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Amyloid β Protein Potentiates Ca^{2+} Influx Through L-Type Voltage-Sensitive Ca^{2+} Channels: A Possible Involvement of Free Radicals

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Abstract: Amyloid β protein (Aβ), the central constituent of senile plaques in Alzheimer's disease (AD) brain, is known to exert toxic effects on cultured neurons. The role of the voltage-sensitive Ca^{2+} channel (VSCC) in β(25–35) neurotoxicity was examined using rat cultured cortical and hippocampal neurons. When L-type VSCCs were blocked by application of nimodipine, β(25–35) neurotoxicity was attenuated, whereas application of ω-conotoxin GVIA (ω-CgTX-GVIA) or ω-agatoxin IVA (ω-Aga-IVA), the blocker for N- or P/Q-type VSCCs, had no effects. Whole-cell patch-clamp studies indicated that the Ca^{2+} current density of β(25–35)-treated neurons is about twofold higher than that of control neurons. Also, β(25–35) increased Ca^{2+} uptake, which was sensitive to nimodipine. The 2′,7′-dichlorofluorescin diacetate assay showed the ability of β(25–35) to produce reactive oxygen species. Nimodipine had no effect on the level of free radicals. In contrast, vitamin E, a radical scavenger, reduced the level of free radicals, neurotoxicity, and Ca^{2+} uptake. These results suggest that β(25–35) generates free radicals, which in turn, increase Ca^{2+} influx via the L-type VSCC, thereby inducing neurotoxicity. **Key Words:** Alzheimer's disease—Amyloid β protein—Ca^{2+} uptake—2′,7′-Dichlorofluorescin diacetate—Free radicals—L-type Ca^{2+} channel—Neurotoxicity—Nimodipine—Patch clamp.
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The accumulation of amyloid β protein (Aβ) in the brain is a characteristic feature of Alzheimer's disease (AD) (Glenner and Wong, 1984; Masters et al., 1985; Selkoe, 1989). Aβ deposits are generally assumed to contribute to progressive neurodegeneration in the disease (Yankner et al., 1990; Kowall et al., 1991; Mattson et al., 1992).

Recent studies in primary culture of dissociated neurons have shown that Aβ neurotoxicity is manifested by its aggregative form (Pike et al., 1991; Mattson et al., 1993*b*; Ueda et al., 1994). The application of aggregated Aβ peptides results in elevation of intracellular Ca^{2+} level ($[Ca^{2+}]_i$) and collapse of Ca^{2+} homeostasis (Mattson et al., 1992, 1993*a,b*; Goodman and Mattson, 1994; Goodman et al., 1994). Aβ neurotoxicity can be reduced by the voltage-sensitive Ca^{2+} channel (VSCC) blocker nimodipine (Weiss et al., 1994). Clearly, Aβ neurotoxicity is linked with increased Ca^{2+} influx via VSCCs. Indeed, Aβ can form Ca^{2+} channels in artificial lipid bilayers (Arispe et al., 1993), increases membrane permeability in PC12 cells (Fukuyama et al., 1994), and potentiates the nimodipine-sensitive cation conductance in N1E-115 cells (Davidson et al., 1994).

The involvement of free radicals in Aβ neurotoxicity is also suggested. The aggregation of Aβ in vitro is catalyzed by oxidation and is inhibited by antioxidants (Dyrks et al., 1992). Aβ neurotoxicity is attenuated by a number of antioxidants (e.g., vitamin E and EUK-8) (Behl et al., 1992; Bruce et al., 1996). Aβ itself induces reactive oxygen species (Behl et al., 1994; Goodman and Mattson, 1994; Goodman et al., 1994).

How might increased $[Ca^{2+}]_i$ be related to free radicals in Aβ neurotoxicity? It is suggested that free radicals generated by Aβ damage cell membranes, thereby initiating lipoperoxidation and impairing membrane proteins (Butterfield et al., 1994; Hensley et al., 1994). In fact, the ability of Aβ to induce cellular oxidation, changes in $[Ca^{2+}]_i$, and neuronal cell death are all closely correlated with the ability of Aβ to produce free radicals (Harris et al., 1995). Thus, it can be hypothesized that Aβ neurotoxicity results from free radical–mediated neuronal membrane damage.

