Critical Role of Calpain-mediated Cleavage of Calcineurin in Excitotoxic Neurodegeneration*

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Calcineurin and calpain, a Ca2+/calmodulin-dependent protein phosphatase and a Ca2+/dependent cysteine protease, respectively, mediate neuronal cell death through independent cascades. Here, we report that during neuroexcitotoxicity, calcineurin A (CnA) is directly cleaved by calpain in vitro and in vivo, resulting in the enzyme being converted to an active form. Mass spectrometry identified three cleavage sites in CnA, two of which were constitutively active forms. Overexpression of the cleaved CnA induced caspase activity and neuronal cell death. Calpain inhibitors and membrane-permeable calpastatin peptides not only blocked the cleavage of CnA, but also protected against excitotoxic neuronal cell death in vitro and in vivo. These results indicate that CnA is a crucial target for calpain, and the calpain-mediated activation of CnA triggers excitotoxic neurodegeneration. This study established a molecular link between calpain and calcineurin, thereby demonstrating a new mechanism for proteolytical regulation of calcineurin by calpain in response to certain pathological states.

Neuronal cell death in stroke, trauma, and neurodegenerative diseases such as Huntington’s disease, Alzheimer’s disease, and Amyotrophic Lateral Sclerosis has been proposed to result from the accumulation of high local concentrations of excitatory amino acids, particularly glutamate (1–7). The accumulation of glutamate leads to an excessive level of Ca2+ influx and cell death. Despite years of investigation, the development of several diseases such as brain injury, Alzheimer’s disease, and focal or global cerebral ischemia (14, 15). Some substrates of the enzyme have been suggested as key molecules in the induction of neuronal cell death (14, 16–18). For example, calpain cleaves p35, a specific activator of cyclin-dependent kinase 5 (Cdck5), to p25, which prolongs the kinase activation and causes its mis-localization, resulting in neuronal cell death in Alzheimer’s disease (16, 19, 20).

Calcineurin is composed of two subunits, a calmodulin-binding 60-kDa catalytic subunit, calcineurin A (CnA),1 and an intrinsic Ca2+-binding 19-kDa regulatory subunit, calcineurin B (CnB) (21, 22). CnA has putative autoinhibitory and calmodulin-binding domains in the C-terminal. The phosphatase becomes active when calmodulin binds to the binding site in CnA because the binding triggers the release of the autoinhibitory domain from the catalytic active site (21, 23). Previous studies (9, 10) have shown that artificial overexpression of the truncated CnA induces apoptosis in neuronal and non-neuronal cells. However, it has not been elucidated whether CnA undergoes cleavage in response to certain pathological conditions and whether the cleaved CnA induces neuronal cell death in vitro. In the present study, we show that CnA undergoes cleavage by calpain in two neuroexcitotoxic models and this cleavage plays a critical role in neuronal degeneration in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Neuronal Cultures—Primary cultures of hippocampal and cortical neurons from E17–19 Wistar rats (Japan SLC Inc.) were prepared as described previously (24). Briefly, the cortex and hippocampus were

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1 The abbreviations used are: CnA, calcineurin A; CnB, calcineurin B; ALLM, N-acetyl-Leu-Leu-methionyl; CS, calpastatin peptide; 11R-CS, 11 poly-arginine calpastatin peptide; 11R-CS-R, 11 poly-arginine calpastatin scrambled peptide; KA, kainate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PMF, the peptide mass fingerprinting; PBS, phosphate-buffered saline; T-CnA, truncated CnA; TUNEL, TdT-mediated dUTP nick-end labeling; TRITC, tetramethylrhodamine isothiocyanate; MS, mass spectrometry; ANOVA, analysis of variance; NFAT, nuclear factor of activated T-cell; ACF, artificial cerebro-spinal fluid.
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dissected on ice in Hank’s balanced salt solution (Sigma) and dissociated with 0.25% trypsin in HBSS. Neurons were seeded to a density of 2.7 × 10^6/mm² on dishes coated with poly-β-histidine (100 μg/ml). After 24 h, neurobasal growth medium (Invitrogen) containing 10% fetal bovine serum was replaced with neurobasal medium containing B27 supplement (Invitrogen) instead of serum. The cultures were maintained in a humidified 5% CO₂ incubator at 37°C for 10–14 days.

