Effects of Amyloid- β Peptides on Voltage-Gated L-Type $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ Ca^{2+} Channels

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Overload of intracellular Ca^{2+} has been implicated in the pathogenesis of neuronal disorders, such as Alzheimer's disease. Various mechanisms produce abnormalities in intracellular Ca^{2+} homeostasis systems. L-type Ca^{2+} channels have been known to be closely involved in the mechanisms underlying the neurodegenerative properties of amyloid- β (A β) peptides. However, most studies of L-type Ca^{2+} channels in A β -related mechanisms have been limited to $\text{Ca}_v1.2$, and surprisingly little is known about the involvement of $\text{Ca}_v1.3$ in A β -induced neuronal toxicity. In the present study, we examined the expression patterns of $\text{Ca}_v1.3$ after A β_{25-35} exposure for 24 h and compared them with the expression patterns of $\text{Ca}_v1.2$. The expression levels of $\text{Ca}_v1.3$ were not significantly changed by A β_{25-35} at both the mRNA levels and the total protein level in cultured hippocampal neurons. However, surface protein levels of $\text{Ca}_v1.3$ were significantly increased by A β_{25-35} , but not by A β_{35-25} . We next found that acute treatment with A β_{25-35} increased $\text{Ca}_v1.3$ channel activities in HEK293 cells using whole-cell patch-clamp recordings. Furthermore, using GTP pulldown and co-immunoprecipitation assays in HEK293 cell lysates, we found that amyloid precursor protein interacts with β_3 subunits of Ca^{2+} channels instead of $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ α_1 subunits. These results show that A β_{25-35} chronically or acutely upregulates $\text{Ca}_v1.3$ in the rat hippocampal and human kidney cells (HEK293). This suggests that $\text{Ca}_v1.3$ has a potential role along with $\text{Ca}_v1.2$ in the pathogenesis of Alzheimer's disease.

INTRODUCTION

Overload of intracellular Ca^{2+} has been implicated in the pathogenesis of neuronal disorders, such as Alzheimer's disease, epilepsy, ischemia, and seizures (Choi, 1995; Kim and Rhim, 2004; Pierrot et al., 2004). In the central nervous system (CNS), mechanisms that produce abnormalities in intracellular Ca^{2+} homeostasis systems include aberrant Ca^{2+} influx through plasma membrane channels including the *N*-methyl-D-aspartic acid receptors and voltage-gated calcium channels (Cano-Abad et al., 2001; Kim et al., 2008; Luo et al., 2005; Sattler and Tymianskin, 2000). Among several types of voltage-gated Ca^{2+}

channels, L-type Ca^{2+} channels have been known to be closely involved in the mechanisms underlying the neurodegenerative properties of amyloid- β (A β) peptides. After a report that amyloid β -protein fragment 25–35 (A β_{25-35}) causes reversible and reproducible increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) along with bursts of excitatory potentials in cultured hippocampal neurons (Brorson et al., 1995), the role of L-type Ca^{2+} channels in A β_{25-35} neurotoxicity was further examined using selective blockers of Ca^{2+} channels (Ueda et al., 1997). When L-type Ca^{2+} channels were blocked by application of nimodipine, a selective blocker for L-type Ca^{2+} channels, A β_{25-35} neurotoxicity was attenuated. In contrast, application of ω -conotoxin GVIA or ω -agatoxin IVA, which act as blockers for N- or P/Q-type Ca^{2+} channels, had no effects on cultured rat cortical and hippocampal neurons. Later, L-type Ca^{2+} channel blockers were also found to have neuroprotective effects against A β -induced neuronal apoptosis in cultured rat cortical neurons (Yagami et al., 2004) and from amyloid precursor protein (APP)-induced neurotoxicity in neuroblastoma cells (Anekonda et al., 2011). Furthermore, increased intraneuronal resting $[\text{Ca}^{2+}]_i$ was reported in neurons obtained from a mouse model of Alzheimer's disease. The increased $[\text{Ca}^{2+}]_i$ was partially reduced by application of a selective L-type Ca^{2+} channel blocker, nifedipine, or a non-L-type channel blocker, SKF-96365 (Lopez et al., 2008).