In the present study, we focused on the role of free radicals in excessive influx of Ca^{2+} through VSCCs.

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Abbreviations used: Aβ, amyloid β protein; AD, Alzheimer's disease; ω-Aga-IVA, ω-agatoxin IVA; $[Ca^{2+}]_i$, intracellular Ca^{2+} level; ω-CgTX-GVIA, ω-conotoxin GVIA; ω-CgTX-MVIIC, ω-conotoxin MVIIC; DCFDA, 2′,7′-dichlorofluorescin diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TTX, tetrodotoxin; VSCC, voltage-sensitive Ca^{2+} channel.

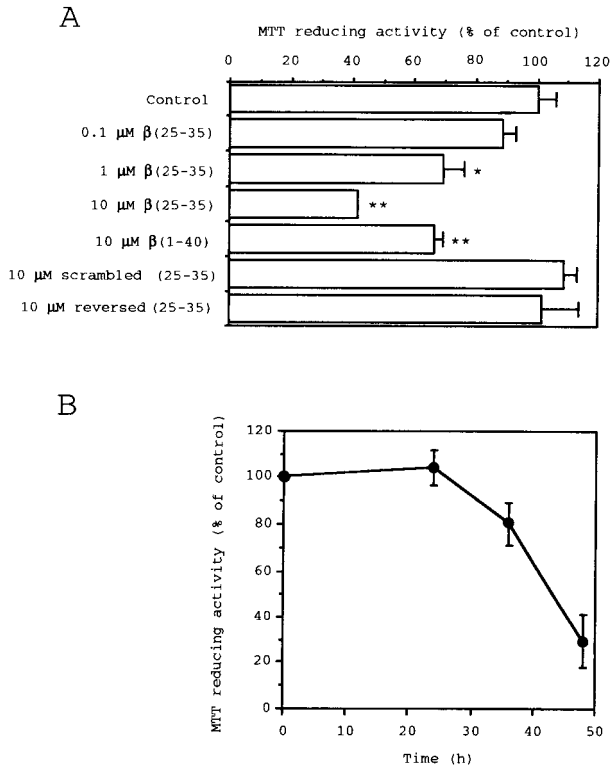

FIG. 1. Neurotoxic responses of cultured cortical cells to Aβ-related peptides. **A:** Cortical cultures were incubated with indicated concentrations of the peptides for 48 h, and neurotoxicity was measured by the MTT assay. Data are expressed as mean ± SEM values (n = 4). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with vehicle-treated conditions by ANOVA followed by Dunnett's test. **B:** Time course of β(25–35) toxicity. β(25–35) (10 μM) was added to cultures and incubated for different time periods for the MTT assay. Data are expressed as mean ± SEM values of the percentages of the vehicle-treated cultures that were incubated for the same amount of time as experimental cultures (n = 4).

For this purpose, we used rat primary culture of cortical and hippocampal neurons. The data suggest that free radicals contribute to the alterations and/or the formation of VSCCs.

MATERIALS AND METHODS

Materials

β(25–35) and β(1–40) were purchased from Bachem. Their scrambled (25–35) and reversed (25–35) forms were obtained from Takara Shuzo. Stock solutions of Aβs were prepared by dissolving the peptides at 1 mM in deionized water and were incubated at 37°C for 2 days to aggregate the peptides (Ueda et al., 1994). 2′,7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Kodak. ω-Conotoxin GVIA (ω-CgTX-GVIA), ω-agatoxin IVA (ω-Aga-IVA), and ω-conotoxin MVIIC (ω-CgTX-MVIIC) were purchased from Peptide Institute. Nimodipine was synthesized in our laboratories.

Tissue culture

Cerebral cortical and hippocampal cultures were prepared from 19-day-old Sprague–Dawley rat embryos (Ueda et al., 1996). The tissue was dissociated in isotonic buffer with 4 mg/ml trypsin and 0.4 mg/ml deoxyribonuclease I. The isotonic buffer contained (in mM) KH_2PO_4 0.22, Na_2HPO_4 0.17, NaCl 137, KCl 5.4, glucose 5.5, and sucrose 59. Cells were plated at a density of 2.5×10^5 cells/cm^2 on poly-L-lysine–coated dishes in Leibovitz's L-15 medium supplemented with fetal calf serum (10%) and horse serum (5%). Cultures were used for the experiments after incubation of 2 days in vitro.