**Western Blotting Analysis**—Western blotting analysis was performed as described previously (25). Briefly, the polyclonal antibodies against CaNα were from StressGen Biotech (Victoria, BC, Canada); the monoclonal CaNβ antibodies were produced as described previously (26); the polyclonal antibodies against cabin-1 and non-erythroid α-spectrin mouse monoclonal antibodies were from Chemicon (Temecula, CA). After incubation with the appropriate secondary antibodies and horseradish peroxidase (Sigma), positive bands were visualized using an enhanced chemiluminescence (ECL) detection system (Amer sham Biosciences).

**Immunocytochemistry**—Immunocytochemistry was performed as described previously (25). Briefly, after being treated under different experimental conditions, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 15 min and then membrane-permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 10% bovine serum albumin at 37°C for 1 h, cells were incubated with anti-microtubule-associated protein 2 antibodies (1:200 dilution, Sigma) or anti-active caspase-3 antibodies (1:200 dilution, R&D Systems, Minneapolis, MN) at 4°C overnight. The secondary antibodies were then incubated with secondary TRITC-conjugated anti-mouse antibodies (1:200 dilution, Sigma). Fluorescent images were obtained using a Zeiss confocal microscope.

**Recombinant Adenovirus Preparation**—The recombinant adenoviruses encoding 48-kDa truncated CaNα isoform (T-CaNα), wild-type CaNβ and lacZ cDNAs were produced as described previously (12). Briefly, rat CnA α isoform cDNA corresponding to T-CaNα (amino acid residues 1–424) and wild-type rat CaNβ cDNA were subcloned into pXCaWt cosmid vector (Takara, Tokyo, Japan). Recombinant adeno virus of T-CaNα (pAdex-CaNα-T) and CaNβ (pAdex-CaNβ) containing chicken β-actin promoter and SV40 polyadenylation signal were produced using the Adenovirus Expression Vector kit (Takara). The recombinant adenoviruses were purified and concentrated using the CsCl step-gradient method as described previously (27). Recombinant adenovirus of lacZ (pAdex-lacZ) was also produced following the manufacturer’s protocol.

**Detection of Neuronal Cell Death**—Immunostained neurons were incubated with Hoechst 33258 (1 μg/ml) for 1 min, and then the morphology of the nucleus was observed with a fluorescence microscope. Apoptotic neuronal cells were identified by the presence of highly condensed or fragmented nuclei. Representative graphs are shown for experiments where at least 4 randomly chosen fields with 150 cells were scored. TUNEL assays were performed following the manufacturer’s protocol. The stained cells were observed using a fluorescence microscope (Zeiss). Representative graphs are shown for experiments where at least 4 randomly chosen fields with 100 cells were scored.

**Luciferase Reporter Assay**—Rat wild-type CaNα isoform, truncated CaNαs encoding 57-kDa, 48-kDa, and 45-kDa CaNα isoform, and the wild-type CaNβ cDNA were subcloned into a mammalian cell expression vector (pcDNA3.1myc-His, Invitrogen). These plasmids (1 μg) were transfected into ~70% confluent HEK 293 cells by LipofectAMINE (Invitrogen) following the manufacturer’s protocol. To measure the NFAT transcriptional activity, 1 μg luciferase reporter vector (pNFATTA-Luc, BD Biosciences Clontech) was also transfected into HEK 293 cells. After 24 h, cells were incubated with Ca²⁺ ionophore A23187 (2 μM, Calbiochem, San Diego, CA) for 5 min and then cultured for 1 h. The cells were then harvested and luciferase activities were measured using a luminometer using a reagent kit (Toyo Ink, Tokyo, Japan). The background luciferase activity was subtracted from all experiments.