Using whole-cell patch-clamp recordings, the involvement of L-type Ca^{2+} channels in A β -induced neuronal apoptosis has been directly demonstrated. Green and Peers (2001) have shown that A β selectively upregulates native L-type Ca^{2+} currents in PC12 cells, while other voltage-gated Ca^{2+} channels are unaffected. In various neuronal cells, bath application of A β produced a reversible increase in Ca^{2+} channel currents. This effect was blocked by treatment with nifedipine (Rovira et al., 2002; Ueda et al., 1997). In the CNS, there are 2 main types of L-type Ca^{2+} channels; $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$. While the involvement of $\text{Ca}_v1.2$ in A β -induced neuronal apoptosis has been previously studied, the relevance of $\text{Ca}_v1.3$ to A β -induced neuronal apoptosis is poorly understood. Therefore, we examined the expression patterns of $\text{Ca}_v1.3$ before and after A β_{25-35} exposure in cultured hippocampal neurons and the regulation of $\text{Ca}_v1.3$ channel currents by acute A β_{25-35} exposure using whole-cell patch-clamp recordings in $\text{Ca}_v1.3$ expressed HEK293 cells.

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MATERIALS AND METHODS

Materials

A β_{25-35} , A β_{35-25} , anti- β -actin, and anti-Flag antibodies were purchased from Sigma Aldrich (USA). Nifedipine was purchased from Tocris (USA). Anti-Cav1.2 and anti-Cav1.3 antibodies were obtained from Alomone Labs (Israel). Anti-GST and anti-myc antibodies were purchased from Novagen (USA) and Cell Signaling Technology (USA), respectively. All other chemicals were purchased from Sigma Aldrich.

RNA isolation and RT-PCR analysis

The total RNA from each sample was extracted using the easy-BLUE™ kit (iNtRON Biotech, Korea) according to the manufacturer's instructions. Total RNA samples were treated with DNase I before cDNA synthesis and quantified using ultraviolet spectrophotometry. Single-strand cDNAs were then synthesized in a reverse transcription (RT) reaction in the presence of 2 μ g of total RNA, 0.1 nmol of specific antisense primer, and first-strand cDNA synthesis mix (iNtRON Biotech) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10 mM dithiothreitol, 0.25 mM of each dNTP, and 100 units of Moloney murine leukemia virus reverse transcriptase. The sequences of the primers and the predicted sizes of PCR products were previously reported by Kim et al. (2007). Using these primers, amplification of cDNA fragments was performed by PCR with 25–28 cycles with 1 μ l of each RT product as template DNA in 60 mM Tris-HCl (pH 9.1) buffer containing 18 mM (NH₄)₂SO₄, 16 mM MgCl₂, 0.25 mM of each dNTP, 0.1 nmol of each primer, and Ex-Taq DNA polymerase (Takara, Japan). Each sample (5 μ l) of final PCR product was separated using a 1.5–2.5% agarose gel and visualized using UV fluorescence after staining with ethidium bromide for 15 min. The β -actin, as a template control, was included in each run.

Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. For electrophysiological recordings of Cav1.3 channel currents, HEK293 cells were transiently transfected with Cav1.3 α_1 and β_3 subunits using Lipofectamine 2000 (Invitrogen, USA). Cultured hippocampal neurons were prepared using a technique modified from Kim et al. (2007). Briefly, the hippocampi were isolated from 18-day-old fetal Sprague-Dawley rats and incubated with 0.25% trypsin in Leibovitz L-15 medium (Invitrogen). Cells were then mechanically dissociated and plated on poly-L-lysine-coated culture dishes. Cultures were maintained in Neurobasal/B27 medium (Invitrogen) containing 0.5 mM L-glutamine, 25 μ M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin under a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Experiments were carried out on neurons after 5–10 days *in vitro*.