Assessment of neurotoxicity

Neurotoxicity was assessed by two different assays, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) assay and morphometric cell counting. The MTT assay reflecting mitochondrial activity was performed as previously described (Ueda et al., 1996). Morphometric cell counting was made for the viability assay, using the presence of neurites and smooth, round cell bodies as criteria (Ueda et al., 1996). Data are given as percentages of corresponding vehicle-treated values.

Electrophysiology

The procedures for whole-cell recordings were performed as previously described (Umemiya and Berger, 1994). Hippocampal pyramidal neurons were visually identified by Nomarski optics. The surface of pyramidal neurons was cleaned by applying a jet stream of artificial CSF through a glass pipette. The patch pipettes were prepared from borosilicate glass capillaries with a PA-91 glass microelectrode puller (Narishige). The pipette resistance ranged from 4 to 5 MΩ. Whole-cell recordings were done with an EPC-9 patch amplifier (HEKA Elektronik) at room temperature (~25°C). Currents elicited by depolarizing pulses were filtered at 3 kHz with a four-pole, low-pass Bessel filter and analyzed using Pulse + Pulsfit software (HEKA Elektronik). For recording voltage-activated Ca^{2+} currents, the pipette solution contained (in mM) cesium methanesulfonate 100, triethylamine 30, $CaCl_2$ 1, $MgCl_2$ 1, ATP 4, GTP 0.3, EGTA 10, and HEPES 10 (pH 7.2 with CsOH). The external solution contained (in mM) NaCl 125, $CaCl_2$ 2.4, $MgCl_2$ 1.5, NaH_2PO_4 1.25, glucose 11, and 0.4 μM tetrodotoxin (TTX). Leak currents were subtracted using P/5 protocol.

Measurements of Ca^{2+} uptake

Ca^{2+} uptake into cultured cells was measured as previously described (Kanemasa et al., 1996). Cortical cells were preincubated for 5 min at 37°C with basal saline containing (in mM) NaCl 145, Tris-HCl 10 (pH 7.4), KH_2PO_4 0.4, $MgCl_2$ 1.2, KCl 3.1, glucose 10, and $CaCl_2$ 0.5. The cells were then exposed to the 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 were solubilized with 1 ml of sodium dodecyl sulfate (0.5%) and deoxycholate (0.05%). Samples were mixed with scintillation fluid (Picofluor 40, Hewlett-Packard) and quantified by a liquid scintillation counter. Data are given as percentages of corresponding vehicle-treated values.

Measurements of reactive oxygen species

Intracellular reactive oxygen species were measured by the DCFDA assay (Chacon and Acosta, 1991). In brief, cultures were loaded with 1 μM DCFDA for 20 min, and then the monolayers were washed twice with 1 ml of phosphate-buffered saline. One milliliter of deoxycholate (1%) was added to lyse the cells. The lysates were transferred to a new