**Calcineurin Activity Assay**—Calcineurin activity was assessed using the Calcineurin Activity Assay kit (Calbiochem) according to the manufacturer’s instruction. Briefly, RII phosphopeptide (DLD-VIPGRFDRWVPSVAAE), the well known substrate for calcineurin, was used as the substrate. Cortical neurons were collected 12 h after 500 μM glutamate treatment for 15 min and were then lysed in lysis buffer (25 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 50 μM EDTA, 50 μM EGTA, 10% glycerol, 0.1% Nonident P-40). The mixture was incubated with RII phosphopeptide (1.64 mg/ml) in assay buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM dithiothreitol, 0.05% Nonident P-40, and 500 μM okadaic acid with or without 10 μM EGF. After 30 min at 30°C, reactions were terminated by adding 100 μl GREEN™ and fluorescence was measured at 620 nm using a microtiter plate reader (Takara, Tokyo, Japan).

**In Vivo Experiment**—Male FVB/N mice (25.0–30.0 g, 8 weeks old) were housed individually on a 12-h light/dark schedule with free access to food and water. N-acetyl-Leu-Leu-methionyl (ALLM) (20 mg/kg, Calbiochem) was injected 30 min prior to kainite (KA, TOCRIS Cookson Inc., Ballwin, MO) injection and injected once a day for five consecutive days. After intraperitoneal (ip) administration of KA (20 mg/kg), mice were continuously monitored for seizure activity for 2 h. For Western blotting analysis, the hippocampi were obtained from the KA-injected mice 1, 3, 6, 12, and 24 h after KA injection.

**Nissl staining** was performed as described previously (28). Briefly, the brains of the KA-injected mice were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 1 h, dehydrated in graded ethanol, and embedded in paraffin following a standard procedure. Thirty-micro meter sections were cut coronally with a microtome at the dorsal hippocampus and then stained with 1% Nissl.

**In Vitro Protoxidation of CaNα by Calpain**—Cleavage of calcineurin by calpain was analyzed by a modified procedure as described previously (29). Purified bovine calcineurin (4 μM, Calbiochem) was incubated with 1 μM recombinant m-calpain (Calbiochem) in a reaction buffer containing 40 mM Tris-HCl (pH 7.5), 20 mM CaCl₂, and 0.2 μM calmodulin at 30°C for 1 h. The reactions were stopped by addition of an SDS-PAGE sample buffer, and the reaction mixtures were then loaded on a 12% SDS-PAGE gel. The cleavage of calcineurin was analyzed by Coomassie Brilliant Blue staining of the gel.

**Mass Spectrometry**—Standard peptides, angiotensin III, and oxidized insulin B chain were obtained from Sigma. For MALDI-TOF/MS analysis, 2,5-dihydroxybenzoic acid (Wako, Osaka, Japan) was used as a matrix. The water used for all experiments was obtained from a MilliQ UV plus water purification system (Millipore, Bedford, MA). Sequencing-grade unmodified trypsin, Glu-C, and Asp-N were obtained from Roche Diagnostics, Lyo-C was from Wako, and n-octyl glucoside was obtained from Dojin (Kumamoto, Japan).

**In-gel Digestion**—Coomassie Brilliant Blue-stained bands were destained by soaking the gel pieces in a mixture of 50 mM ammonium bicarbonate and 50% acetonitrile. Then, the gel pieces were vigorously shaken with 400 μl of methanol/water/acetic acid = 50:40:10 (MWA) for 30 min as a washing step. After four replacements of MWA, the gel pieces were soaked with 400 μl of 100 mM ammonium bicarbonate solution for 5 min, then with 400 μl of acetonitrile for 5 min, and dried completely in a Speedvac evaporator (Thermo Electron, San Jose, CA). The dried gel pieces were re-swollen in 2 μl of 100 mM ammonium bicarbonate containing 0.02 μg of protease and 0.1% n-octyl glucoside. Then, 2 μl of 100 mM acetonitrile were further added and the mixture was incubated at 37°C overnight. After incubation, the digested peptides were extracted twice with 30 μl of acetonitrile/water/trifluoroacetic acid = 50:25:0.1 by sonication for 15 min. The combined extracts were dried in a Speedvac.