Western blot analysis of Cav1.2 or Cav1.3 protein expression

For the detection of Cav1.2 or Cav1.3, cultured hippocampal neurons were treated with A β_{25-35} or A β_{35-25} for 24 h, and the prepared protein samples were separated on 6% SDS-PAGE. The proteins then transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was first blocked with PBS containing 5% skim milk and 0.1% Tween 20 for 1 h. After 3 washes, the membranes were incubated with anti-Cav1.2 (1:150 dilution) or anti-Cav1.3 (1:150 dilution) antibodies for 20 h at room temperature in PBS containing 1% skim milk

and 0.1% Tween 20. For a positive control, anti- β -actin antibodies (1:2500 dilution) were incubated for 1 h. After 3 washes, the membranes were incubated with peroxidase-conjugated secondary antibodies in PBS containing 3% skim milk and 0.1% Tween 20 for 2 h at room temperature. The signal resulting from the immunoreactivity was detected using a western blot detection reagent (EliPis Biotech, Korea). For the detection of each channel's surface protein, biotinylation of cell surface proteins was performed using the Pinpoint Cell Surface Protein Isolation kit (Pierce) according to the manufacturer's instructions as previously described by Yun et al. (2007).

Electrophysiological recording

Cav1.3 channel currents were measured in HEK293 cells transfected with Cav1.3 α_1 subunits using Ba²⁺ as a charge carrier with standard whole-cell patch-clamp techniques. Borosilicate glass electrodes were filled with the internal solution containing (in mM): 135 CsCl, 10 EGTA, 4 MgCl₂, 10 HEPES, and 4 Mg-ATP (pH 7.4). The external solution contained (in mM): 135 CholineCl, 5 BaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). Cells were held at a holding potential of -70 mV, and currents were elicited with step depolarizations from -60 mV to +30 mV (10 mV increments) for 200 ms. All of the data were obtained and analyzed using an EPC-10 amplifier and Pulse/Pulsefit software program (HEKA, Germany).

GST pulldown assays

GST pulldown assays were performed with purified GST and various GST-tagged proteins using the ProFound Pull-Down GST Protein:Protein Interaction Kit (Pierce, USA). The immobilized GST or various GST-tagged proteins were incubated with cell lysates prepared from HEK293 cells. Glutathione-bound proteins were eluted by boiling at 95°C in SDS sample buffer for 10 min, and detected by immunoblotting with anti-Cav1.2, anti-Cav1.3, anti-GST, or anti-Flag antibodies.

Co-immunoprecipitation assay

Co-immunoprecipitation (co-IP) assays were performed using the ProFound Mammalian Co-Immunoprecipitation Kit (Pierce). Briefly, lysates from HEK293 cells were precleared, immunoprecipitated with 10 μ g of a specific antibody and 50 μ l of AmniLink® Plus Coupling Gel at 4°C for 6 h or overnight, and washed 5 times. Immune complexes were eluted using IgG Elution Buffer (Pierce) or by boiling at 95°C in SDS-sample buffer for 10 min, and detected by immunoblotting with anti-Cav1.2, anti-Cav1.3, anti-myc, or anti-Flag antibodies.

RESULTS AND DISCUSSION

To elucidate the involvement of Cav1.3 L-type Ca²⁺ channels in the mechanisms underlying the neurodegenerative properties of A β peptides, we examined the expression profiles of Cav1.2 and Cav1.3 before and after treatment with A β_{25-35} in cultured rat hippocampal neurons. Treatment with 25 nM A β_{25-35} for 24 h significantly increased both total and surface Cav1.2 protein levels as well as Cav1.2 mRNA levels while there was no significant change observed upon treatment with the inverted sequence of A β_{25-35} , amyloid β -protein fragment 35–25 (A β_{35-25}) (Figs. 1A–1C). However, the expression levels of Cav1.3 were not significantly changed by either A β_{25-35} or A β_{35-25} both at the mRNA and total protein levels. Interestingly, Cav1.3 proteins were significantly increased only at the surface level by A β_{25-35} , but not by A β_{35-25} (Fig. 1C). For Cav1.2, we also observed the increased surface level by A β_{25-35} , but not by A β_{35-25} . Figure 1D displays the summarized data showing the effects of A β_{25-35} or