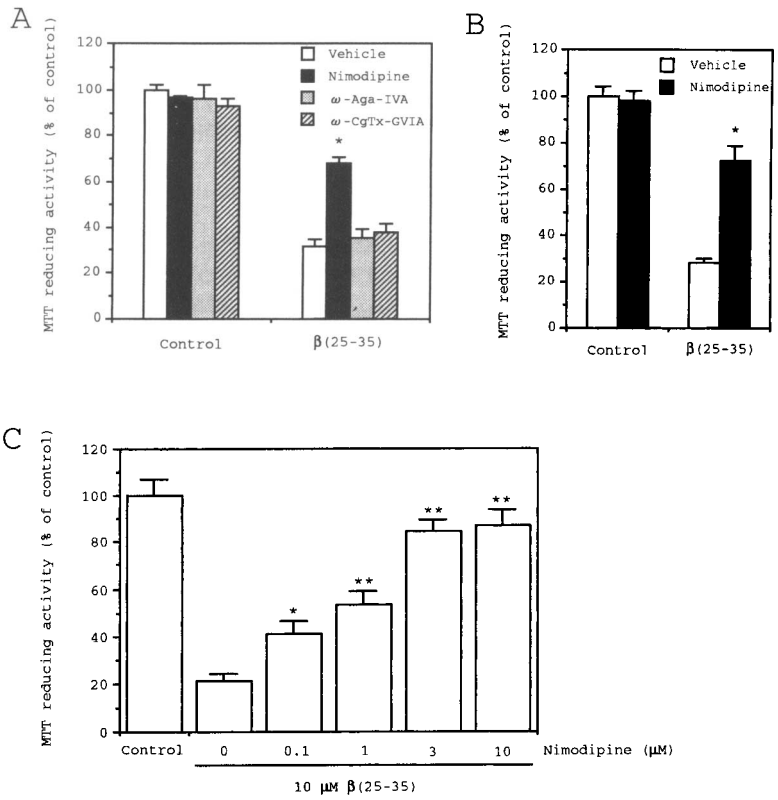

FIG. 2. Effects of Ca^{2+} channel blockers on β(25–35) neurotoxicity. **A:** Cortical cultures. **B:** Hippocampal cultures. Cultures were incubated for 48 h with 10 μM β(25–35) alone or in combination with 1 μM Ca^{2+} channel blockers. Data are expressed as mean ± SEM values (n = 3). *$p < 0.05$, compared with β(25–35) conditions by ANOVA followed by Dunnett's test. **C:** Concentration dependence of nimodipine for β(25–35) neurotoxicity. Cortical cultures were exposed to 10 μM β(25–35) in the presence of different concentrations of nimodipine, and the MTT assay was performed 48 h later. Data are expressed as mean ± SEM values (n = 3). *$p < 0.05$; **$p < 0.01$, compared with the β(25–35) conditions by ANOVA followed by Dunnett's test.

tube and chilled on ice. The dishes were washed with 1 ml of distilled water, and the liquid used for washing was added to the lysate. The fluorescent intensity of the lysate was determined with a spectrofluorometer using excitation and emission wavelengths of 488 and 525 nm, respectively. Data are given as percentages of DCFDA fluorescence of corresponding vehicle-treated values.

Statistical analysis

Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett's test. For single comparison, the significance of differences between means was determined by Student's *t* test. In some of the present experiments, sample size is so small that nonparametric analysis cannot be performed. Because variations of each values were relatively small, we consider that the parametric analysis is enough for evaluating the data.

RESULTS

Effects of VSCC blockers on Aβ neurotoxicity in cultured cortical and hippocampal neurons

Primary cultures of dissociated cortical cells were exposed to Aβ-related peptides for 48 h, and their toxicity was quantified by the MTT assay. β(25–35), the toxic fragment of Aβ (Yankner et al., 1990), showed neurotoxicity dose-dependently at micromolar concentrations (Fig. 1A). The application of 10 μM β(1–40), a native form of Aβ, resulted in a 44% decrease of the MTT activity, whereas no neurotoxicity was observed by either scrambled (25–35) (Mattson et al., 1992) or reversed (25–35), the reverse sequence of β(25–35).

The time course of β(25–35) (10 μM) neurotoxicity is shown in Fig. 1B, as measured by the MTT assay after different incubation periods. Neurotoxic effects were not observed during the first 24 h but became evident at longer time periods (36–48 h); at 48 h, the MTT value was reduced by 70%.

As noted previously (see introductory section), Aβ neurotoxicity appears to be associated with an increase in $[Ca^{2+}]_i$. We investigated the effects of several Ca^{2+} channel blockers, including nimodipine, ω-Aga-IVA, and ω-CgTX-GVIA. Cortical cultures were incubated with 1 μM concentration of the Ca^{2+} blocker together with 10 μM β(25–35) for 48 h. Nimodipine attenuated β(25–35) neurotoxicity significantly, whereas the two other toxins had no effect (Fig. 2A). The protective effect of nimodipine was also confirmed in hippocampal cultures (Fig. 2B). Each Ca^{2+} blocker used per se showed no effect on the MTT activity of vehicle-treated control cultures (Fig. 2A and B). Figure 2C presents the dose dependence of nimodipine; the effective doses were between 0.1 and 10 μM.