**MALDI-TOF/MS**—The dried peptides were re-dissolved in 5 μl of acetonitrile/water/trifluoroacetic acid = 95:5:0.1 and a 0.5-μl aliquot was used for MALDI-TOF/MS analysis. Matrix solution was prepared by saturating 2,5-dihydroxybenzoic acid in acetonitrile/water/trifluoroacetic acid = 33:67:0.1 and 4-fold-diluted before being mixed with the peptide solution. Preparation of co-crystals was done by applying 0.5 μl of the peptide solution and 0.5 μl of the diluted matrix solution together on the same spot on a MALDI plate and allowing the mixture to dry at room temperature. MALDI-TOF/MS spectra were obtained from a Voyager linear DE or oMALDI-QSTAR pulsar I instrument (Applied Biosystems) operated in delayed-extraction mode. The spectra were calibrated using internal standards, angiotensin III, and oxidized insulin B chain.

**Biochemical Analysis**—Values are reported as the mean ± S.E. Data were analyzed using either the Student’s t test to compare the two conditions or the Scheffe’s post hoc analysis following two-way ANOVA to compare the multiple conditions, and a p value less than 0.05 was considered significant.

**RESULTS**

**Calpain-mediated Cleavage of CaNα in Glutamate- and Kainate-treated Cultures of Primary Cortical Neurons**—To demonstrate whether calcineurin is involved in neuronal excitotoxicity, we investigated the expression changes of calcineurin in cultures of primary cortical neurons during glutamate and KA excitotoxicity. In glutamate-exposed neurons, interestingly, we detected two or three truncated products of CaNα by Western

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Glutamate exposure induced the cleavage of protein and a well-defined physiological substrate of calpain by Western blotting to detect CnA (upper panel). At 24 h, the neurons were analyzed by Western blotting analysis using anti-CnA antibodies. As a control, ACSF was added instead of glutamate. Time-dependent changes of the cleavage of non-erythroid α-spectrin after glutamate exposure. A band corresponding to 240 kDa represents wild-type α-spectrin. Calpain-dependent truncated α-spectrin was detected as 145-kDa and 150-kDa molecules. Effect of calpain inhibitors on KA-induced cleavage of CnA and calpain activity. Neurons were incubated with a 15-min application of 500 μM of KA in the presence or absence of ALLM (25 μM), 11R-CS (2.5 μM), or 11R-CS-R (2.5 μM) 30 min before KA. After 3 and 24 h, the neurons were analyzed by Western blotting to detect CnA (upper panel) and α-spectrin (lower panel). Cont., control.

Calpain-mediated cleavage of CnA was partially inhibited in glutamate-exposed neurons. Cortical neurons cultured for 10 days were incubated with 500 μM of glutamate for 15 min and then washed with PBS and returned to the original culture medium. Calpain inhibitor II (ALLM, 25 μM) was added 30 min before stimulation with glutamate. Neurons were collected at each indicated time, and equal quantitative protein was analyzed by Western blotting analysis using anti-CnA antibodies. As a control, ACSF was added instead of glutamate. Time-dependent changes of the cleavage of α-spectrin after glutamate exposure. A band corresponding to 240 kDa represents wild-type α-spectrin. Calpain-dependent truncated α-spectrin was detected as 145-kDa and 150-kDa molecules. Effect of calpain inhibitors on KA-induced cleavage of CnA and calpain activity. Neurons were incubated with a 15-min application of 500 μM of KA in the presence or absence of ALLM (25 μM), 11R-CS (2.5 μM), or 11R-CS-R (2.5 μM) 30 min before KA. After 3 and 24 h, the neurons were analyzed by Western blotting to detect CnA (upper panel) and α-spectrin (lower panel). Cont., control.

