefore, we evaluated the neuroprotective effect of *S*-312-*d* by morphologic criteria. In control (Figs. 4A and 5) and *S*-312-*d*-treated neurons (Figs. 4D and 5), neurons had extended neurites and smooth, round cell bodies. Some cell bodies shrank and lost their bright phase-contrast appearance in Aβ(25–35)-treated neurons (Figs. 4B and 5). There were markedly fewer cells, and extensive debris was seen attached to the substratum in sPLA$_2$-IIA-treated neurons (Figs. 4C and 5). *S*-312-*d* completely reverted the morphologic disruption in Aβ(25–35)-treated (Figs. 4E and 5) and sPLA$_2$-IIA-treated neurons (Figs. 4F and 5).

3.5. Effects of S-312-d on Aβ(25–35)- and sPLA$_2$-IIA-induced chromatin condensation

Previously, we have reported that Aβ(25–35)- [20] and sPLA$_2$-IIA-induced neuronal cell death [28] was

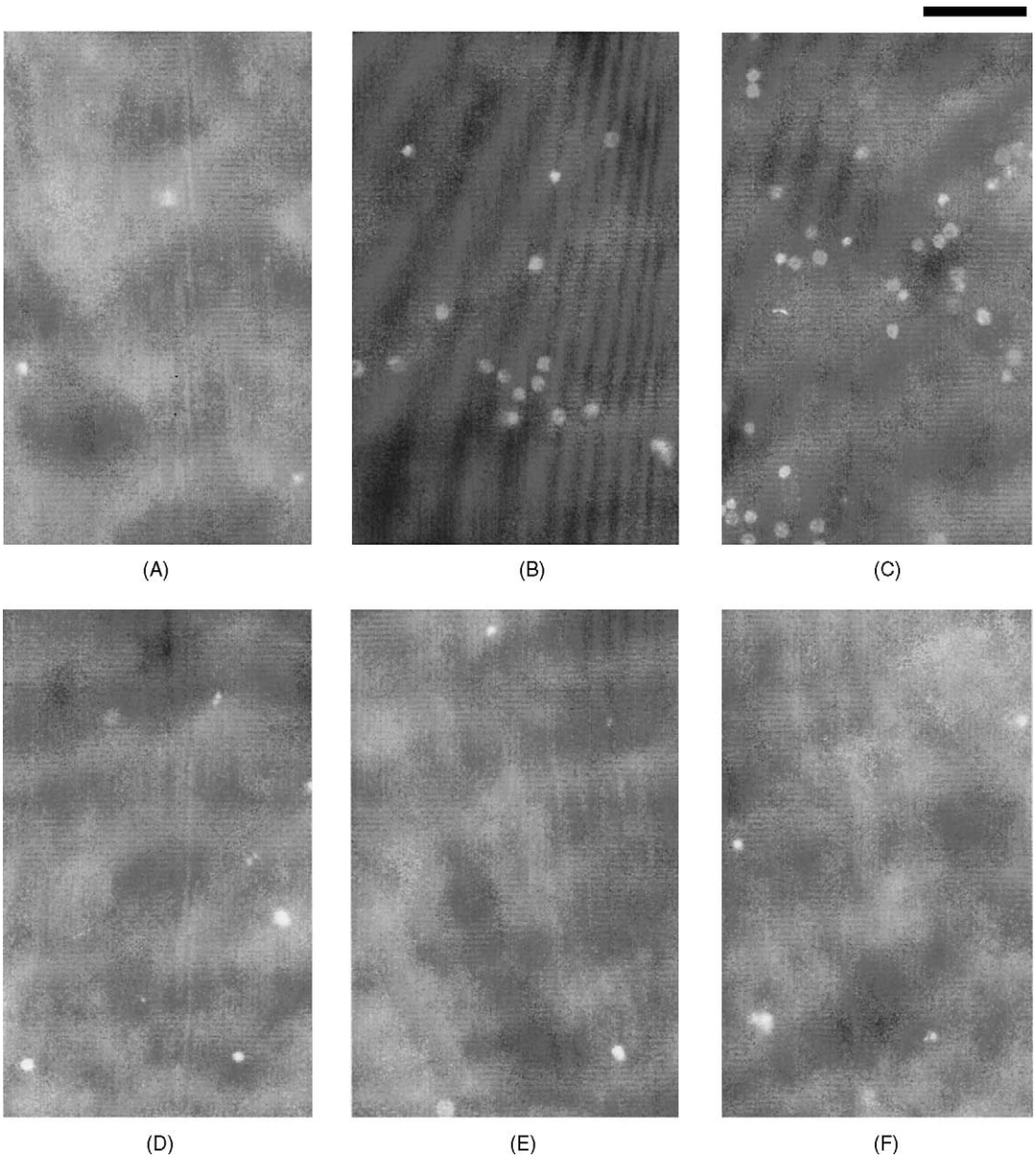

Fig. 6. Effects of *S*-312-*d* on sPLA$_2$-IIA- and Aβ(25–35)-induced chromatin condensation. Cortical neurons were treated with control (A), 1 μM sPLA$_2$-IIA (B), 10 μM Aβ(25–35) (C), 1 μM *S*-312-*d* (D), 1 μM sPLA$_2$-IIA + 1 μM *S*-312-*d* (E), or 10 μM Aβ(25–35) + 1 μM *S*-312-*d* (F). Neurons were stained with 1 μM Hoechst 33258 for 10 min 48 hr later. Scale bar = 100 μm.