Fig. 1. Effects of Aβ exposure on expression profiles of Cav1.2 and Cav1.3 in hippocampal neurons. (A) Expression levels of Cav1.2 and Cav1.3 mRNAs in the presence of Aβ₂₅₋₃₅ (25 nM) for 24 h. Cav1.2 and Cav1.3 mRNAs were determined using RT-PCR analysis from cell lysates obtained from cultured hippocampal neurons. (B and C) Expression levels of total (B) or surface (C) Cav1.2 and Cav1.3 proteins in the presence of Aβ₂₅₋₃₅ or Aβ₂₅₋₃₅. Cav1.2 (240 kDa) and Cav1.3 (260 kDa) proteins were determined using western blot analysis with anti-Cav1.2 and anti-Cav1.3 antibodies, respectively. β-Actin was used as a positive control for RT-PCR and Western blot analyses. (D) The bar graph indicates the mean values of relative mRNA or protein levels from each group ($n = 3$). The level of control mRNA or protein was counted as an arbitrary control (100%). ***, $p < 0.001$ compared with the control level.

Aβ₃₅₋₂₅ on relative mRNA or protein levels of Cav1.2 and Cav1.3. These results suggest that Aβ₂₅₋₃₅-induced expression profiles of Cav1.3 mRNA and total proteins are different from the profiles of Cav1.2, and both channels are similarly upregulated at the surface level only in an Aβ₂₅₋₃₅ specific manner in cultured hippocampal neurons.

We next examined whether acute treatment with Aβ₂₅₋₃₅ modulates Cav1.3 channel activities using whole-cell patch-clamp recordings. When Cav1.3 α_1 and β_3 subunits were transiently transfected in HEK293 cells, GFP was also co-transfected to identify positively transfected cells. Using Ba²⁺ as a charge carrier, Cav1.3 channel currents were measured in transfected cells only as previously described in Park et al. (2010). When Aβ₂₅₋₃₅ was applied to the perfusate, Ba²⁺ currents were rapidly increased. This was maximally observed at a test pulse of -10 mV (Fig. 2A). In Fig. 2B, upper representative traces of the Cav1.3 recordings show an increase in Ba²⁺ currents upon addition of 25 nM Aβ₂₅₋₃₅. Lower traces show a decrease in Ba²⁺ currents upon addition of 10 μ M nifedipine, a selective L-type Ca²⁺ channel blocker. These results suggest

Fig. 2. Effects of Aβ acute exposure on Ca²⁺ channel currents in HEK 293 cells stably expressing the human Cav1.3 α_1 subunit. (A) Current-voltage (I - V) relationships of peak Cav1.3 currents in the absence (open circles) or presence (filled triangles) of 25 nM Aβ₂₅₋₃₅. (B) Upper traces: representative example currents showing Aβ₂₅₋₃₅-induced upregulation of Cav1.3 currents. Lower traces: example currents showing the effects of bath application of nifedipine (Nif, 10 μ M). Ca²⁺ channel currents were evoked by a step depolarization applied from -70 mV to -10 mV for 200 ms. (C) Mean current density-voltage relationships obtained from control cells (open circles) and from cells acutely exposed to 25 nM Aβ₂₅₋₃₅ during whole-cell patch-clamp recordings (filled triangles). (D) Pooled results illustrating the mean current density of peak Cav1.3 channel currents in the presence of nifedipine, Aβ₂₅₋₃₅ or Aβ₃₅₋₂₅. ***, $p < 0.001$; **, $p < 0.01$ compared with the control level. NS, not significant.

that Ba²⁺ currents were predominantly recorded via an activation of Cav1.3 channels. Aβ₂₅₋₃₅ significantly increased the peak currents of Cav1.3 (8.93 ± 0.65 pA/pF in control; 16.40 ± 1.97 pA/pF in the presence of Aβ₂₅₋₃₅, $n = 10$), but did not cause any shift in the current-voltage curve in Fig. 2C. Addition of Aβ₃₅₋₂₅ at the same concentration as Aβ₂₅₋₃₅ (25 nM) did not produce any effect on peak currents of Cav1.3 ($n = 5$). This is summarized in Fig. 2D. These results provide the first experimental evidence that acute treatment with Aβ₂₅₋₃₅ increases Cav1.3 L-type Ca²⁺ currents in HEK293 cells.