Figure 1. Calpain-mediated cleavage of CnA during glutamate and KA excitotoxicity in cortical neurons. A, cortical neurons cultured for 10 days were incubated with 500 μM of glutamate for 15 min and then washed with PBS and returned to the original culture medium. Calpain inhibitor II (ALLM, 25 μM) was added 30 min before stimulation with glutamate. Neurons were collected at each indicated time, and equal quantitative protein was analyzed by Western blotting analysis using anti-CnA antibodies. As a control, ACSF was added instead of glutamate. B, time-dependent changes of the cleavage of α-spectrin after glutamate exposure. A band corresponding to 240 kDa represents wild-type α-spectrin. Calpain-dependent truncated α-spectrin was detected as 145-kDa and 150-kDa molecules. C, effect of calpain inhibitors on KA-induced cleavage of CnA and calpain activity. Neurons were incubated with a 15-min application of 500 μM of KA in the presence or absence of ALLM (25 μM), 11R-CS (2.5 μM), or 11R-CS-R (2.5 μM) 30 min before KA. After 3 and 24 h, the neurons were analyzed by Western blotting to detect CnA (upper panel) and α-spectrin (lower panel). Cont., control.

Full-length cabin-1, which has previously been shown to play a role in calcineurin activation, was decreased by 15-min application of either glutamate (500 μM) or KA (500 μM) (Fig. 2A, a), and ALLM inhibited the reduction of the expression of full-length cabin-1 (Fig. 2A, b). We compared the quantitative cleavage of cabin-1 and CnA in response to glutamate/KA excitotoxicity. Quantitative assay of the relative expression revealed that both full-length cabin-1 and CnA decreased at the similar degree (full-length cabin-1, glutamate, 70.67% at 3 h, 65.57% at 24 h; KA, 56.16% at 3 h, 18.36% at 24 h (Fig. 2B); full-length CnA, glutamate, 72.42% at 3 h, 47.24% at 24 h; KA, 48.88% at 3 h, 27.31% at 24 h (Fig. 2C)). This result may suggest that calpain-mediated cleavage of CnA is physiologically as relevant to glutamate/KA-induced neuroexcitotoxicity as calpain-mediated cleavage of cabin-1.

Present results showed that glutamate exposure induced the cleavage of CnA but the cleavage was partial (Fig. 1). To investigate whether the partial cleavage of CnA influence the calcineurin activity in neurons, calcineurin activity in cultured neurons during glutamate excitotoxicity was examined in vitro. There was no change in calcineurin activity between control neurons and glutamate-exposed neurons in the presence of Ca²⁺ (Fig. 2D, control, 0.34 ± 0.03; glutamate (Glu.), 0.39 ±
Cortical neurons cultured for 10 days were incubated with 500 μM of glutamate for 15 min and then washed with PBS and returned to the original culture medium. Neurons were collected at each indicated time, and equal quantitative protein (50 μg) was analyzed by Western blotting analysis using anti-cabin-1 antibodies. As a control (Contr.), ACSF was added instead of glutamate. b, the effect of ALLM on calpain-mediated degradation of cabin-1 shown in A, α. For evaluating the quantitative expression, the scanned digital images of full-length cabin-1 were quantified using NIH image software. The ratio of the expressions was normalized to the control to derive the percentage of control values. C, quantitative analysis of the expression changes of full-length CnA shown in Fig. 1, A and C. D, in vitro calcineurin activity in glutamate-treated culture neurons. Cortical neurons cultured for 10 days were incubated with 500 μM of glutamate for 15 min and then washed with PBS and returned to the original culture medium. Neurons were collected 12 h after glutamate treatment and the cell lysates were assayed for calcineurin activity as described under “Experimental Procedures.” As a control, ACSF was added instead of glutamate.