accompanied by characteristic features of apoptosis, such as chromatin condensation. Therefore, chromatin condensation was examined with Hoechst 33258 fluorescent dye (Fig. 6) and Hoechst 33258-positive cells were counted (Fig. 8A). In control (Fig. 6A) and *S*-312-*d*-treated neurons (Fig. 6D) showed little fluorescence in the nucleus. On the other hand, condensed and fragmented chromatin was markedly increased in 10 μM Aβ(25–35) (Fig. 6B) and 1 μM $sPLA_2$-IIA-treated neurons (Fig. 6C). One μM *S*-312-*d* significantly decreased the amount of condensed chromatin in Aβ(25–35) (Fig. 6E) and $sPLA_2$-IIA-treated neurons (Fig. 6F).

3.6. Effects of S-312d on Aβ(25–35)- and $sPLA_2$-IIA-induced DNA fragmentation

Fragmentation of DNA was another apoptotic features, and also estimated with the TUNEL technique (Fig. 7), and TUNEL-positive cells were counted (Fig. 8B). In control (Fig. 7A) and *S*-312-*d*-treated neurons (Fig. 7D), few TUNEL-positive nuclei were detected. On the other hand, TUNEL-positive nuclei were clearly observed in 10 μM Aβ(25–35) (Fig. 7B) and 1 μM $sPLA_2$-IIA-treated neurons (Fig. 7C). The amount of fragmented DNA in Aβ(25–35) (Fig. 7E) and $sPLA_2$-IIA-treated neurons (Fig. 7F) was