Potentiation of Ca^{2+} currents by β(25–35)

To examine whether Ca^{2+} currents can be modulated by β(25–35), we performed patch-clamp recording studies. Ca^{2+} currents were recorded under voltage-clamp conditions in the presence of 0.4 μM TTX, a blocker for voltage-activated Na^+ currents. As

shown in Fig. 3A, a depolarizing pulse from a holding potential at −70 to 0 mV showed inward currents. These currents were blocked by nimodipine. Ca^{2+} currents were recorded from several dissociated hippocampal neurons treated with β(25–35) or the vehicle solution. The Ca^{2+} currents (in picoamperes) so recorded were normalized to cell capacitance (in picofarads) to control for the difference in cell size. The Ca^{2+} current densities of β(25–35)-treated cells and vehicle-treated cells were 8.5 ± 1.7 and 4.1 ± 1.2 pA/pF (mean ± SEM), respectively. Thus, the Ca^{2+} current density of the β(25–35)-treated group was about twofold greater than that of the control group (Fig. 3B).

Potentiation of nimodipine-sensitive Ca^{2+} influx by β(25–35)

Effects of β(25–35) on electrophysiologically recorded Ca^{2+} currents were examined further by measuring $^{45}Ca^{2+}$ influx. Application of β(25–35) (10 μM) caused a significant increase in Ca^{2+} uptake, compared with the vehicle-treated cultures (Table 1). The reversed peptide (25–35) (10 μM) had no effect (95 ± 19% of that in the vehicle-treated cultures).

The potentiation of Ca^{2+} uptake by β(25–35) was reduced by nimodipine (Table 1), whereas other Ca^{2+} channel blockers, ω-CgTX-MVIIC, ω-CgTX-GVIA, or ω-Aga-IVA, had no effects (Table 1). Each Ca^{2+} blocker used showed no effect on Ca^{2+} uptake of vehicle-treated control cultures (Table 1).

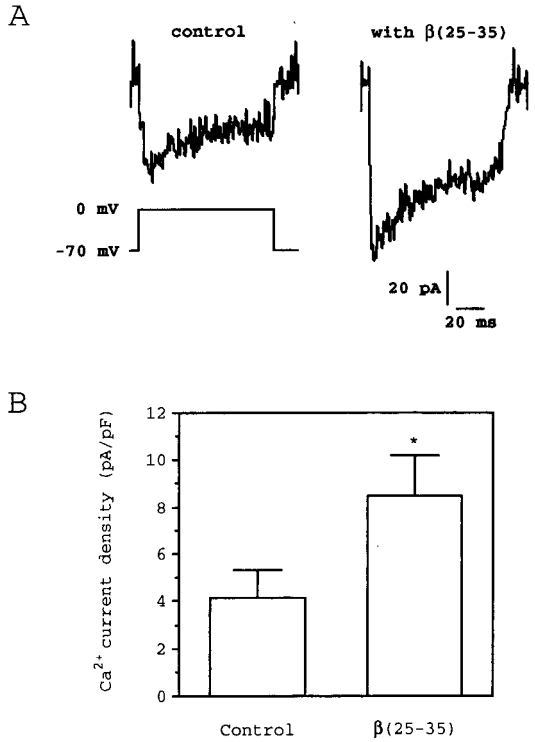

FIG. 3. Effects of β(25–35) on Ca^{2+} currents in hippocampal neurons. Dissociated hippocampal neurons were incubated with 10 μM β(25–35) for 16 h, and visually identified pyramidal neurons were used for the experiment. **A:** Sample traces of currents elicited a voltage step from −70 to 0 mV. **B:** Ca^{2+} current density. Data are expressed as mean ± SEM values. n = 11 and 10 for vehicle-treated control and β(25–35) groups, respectively. *$p < 0.05$, compared with control value by Student's t test.

TABLE 1. *Effects of VSCC blockers on β(25–35)-induced Ca^{2+} uptake*

Treatment	Ca^{2+} uptake (% of vehicle control cultures)	
	Vehicle	β(25–35)
Control	100 ± 7	159 ± 9[a]
3 μM nimodipine	111 ± 15	120 ± 17[b]
3 μM ω-CgTX-MVIIC	88 ± 6	147 ± 5
1 μM ω-CgTX-GVIA	104 ± 5	146 ± 11
1 μM ω-Aga-IVA	112 ± 9	155 ± 8

Cortical cultures were incubated with 10 μM β(25–35) for 16 h, and Ca^{2+} uptake was measured in the presence or absence of Ca^{2+} channel blockers. Data are presented as mean ± SEM values (n = 4–12).