Interestingly, calcineurin activity in glutamate-treated neurons was significantly higher than that of control neurons in the absence of Ca^{2+} (Fig. 2D, control, 0.03 ± 0.003; glutamate (Glu.), 0.21 ± 0.007, n = 4 each, p < 0.05). These results may suggest that the partial cleavage of CnA after glutamate treatment induced Ca^{2+}-independent calcineurin activity but not the Ca^{2+}-dependent activity.

Calpain-mediated Truncations of CnA in Vitro and Determination of the Cleavage Sites—To examine whether calpain cleaved CnA, we digested purified calcineurin with recombinant m-calpain under various conditions in vitro (Fig. 3). Incubation of calcineurin with recombinant m-calpain for 1 h at 30 °C led to the complete digestion of full-length CnA as determined by Coomassie Brilliant Blue staining (Fig. 3A) and Western blotting analysis (Fig. 3B). The cleaved sites depended on the presence of calmodulin. One truncation product of CnA with an apparent molecular mass of ~45 kDa was generated in the absence of calmodulin (black arrow in Fig. 3, A and B), whereas one more truncation product of CnA with a molecular mass of ~48 kDa appeared in the presence of calmodulin (black arrowhead in Fig. 3, A and B).

To clarify whether calpain-dependent cleavage sites of CnA were calmodulin-dependent or whether calmodulin only delayed the cleavage of CnA, we investigated the time-dependent cleavage of CnA by m-calpain in the presence of calmodulin (Fig. 3C). CnA was immediately cleaved by calpain within 30 s (Fig. 3C). Interestingly, we detected three cleavage products of CnA, the molecular masses of which were 57 kDa (gray arrow), 48 kDa (black arrowhead), and 45 kDa (black arrow), when the phosphatase was incubated with calpain for a short time (30 s–12 min). The 57-kDa product disappeared 24 min after incubation with calpain, and the two other truncations remained until 3 h after incubation, indicating that both CnA products were stable in the presence of calmodulin.

We further examined whether the calpain-mediated cleavage of CnA responded to the concentration of calpain. As shown in Fig. 3D, m-calpain at the concentration of 1 μM completely cleaved CnA into two products with molecular masses of 48 kDa (black arrowhead) and 45 kDa (black arrow). When the concentration was reduced to 0.1 μM, the expressions of both the 45 and 48 kDa products were weak, and a cleavage product appeared to be close to full-length CnA (gray arrow in Fig. 3D). When the concentration of m-calpain was below 0.01 μM, however, no truncation product of CnA appeared.

To determine the precise cleavage sites of CnA by calpain, we next performed MS analysis. The three fragments of cleaved CnA by calpain in vitro shown in Fig. 3 were digested with trypsin in a gel, and the tryptic peptides were loaded onto a MALDI-TOF. The results of the peptide mass fingerprinting (PMF) are shown in Fig. 4. When we compared PMF of the
truncated 48-kDa CnA (middle spectrum of Fig. 4A) with that of full-length CnA (upper spectrum of Fig. 4A), four additional peaks (1–3 and 5) were found on the middle spectrum. They were non-tryptic peptides and corresponded to 409–421, 409–422, 409–423, and 409–425 (VFSVLREESYSVTLKG) residues, respectively. Then 426–521 residues were not detected on the middle spectrum (48-kDa CnA) compared with the full-length CnA (upper spectrum). These observations showed that the cleavage sites on CnA by calpain were heterogeneous, and in the range from 421 to 425 residues. However, the truncated 45-kDa CnA lost this region (lower spectrum of Fig. 4A, peak 4, 409–424 disappeared), and 361–392 residues (mass 3427.8 = oxidized Met form) still remained. On the other hand, the truncated 57-kDa CnA showed 467–501 residues and the 502–521 regions were not detected on PMF (data not shown). Moreover, to confirm the exact cleavage sites of CnA by calpain, we digested the full-length 60 kDa and truncated 45 kDa CnAs with four different proteases and compared the PMFs. The results using each different protease are shown in Fig. 4B. The full-length 60 kDa of CnA covered up to a total of 96.5% of the residues by MALDI-TOF analysis in combination with the different proteases. On the other hand, the C-terminal residues (395–521 residues) of the truncated 45-kDa CnA were not found in the digested peptides by any of the proteases. These findings suggest that calpain cleaves off the C-terminal region of CnA and produces a 1–393 or 1–392 residue truncated CnA.