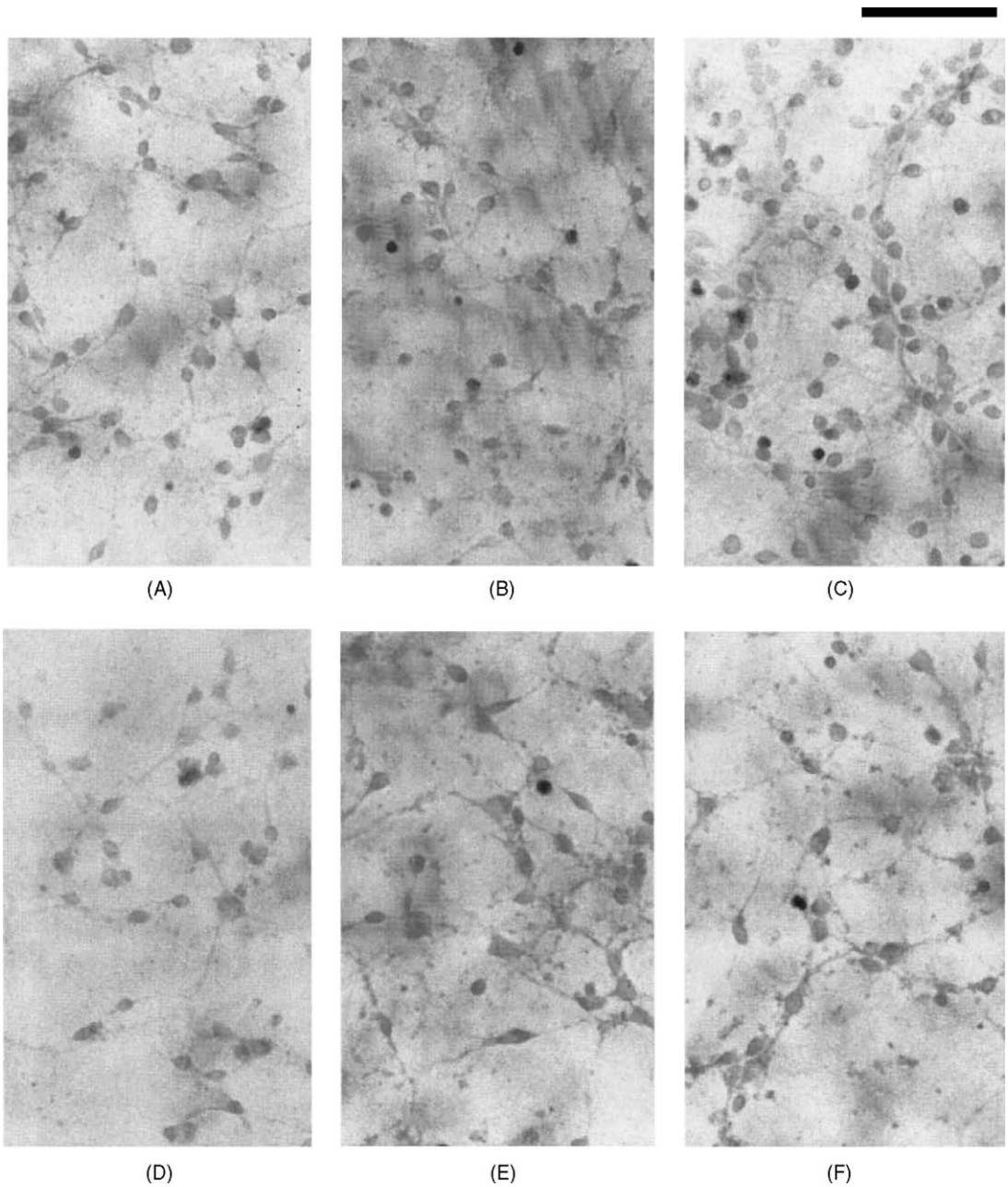

Fig. 7. Effects of *S*-312-*d* on $sPLA_2$-IIA- and Aβ(25–35)-induced DNA fragmentation of cortical neurons. Cortical neurons were treated with control (A), 1 μM $sPLA_2$-IIA (B), 10 μM Aβ(25–35) (C), 1 μM *S*-312-*d* (D), 1 μM $sPLA_2$-IIA + 1 μM *S*-312-*d* (E), or 10 μM Aβ(25–35) + 1 μM *S*-312-*d* (F). Neurons were fixed with 4% paraformaldehyde, washed twice with PBS, and stained by the TUNEL technique 48 hr later. Scale bar = 100 μm.