For the possible mechanisms of Aβ-induced upregulation of Cav1.3, we attempted to determine whether a direction association occurs between α_1 subunits of L-type Ca²⁺ channels and full length amyloid precursor protein (APP695). We performed GST pulldown assays using GST-tagged APP695 and a GST-tagged fragment of APP695, which contains the amino acid sequence from 306 to 695 (APP390, Fig. 3A). After GST, GST-APP695, and GST-APP390 were expressed in *Escherichia coli*, they were immobilized by glutathione-agarose 4B. Then, the lysates from the HEK293 cells overexpressing α_1 subunits of Cav1.2 or Cav1.3 were incubated with the glutathione-agarose 4B bound to GST-APP695 or GST-APP390. As shown in Fig. 3B and 3C, GST-APP695 was shown not to interact with either Cav1.2 or Cav1.3. Using GST-APP390, we also found that there is no interaction of Cav1.2 or Cav1.3 with APP390. There-

Fig. 3. The GST pull-down assay between APP and Ca_v1.2 or Ca_v1.3. (A) Schematic diagram of GST-tagged full-length amyloid precursor protein (APP695) and a fragment of APP695, which contains the amino acid sequence from 306 to 695 (APP390). (B) Immobilized GST, GST-APP695, or GST-APP390 was incubated with cell lysate, and then the retained proteins were analyzed with anti-Ca_v1.2 or anti-GST antibodies. (C) Immobilized GST, GST-APP695, or GST-APP390 was incubated with cell lysate, and then the retained proteins were analyzed with anti-Ca_v1.3 or anti-GST antibodies. (D) Schematic diagram of myc-tagged APP695, Ca_v1.2 and Ca_v1.3. (E, F) *In vivo* co-IP assays. Where specified, the solubilized total proteins (100 μ g) from HEK293 cells were immunoprecipitated with anti-Ca_v1.3, anti-Ca_v1.2, or anti-APP antibodies. The immune complexes were then analyzed by SDS-PAGE, followed by immunoblotting with anti-APP antibodies (E, F), anti-Ca_v1.2 (E) or anti-Ca_v1.3 (F) antibodies.

fore, using an *in vivo* co-IP assay, we further examined a direct interaction between L-type Ca²⁺ channels and APP695 in HEK293 cells overexpressing myc-tagged APP695 and one of the L-type Ca²⁺ channels (Fig. 3D). For the Ca_v1.2 channels, cell lysates were prepared, immunoprecipitated with anti-Ca_v1.2 antibodies, and subsequently immunoblotted with anti-myc antibodies. As shown in Fig. 3E, no positive signal was detected. When co-IP was performed in reverse with anti-myc antibodies followed by immunoblotting with anti-Ca_v1.2, the result was the same. For the examination of a direct interaction between Ca_v1.3 with APP695, cell lysates were prepared, immunoprecipitated with anti-Ca_v1.3 antibodies, and subsequently immunoblotted with anti-myc antibodies. In addition, co-IP was performed in reverse with anti-myc antibodies followed by immunoblotting with anti-Ca_v1.3. However, we found that there is no association between Ca_v1.3 and APP695 in HEK293 cells (Fig. 3F).

Fig. 4. Interaction between APP and β_3 subunit using GST pull-down and co-IP assays. (A) Schematic diagram of GST-tagged APP695, GST-tagged APP390, myc-tagged APP695, and Flag-tagged β_3 subunit. (B) Immobilized GST, GST-APP695, or GST-APP390 was incubated with cell lysate, and then the retained proteins were analyzed with anti- β_3 subunit or anti-GST antibodies. (C) Solubilized total proteins (100 μ g) from HEK293 cells were immunoprecipitated with anti-APP antibodies, and the immune complexes were detected with anti- β_3 antibodies.