When the results of Fig. 4, A and B, are taken into consideration together, calpain cleaves off three sites in the C-terminal region of CnA, and the remaining parts are 1–392 residues, 1–424 residues, and 1–501 residues, which correspond to 45, 48, and kDa truncated CnA α isoform, respectively (Fig. 5A). A summary of the results of mass spectrometric analysis is shown in Fig. 5E. CnA has highly conserved functional domains (21). The catalytic domain (residues 70–328) localized in the N-terminal is followed by the regulatory domain, which includes CnB-binding (residues 348–368), calmodulin-binding (residues 391–414), and autoinhibitory domains (residues 468–490) (Fig. 5A). The present results showed that the calpain-dependent truncated 45-kDa CnA included neither the calmodulin-binding domain nor the autoinhibitory domain, and the 48-kDa truncation was cleaved on the C-terminal of the calmodulin-binding domain (Fig. 5, A and B). Moreover, 57-kDa truncated CnA was cleaved on the C-terminal of the autoinhibitory domain, indicating the inclusion of CnB-binding, calmodulin-binding and autoinhibitory domains (Fig. 5, A and B). These calpain-dependent cleavage sites are also conserved in CnA β isoform, suggesting that calpain may cleave both isoforms of CnA.

Calpain-mediated Activation of Calcineurin Phosphatase Activity—Previous studies have shown (21, 35) that removal of residues 390–521 or 420–521 from CnA results in the full Ca"^{2+}/calmodulin-independent activity in vitro. To investigate whether the calpain-dependent truncations of CnA have Ca"^{2+}-independent full activity in cells, we transfected each truncated construct of CnA α isoform cDNA encoding 57-, 48-, and 45-kDa CnAs in 293 cells, and the luciferase reporter activity representing calcineurin-mediated-NFAT gene transduction was measured. In the full-length and 57-kDa truncated CnA-overexpressing cells, the calcineurin activities were extremely low in the absence of calcium ionophore (Fig. 6A, a). In contrast, the relative luciferase activities in 48- and 45-kDa CnA-overexpressing cells were significantly higher than that of mock-infected cells (48.2 ± 4.8- and 44 ± 6.6-fold compared with mock; p < 0.0001 versus mock) in the absence of calcium ionophore (Fig. 6A, a). An application of calcium ionophore markedly induced calcineurin activity in full-length and 57-kDa CnA-overexpressing cells compared with that in mock-infected cells (full-length, 37.1 ± 4.5-fold; 57 kDa, 39.5 ± 3.9-fold compared with mock in the absence of calcium ionophore). The calcineurin activities in the 48- and 45-kDa CnA-overexpressing cells were significantly higher than that of the full-length CnA-overexpressing cells in the presence of calcium ionophore (Fig. 6A, a; 48 kDa, 53.3 ± 7.2%; 45 kDa, 55.1 ± 6.6%; p < 0.01 versus full-length). Moreover, FK506, a potent calcineurin inhibitor, significantly inhibited calcineurin activities in full-length and 48-kDa CnA-overexpressing cells. These results suggest that calpain-dependent 48- and 45-kDa trun-
cated CnAs have full enzyme activity and are constitutive active forms requiring only a physiological level of calcium for their activity.