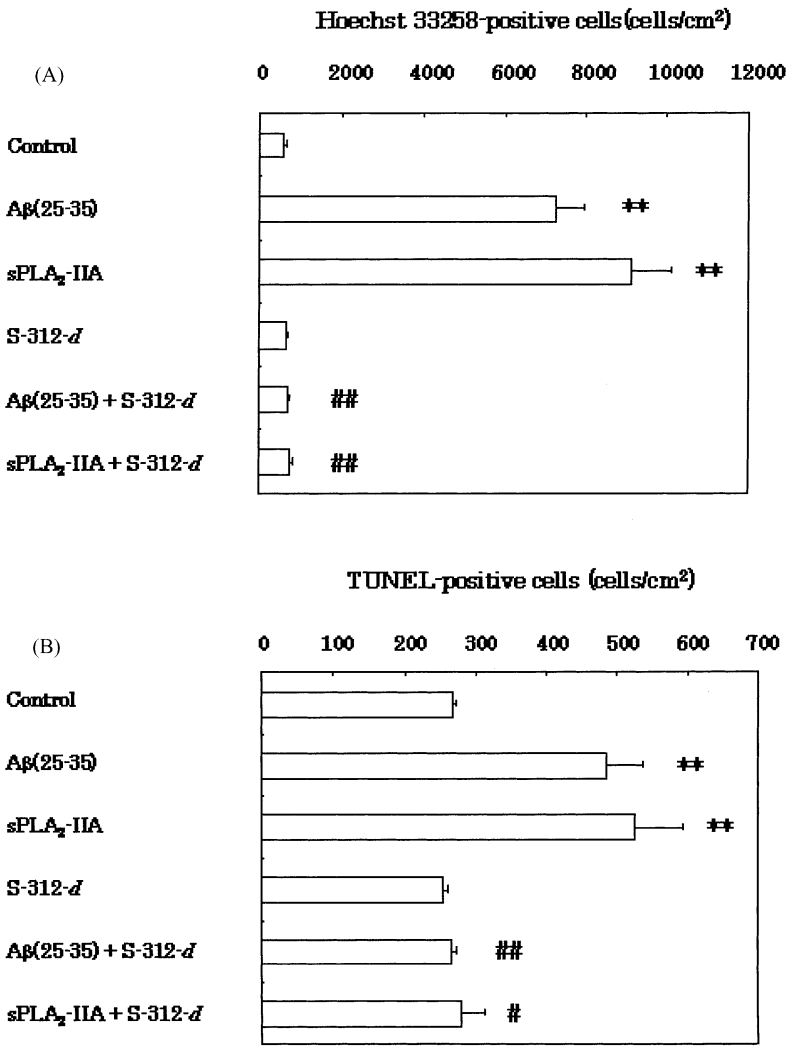

Fig. 8. Effects of *S*-312-*d* on Aβ(25–35)- and $sPLA_2$-IIA-induced apoptotic features. Cortical neurons were treated with control (A), 1 μM $sPLA_2$-IIA (B), 10 μM Aβ(25–35) (C), 1 μM *S*-312-*d* (D), 1 μM $sPLA_2$-IIA + 1 μM *S*-312-*d* (E), or 10 μM Aβ(25–35) + 1 μM *S*-312-*d* (F). Hoechst 33258- (A) and TUNEL-positive neurons (B) were detected 48 hr later. Data are expressed as means ± SEM (N = 4). $^{**}P < 0.01$, compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Aβ(25–35) alone, by ANOVA followed by Dunnett's test.

reduced significantly by 1 μM *S*-312-*d*. These results indicated that *S*-312-*d* ameliorated apoptotic features of Aβ(25–35)- and $sPLA_2$-IIA-induced neuronal cell death.

3.7. Effects of S-312-d on Aβ(25–35)- and sPLA2-IIA-induced Ca2+ influx

Before neuronal cell death, Aβ(25–35) increases the influx of Ca^{2+} into neurons [21]. Application of Aβ(25–35) caused a significant increase in Ca^{2+} uptake at 16 hr (Fig. 9A). *S*-312-*d* inhibited the potentiation of Ca^{2+} uptake in a concentration-dependent manner (Fig. 9A). Nimodipine also significantly reduced the potentiation of Ca^{2+} uptake (Fig. 9B). On the other hand, neither an N type VSCC blocker (ω-CgTX-GVIA) nor P/Q type VSCC blocker (ω-CgTX-MVIIC and ω-Aga-IVA) affected Aβ(25–35)-induced Ca^{2+} uptake (Fig. 9B).

Prior to neuronal cell death, $sPLA_2$-IIA also potentiates the uptake of Ca^{2+} into neurons [39]. *S*-312-*d* significantly reduced the elevation of Ca^{2+} uptake at 18 hr (Fig. 10A). Although nimodipine also significantly decreased the Ca^{2+} uptake (Fig. 10B), ω-CgTX-GVIA, ω-CgTX-MVIIC and ω-Aga-IVA had no effect on $sPLA_2$-IIA-induced Ca^{2+} influx (Fig. 10B). Thus, *S*-312-*d* prevented neurons from Aβ(25–35)- and $sPLA_2$-IIA-induced Ca^{2+} influx.