Then, what is the mechanism of A β -induced upregulation of Ca_v1.3 evidenced by the data shown in Figs. 1 and 2? In native brain tissues, α_1 subunits of Ca²⁺ channels interact with other auxiliary subunits, e.g., $\alpha_2\delta$, β , and γ subunits, to provide complete activity and functional regulation. In a heterologous expression system such as the HEK293 cells used in this study, other auxiliary subunits of Ca²⁺ channels are required when α_1 subunits are expressed for their full activation. In the present study, we used HEK293 cells transfected with Ca_v1.3 α_1 and β_3 subunits. Therefore, we examined the possible involvement of β_3 subunits as modulators of A β -induced upregulation of L-type Ca²⁺ channels. We performed GST pull-down assays to observe the specific interaction between APP and β_3 subunits. As shown in Figs. 4A and 4B, GST-APP695 or GST-APP390 interacts with Flag-tagged β_3 subunits in HEK293 cell lysates expressing β_3 subunits while GST alone (a negative control) was found to not interact with the β_3 subunits. Subsequently, using an *in vivo* co-IP assay, we confirmed the association of APP and β_3 subunits in HEK293 cells expressing myc-tagged APP695 and Flag-tagged β_3 subunits. Cell lysates prepared from HEK293 cells were immunoprecipitated with anti-myc antibodies, and subsequently immunoblotted with anti-Flag antibodies. In Fig. 4C, APP binds to endogenous β_3 subunits in HEK293 cells whereas no signal was detected in an IgG control. These results strongly suggest that a specific interaction between APP and β_3 subunits occurs in mammalian cells.

Herein, we present 3 principal findings regarding possible roles of Ca_v1.3 in A β -induced neuronal toxicity. First, we found that the expression levels of Ca_v1.3 are moderated only at the surface protein level by chronic treatment with A β . This pattern is different from the Ca_v1.2 pattern. In the current study, the 24 h treatment with A β_{25-35} significantly increased both total and surface Ca_v1.2 protein levels as well as Ca_v1.2 mRNA levels while there was no significant change when A β_{35-25} , the reverse amino acid sequence peptide of A β_{25-35} , was used. In addition,

A β -mediated upregulation of Cav1.2 was previously reported with respect to mRNA and total protein levels (Chiou, 2006) as well as surface protein levels (Scragg et al., 2004). Our results with respect to Cav1.3 mRNA and total protein levels were consistent with the previous findings using human SK-N-SH neuroblastoma cells. When the expression patterns of 4 α_1 subunits of P/Q (Cav2.1)-, N (Cav2.2)-, and L (Cav1.2 and Cav1.3)-type Ca²⁺ channels before and after A β exposure were investigated in SK-N-SH cells, no significant changes were observed with respect to Cav1.3 mRNA and total protein levels (Chiou, 2006). Because the surface protein levels were not examined by Chiou (2006), we first reported that the 24 h treatment with A β selectively upregulates the Cav1.3 surface expression in cultured hippocampal neurons.

Secondly, we found that acute treatment with A β_{25-35} increased Cav1.3 channel activities using whole-cell patch-clamp recordings in HEK293 cells. Direct evidence for the involvement of L-type Ca²⁺ channels in A β -induced neuronal toxicity was obtained from the electrophysiological studies of endogenous L-type Ca²⁺ channels in neurons or cell lines expressing Cav1.2 in heterologous systems (Green and Peers, 2001; Rovira et al., 2002; Ueda et al., 1997). Because there were no reports examining the acute effects of A β on Cav1.3 channel activities using electrophysiological tools, we attempted to examine them in HEK293 cells transiently expressing Cav1.3 α_1 and β_3 subunits. On the basis of our data, it appears that Cav1.3 could be directly modulated by A β , and the degree of upregulation is very similar to that of Cav1.2 determined in HEK293 cells (Scragg et al., 2004). Although the augmentation of currents by A β_{25-35} is apparent in the present study, it is also interesting to examine effects of the predominant 2 toxic forms of A β peptides, A β_{1-40} and A β_{1-42} , on Cav1.3 channel activities.