[a] $p < 0.01$, compared with vehicle-treated condition.

[b] $p < 0.05$, compared with β(25–35) condition by ANOVA followed by Dunnett's test.

Involvement of free radicals in β(25–35) neurotoxicity

To examine whether free radicals may mediate β(25–35) neurotoxicity, accumulation of the intracellular reactive oxygen species was measured by the DCFDA assay. Application of β(25–35) (10 μM) for 12 or 24 h significantly increased the intracellular reactive oxygen species (Fig. 4A). The radical scavenger, vitamin E (100 μM), potently reduced accumulation of reactive oxygen species (Fig. 4B).

To examine the effects of vitamin E on β(25–35) neurotoxicity, we performed morphometric cell counting to quantify the magnitude of cell death. The presence of vitamin E may interfere with the MTT assay (Behl et al., 1992). Therefore, neurotoxicity was measured morphologically. Vitamin E (100 μM) was added to cortical cultures simultaneously with β(25–35). Morphometric cell counting was then performed 48 h later. Vitamin E significantly protected neurons from β(25–35)-induced cell death (Fig. 4C).

Involvement of free radicals in β(25–35)-induced Ca^{2+} uptake

The present data indicate the involvement of Ca^{2+} influx through L-type VSCCs and/or free radicals in β(25–35) neurotoxicity. To investigate whether free radicals are involved in potentiation of Ca^{2+} uptake induced by β(25–35), we examined the effect of vitamin E on Ca^{2+} uptake. Cultures were treated simultaneously with vitamin E and β(25–35) for 16 h, and Ca^{2+} uptake was measured. Vitamin E inhibited β(25–35)-induced potentiation of Ca^{2+} uptake significantly (Fig. 5).