4. Discussion

In the present study, we demonstrated that *S*-312-*d* rescued cortical neurons from Aβ(25–35)- and $sPLA_2$-IIA-induced apoptosis. *S*-312-*d* also ameliorated the two death ligands-induced apoptotic features such as the condensation of chromatin and the fragmentation of DNA. Previously, we have reported that the compositions of neurons, astrocytes and microglias were determined by use of antibodies for MAP2, GFAP, and microglial antigen, which are specific for neurons, astrocytes, and microglias,

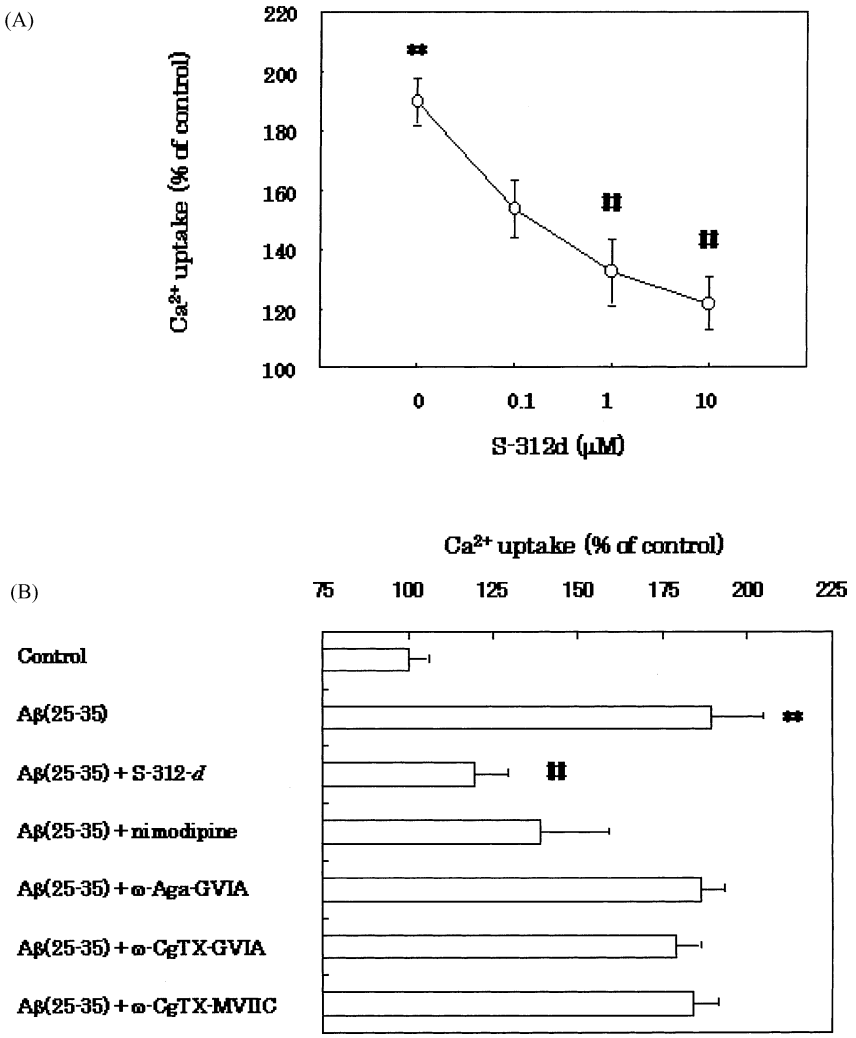

Fig. 9. Effects of *S*-312-*d* on Aβ(25–35)-induced influx of Ca^{2+} into neurons. (A) Cortical neurons were treated with *S*-312-*d* at the indicated concentrations in the presence of 10 μM Aβ(25–35). (B) Cortical neurons were treated with 3 μM *S*-312-*d* or 3 μM VSCC blockers in the presence of 10 μM Aβ(25–35). Ca^{2+} uptake was measured 16 hr later. Data are expressed as means ± SEM (N = 6). $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with Aβ(25–35) alone, by ANOVA followed by Dunnett's test.

respectively in the present cortical cultures. Approximately 95% of the cells were stained with anti-MAP2 antibody, whereas there were few anti-GFAP- and anti-microglial antigen-positive cells [28]. Thus, the present cortical cultures contained few non-neuronal cells, indicating that *S*-312-*d* exhibited a neuroprotective effect directly, but not indirectly via non-neuronal cells.

The influx of Ca^{2+} into neurons was evaluated by measuring radioactivity in cells exposed to radiolabeled calcium. It should be noted that the levels of radioactivity could be determined not only by calcium influx, but also by calcium extrusion and binding to intracellular proteins. We have reported the Aβ-induced inward current of Ca^{2+} by patch-clamp recording studies [21,36,39]. A depolarizing pulse from a holding potential at −70 to 0 mV showed inward currents. These currents were blocked by nimodipine. The Ca^{2+} current density of the Aβ(25–35)-treated neurons was about 2-fold greater than that of the control ones. There was a close correlation between the radiolabeled Ca^{2+} in cells and the inward Ca^{2+} current. Thus, the elevated level of radioactivity in cells reflected the influx of Ca^{2+} into neurons.