What is a possible mechanism of A β -induced upregulation of Cav1.2 and Cav1.3 both chronically and acutely? As the third and final piece of evidence on the possible roles of Cav1.3 in A β -induced neuronal toxicity, we found that β_3 auxiliary subunits of Ca²⁺ channels may play an important role in A β -induced upregulation of Cav1.2 and Cav1.3. On the basis of experiments using GST pulldown and co-IP assays, we clearly determined that neither Cav1.2 nor Cav1.3 binds to APP in both the GST pulldown and the co-IP experiments. However, we demonstrated that β_3 subunits of Ca²⁺ channels interact with APP695 or APP390. This suggests a possible role of β_3 subunits of Ca²⁺ channels in A β -induced neuronal toxicity. It is possible that APP, which contains the A β fragment, increases surface expression of Cav1.3 or functional activities of Cav1.3 via an interaction with β_3 subunits. However, for the detailed mechanism of A β -induced upregulation of Cav1.3, it is necessary to demonstrate whether A β interacts with β_3 subunits in the brain using binding assays or to elucidate binding sequences of APP using mapping studies.

A number of recent studies have shown that A β can disrupt neuronal Ca²⁺ homeostasis by inducing influx of extracellular Ca²⁺ into the neuronal cytoplasm (Anekonda et al., 2011; Small et al., 2009; Supnet and Bezprozvanny, 2010). Among the functions of voltage-gated Ca²⁺ channels regulating neuronal excitability, synaptic plasticity, and neurotoxicity, functions of the neuronal L-type Ca²⁺ channels are distinguished from the functions of the N- and P/Q-type Ca²⁺ channels. N- and P/Q-type Ca²⁺ channels clearly play central roles in controlling Ca²⁺ entry into, and thus neurotransmitter release from, neuronal synaptic terminals in the CNS (Catterall, 1999; Dunlap et al., 1995). On the other hand, neuronal L-type Ca²⁺ channels are known to be involved in translating synaptic activity into alterations in gene expression and neuronal cell death (Cano-Abad

et al., 2001; Finkbeiner and Greenberg, 1997; Fuchs, 1996; Luo et al., 2005). This is related to the A β specific increases in the quantity and activity of L-type Ca²⁺ channels caused by A β . Among the possible mechanisms underlying A β -induced Ca²⁺ dysregulation, A β can increase ion permeability in lipid membranes or activate endogenous ion channels on the cell surface (Small et al., 2009). In the present study, we showed that A β does not change the amount of mRNA of Cav1.3 but dramatically alters the quantity of surface proteins of Cav1.3. This provides further support for the results showing that A β activates endogenous ion channels on the cell surface. Therefore, the A β -induced increase in surface proteins of Cav1.3 could provide more surface-expressed L-type Ca²⁺ channels, which deteriorate and cause neuronal cell death via Ca²⁺ overload.

In summary, using expression profiling and electrophysiological studies as well as *in vivo* binding assays, we showed the first evidence of A β -mediated upregulation of Cav1.3 surface proteins and Cav1.3 channel activities and further originally demonstrated direct binding between APP and β_3 subunits of Ca²⁺ channels. These results may help elucidate pivotal roles of L-type Ca²⁺ channels in intracellular Ca²⁺ disturbance in Alzheimer's disease. It is likely that elucidation of the mechanism by which A β cause Ca²⁺ dysregulation via L-type Ca²⁺ channels will help identify new drug targets for the treatment of Alzheimer's disease.

ACKNOWLEDGMENTS

This work was supported by a Mid-Career Researcher Program Grant (No. 20110000094), a Pioneer Grant (No. 20110002117), and the BRC of the 21st Century Frontier Research Program (No. 2011K000272) to H. Rhim and a Basic Science Research Program (2009-0076837) to S. Kim from the Ministry of Education, Science and Technology, the Republic of Korea.

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