To find whether the generation of free radicals in-

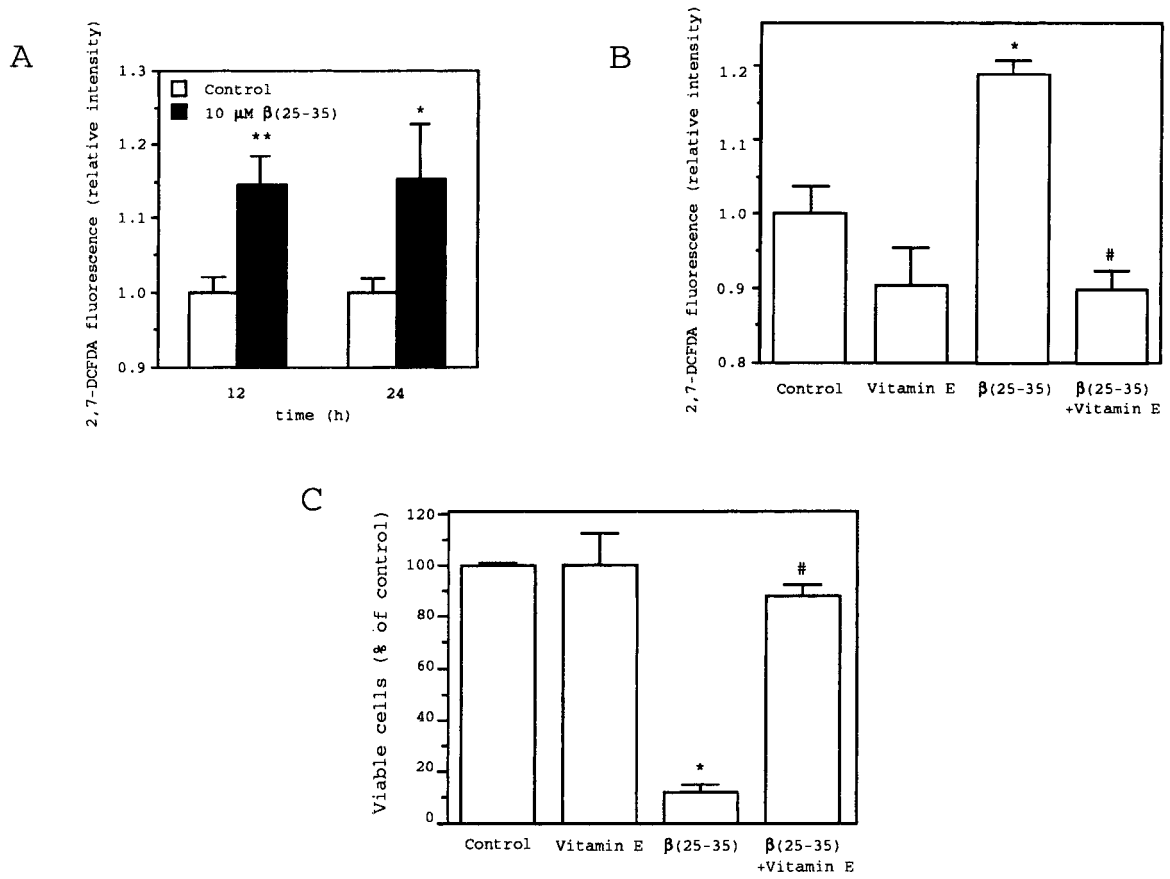

FIG. 4. Involvement of free radicals in β(25–35) neurotoxicity. **A:** Levels of free radicals measured by DCFDA assay. Cortical cultures were exposed to 10 μM β(25–35), and the DCFDA assay was performed 12 or 24 h later. Data are expressed as mean ± SEM values of vehicle-treated cultures, which were incubated for the same amount of time as experimental cultures (n = 4). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with control value by Student's *t* test. **B:** Inhibition of β(25–35)-induced generation of free radicals by vitamin E. Cultures were exposed to 10 μM β(25–35) with or without 100 μM vitamin E, and the DCFDA assay was performed 24 h later. Data are expressed as mean ± SEM values (n = 4). $^{*}p < 0.01$, compared with control value; $^{\#}p < 0.01$, compared with β(25–35) condition by ANOVA followed by Dunnett's test. **C:** Inhibition of β(25–35)-induced neurotoxicity by vitamin E. Cultures were exposed to 10 μM β(25–35) with or without 100 μM vitamin E, and morphometric cell counting was performed 48 h later. Data are expressed as mean ± SEM values (n = 4). $^{*}p < 0.01$, compared with control values; $^{\#}p < 0.01$, compared with β(25–35) conditions by ANOVA followed by Dunnett's test.

duced by β(25–35) depends on Ca^{2+}, we tested the effect of nimodipine. Cultures were treated simultaneously with nimodipine and β(25–35) for 24 h, and the DCFDA assay was performed. Nimodipine did not inhibit β(25–35)-induced free radical accumulation (Fig. 6).

DISCUSSION

A rise of $[Ca^{2+}]_i$ has been suggested to be responsible for toxic effects of Aβ, based on two pieces of information obtained from primary cultures of cortical or hippocampal neurons. First, the presence of extracellular Ca^{2+} is required for Aβ neurotoxicity (Mattson et al., 1993*b*); second, application of Aβ elevates $[Ca^{2+}]_i$, thereby rendering the neurons vulnerable to excitotoxicity (Mattson et al., 1992, 1993*b*). These studies indicate that chronic treatment with Aβ (>24 h) is needed to impair the Ca^{2+} homeostasis in cultured neurons. In agreement with these results, we found that β(25–35) induced excessive Ca^{2+} influx after 16-h exposure. On the other hand, there was no evidence that $[Ca^{2+}]_i$ increases 10 min after application of β(25–35), when measured with fura-2, a Ca^{2+} indicator (unpublished data).

Another support for a role of Ca^{2+} entry in Aβ neurotoxicity comes from the finding that the VSCC blocker nimodipine attenuates the neurotoxicity (Weiss et al., 1994). These findings imply that Aβ-induced Ca^{2+} influx via VSCCs may be related directly to neuronal death.

We tried to specify the types of VSCCs that are involved in Aβ neurotoxicity. As expected, nimodipine reduced β(25–35)-induced cell death significantly. By contrast, ω-Aga-IVA, a P/Q-type Ca^{2+} channel

blocker, or ω-CgTX-GVIA, an N-type Ca^{2+} channel blocker, had little effect. Thus, it appears reasonable to conclude that the L-type VSCC specifically mediates β(25–35) neurotoxicity.