In the present study, we used *S*-312-*d* at 3 μM in the assay of Ca^{2+} influx, and did it at 1 μM in the other assays. At 1 μM, *S*-312-*d* suppressed neurons from Aβ- and $sPLA_2$-IIA-induced Ca^{2+} influx significantly, but not completely. It has been well established that excessive intracellular calcium can induce cell death [40,41]. These reports support the "set point theory" that calcium homeostatic mechanisms, including L-VSCC-mediated influx, regulate the intracellular calcium levels at or near an optimal set point [42,43]. Thus, 1 μM *S*-312-*d* appeared to stabilize the free cytosolic calcium concentration at optimal levels, even if it suppressed incompletely Ca^{2+} influx via L-VSCC.

Previously, we have reported that nimodipine, another L-VSCC blocker, prevented neurons from Aβ-induced apoptosis and Ca^{2+} influx [21]. Recently, we have reported

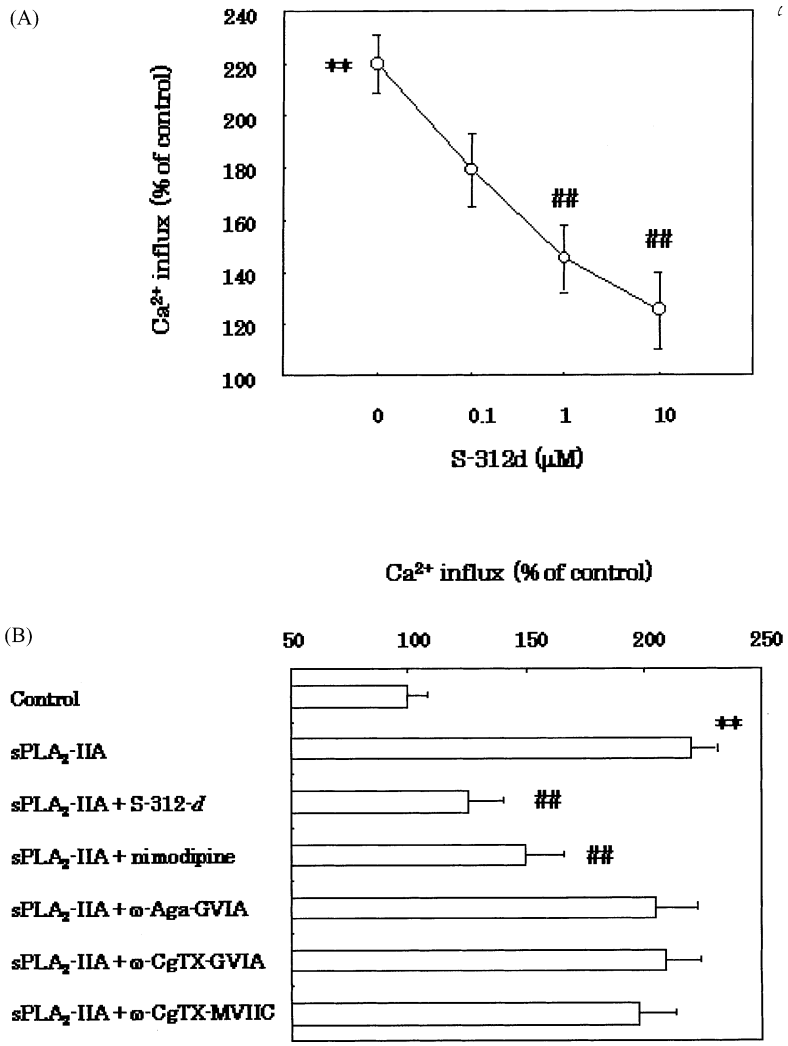

Fig. 10. Effects of *S*-312-*d* on sPLA$_2$-IIA-induced influx of Ca^{2+} into neurons. (A) Cortical neurons were treated with *S*-312-*d* at the indicated concentrations in the presence of 1 μM sPLA$_2$-IIA. (B) Cortical neurons were treated with 3 μM *S*-312-*d* or 3 μM VSCC blockers in the presence of 1 μM sPLA$_2$-IIA. Ca^{2+} uptake was measured 18 hr later. Data are expressed as means ± SEM (N = 6). $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with sPLA$_2$-IIA alone, by ANOVA followed by Dunnett's test.

that endothelin [29], prostaglandin E_2 [44] and the *growth arrest-specific 6* gene product [34] attenuated Aβ neurotoxicity by suppressing the activity of L-VSCCs. In the present study, we confirmed protective effects of the L-VSCC blocker, *S*-312-*d*, on cortical neurons. Furthermore, the concentration of *S*-312-*d* required for the neuroprotective effect was lower than that of nimodipine.

It has been believed that the neuroprotective effect of *S*-312-*d* is mediated by the relaxation of cerebral microvessels [4,5]. For example, intraperitoneal administration of *S*-312-*d* significantly attenuates the brain damage in the cerebral cortex in the *in vivo* model of focal ischemia following PIT-MCA occlusion [45]. Recently, we have established a novel *in vitro* model for stroke [28]. The activity of sPLA$_2$-IIA was elevated in cerebral cortex of photochemical-induced thrombotic (PIT)-MCA occluded rats, an *in vivo* model for stroke. An sPLA$_2$ inhibitor significantly reduced not only the elevated activity of the sPLA$_2$-IIA, but also the neurodegenerative volume in the cerebral cortex. sPLA$_2$-IIA induced neuronal apoptosis by the activation of L-VSCC [39,46]. In this *in vitro* model, *S*-312-*d* exhibited neuroprotective effects more efficiently than nimodipine. Combined with the previous reports, the present study suggested that *S*-312-*d* rescued neurons in ischemic brain directly and indirectly.

Recent epidemiologic and clinico-pathologic data suggest overlaps between AD and cerebrovascular lesions that may magnify the effect of mild AD pathology and promote progression of cognitive decline or even may precede neuronal damage and dementia. In large autopsy series of demented aged subjects, around 80% show Alzheimer type pathology, 20–40% with additional, often minor vascular lesions, 7–10% pure vascular dementia, and 3–5% mixed dementia (combination of AD and vascular encephalopathy). Vascular lesions in AD include cortical microinfarcts, subcortical lacunes, white matter lesions/leukoencephalopathy, small hemorrhage

and corticsubcortical infarcts, while in mixed type dementia multiple larger or hemispheral infarcts are more frequent [47].

The mechanisms of AD and stroke converged to the influx of $[Ca^{2+}]_i$ into neurons. A transient increase of $[Ca^{2+}]_i$ resulting from electrochemical stimulation and opening of voltage-gated Ca^{2+} channels mediates information-coding processes in neural circuits [48] and regulates growth cone behaviors in developing neurons [49]. However, uncontrolled prolonged elevation of $[Ca^{2+}]_i$ can result in neuronal degeneration and cell death [50]. Calcium appears to damage cellular proteins and membranes by activating several enzymes such as proteinase [51], endonuclease [52] and protein kinase [20], and by promoting free radical production via activation of lipase [53] or nitric oxide synthase [54].

Recently, AA cascade has been reported to be involved in AD and stroke. AA is liberated from cell membrane lipids by PLA_2, and prostaglandins are metabolized from AA by cyclooxygenase (COX). Ca^{2+}-dependent PLA_2 is upregulated in the AD brain [55]. A clinical trial of AD patients with a COX inhibitor, indomethacin, indicated a beneficial effect [56]. In the brain, both COX-1 and COX-2 are expressed [57]. COX-2 is up-regulated in AD brain and in Aβ-treated SH-SY5Y neuroblastoma cells [58]. Furthermore, we have reported beneficial effects of S-2474, a specific COX-2 inhibitor, on the Aβ neurotoxicity [31], suggesting the involvement of COX-2 in AD. Collectively, we proposed the hypothesis that the prolonged activation of L-VSCC, the overinflux of Ca^{2+}, and the subsequent stimulation of AA cascade contribute to the pathology of AD and stroke.

In conclusion, we demonstrated that *S*-312-*d* exhibited the neuroprotective effect directly as well as indirectly by the relaxation of cerebral microvessels. Furthermore, the present study sheds light on the therapeutic potential of *S*-312-*d* for dementia ascribed to AD and stroke.

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