Might Ca^{2+} influx actually be increased by β(25–35)? To address this question, patch-clamp or Ca^{2+}-uptake studies were performed. Incubation of cultured neurons with β(25–35) for 16 h significantly increased the Ca^{2+} current density. In a similar manner, neuronal Ca^{2+} uptake was potentiated by treatment with β(25–35), and this increased uptake could be reversed by nimodipine but not by other VSCC blockers. These results suggest that β(25–35) enhances ionic permeation through the L-type VSCCs or increases the number of these channels in cultured neurons. Aβ-induced neurotoxicity is known to be accompanied by apoptotic morphology (Forloni et al., 1993; Loo et al., 1993; Ueda et al., 1996). It is likely that Ca^{2+} influx induced by Aβ triggers apoptosis.

In agreement with previous suggestions (Behl et al., 1992, 1994), we also observed that β(25–35) induces reactive oxygen species and that vitamin E reduces both the generation of free radicals and neurotoxicity. Thus, all results imply two risk factors; one is elevation of $[Ca^{2+}]_i$ and the other is formation of free radicals (Choi, 1988; Jesberger and Richardson, 1991; Mattson et al., 1993*a*). How are increased $[Ca^{2+}]_i$ and free radicals linked to Aβ neurotoxicity? Might free radicals trigger Ca^{2+} influx? Or, as an alternative, might Ca^{2+} influx trigger the generation of free radicals? To address these questions, we performed two experiments. First, we examined the effects of vitamin E on β(25–35)-induced Ca^{2+} uptake; second, we examined the effects of nimodipine on β(25–35)-induced free radicals. As shown in Fig. 5, vitamin E reduced the Ca^{2+} uptake as well as the generation of free radicals. In contrast, nimodipine did not inhibit free radical generation (Fig. 6). Thus, free radicals generated by

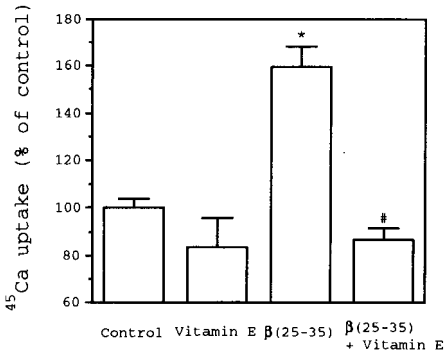

FIG. 5. Effects of vitamin E on Ca^{2+} uptake induced by β(25–35). Cortical cultures were treated with β(25–35) (10 μM) in the presence or absence of vitamin E (100 μM) for 16 h, and Ca^{2+} uptake was measured. Data are expressed as mean ± SEM values (n = 4). $^*p < 0.01$, compared with control value; $^\#p < 0.01$, compared with β(25–35) alone by ANOVA followed by Dunnett's test.

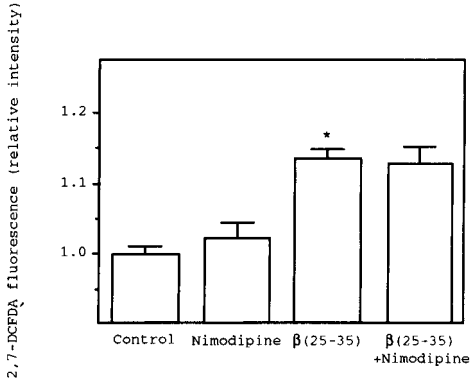

FIG. 6. Effects of nimodipine on free radical levels. Cortical cultures were treated with β(25–35) (10 μM) in the presence or absence of nimodipine (10 μM) for 24 h, and the DCFDA assay was performed. Data are expressed as mean ± SEM values (n = 4). $^*p < 0.01$, compared with control values by ANOVA followed by Dunnett's test.

β(25–35) appear to be responsible for Ca^{2+} influx. This scheme is opposite to the process proposed for NMDA neurotoxicity, in which the free radical formation induced by NMDA is assumed to depend on Ca^{2+} influx (Lafon-Cazal et al., 1993).

We conclude that aggregated Aβ induces excessive Ca^{2+} influx via nimodipine-sensitive L-type VSCCs in cultured neurons and that Aβ-induced influx of Ca^{2+} presumably responsible for cell death is mediated by free radicals.

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