**Induction of Neuronal Cell Death and the Activation of Caspase-3 by Overexpression of 48-kDa CnA in Primary Hippocampal Neurons**—Previous studies (10, 36–38) have suggested that high levels of calcineurin activity activate the caspase cascade and predispose thymocytes, T cells, Jurkat cells, and neuronal cells to apoptosis induced by various stimulation which increase the cytosolic Ca\(^{2+}\) concentration. Next, we investigated whether calpain-dependent truncations of CnA activate the caspase activity and induce neuronal apoptosis in primary cultured neurons. We infected recombinant adenoviruses encoding 48-kDa CnA (pAdex-CnA-T) and CnB (pAdex-CnB) at a multiplicity of infection (MOI) of 100 in primary cultured hippocampal neurons and examined the expression of active caspase-3 and TUNEL-positive cells. As a control, recombinant adenoviruses encoding lacZ (pAdex-lacZ) were infected at a MOI of 100. In both pAdex-lacZ- and pAdex-CnB-overexpressing neurons, the expression of active caspase-3 (red) was low and few TUNEL-positive cells (green) were detected (Fig. 6, B–D). In pAdex-CnA-T- and pAdex-CnB-overexpressing neurons, on the other hand, the number of active caspase-3-positive neurons significantly increased compared with that of pAdex-lacZ-infected neurons (Fig. 6, B and C; pAdex-lacZ + pAdex-CnB, 3 ± 0.4; pAdex-CnA-T + pAdex-CnB, 15.1 ± 2.5; *p* < 0.005), and the number of TUNEL-positive neurons also significantly increased (Fig. 6, B and D; pAdex-lacZ + pAdex-CnB, 3.4 ± 0.5; pAdex-CnA-T + pAdex-CnB, 17.1 ± 2.1; *p* < 0.005). In contrast, overexpression of full-length CnA affected neither the induction of caspase activity nor the induction of apoptosis without glutamate exposure in neurons (data not shown). FK506 significantly inhibited the expression of both active caspase-3 and TUNEL-positive neurons in pAdex-CnA-T- and pAdex-CnB-infected neurons (Fig. 6, B–D; FK506 + pAdex-CnA-T + pAdex-CnB, 6.1 ± 1.8; *p* = 0.03 versus pAdex-CnA-T + pAdex-CnB). These results suggest that calpain-mediated truncated CnA fully activates the caspase cascade and causes neuronal cell death in primary cultured neurons.

**Protective Effects of Calpain Inhibitors and Calcineurin Inhibitor on Glutamate- and KA-induced Excitotoxic Neuronal Cell Death in Hippocampal Neurons**—In the present study, we showed that ALLM and 11R-CS blocked the glutamate- and KA-induced truncation of CnA in cultured neurons (Fig. 1). We next investigated whether the calpain inhibitors blocked gluta
tamate-induced neuronal apoptosis in cultured neurons. Fig. 7A, a, reveals the time-dependent changes of glutamate-in
duced neuronal apoptosis and the effect of the calpain inhibitors and calcineurin inhibitor on apoptosis. We observed severe neuronal apoptosis 6 h after an exposure of 500 μM glutamate for 15 min (Fig. 7A, a; glutamate, 43.5 ± 2.1%). The number of apoptotic cells increased in a time-dependent manner and was prominent 12 h and 24 h after glutamate exposure (Fig. 7, A, a, and B, b and g; glutamate; 12 h, 77.7 ± 3.8%; 24 h, 87.1 ± 4%). Both calpain inhibitors and calcineurin inhibitor significantly inhibited the glutamate-induced apoptosis after 12 and 24 h (Fig. 7, A, a, and B; ALLM: 12 h, 44.9 ± 3.8%; 24 h, 50.7 ± 3.9%; 11R-CS + Glu.: 12 h, 42 ± 3.9%; 24 h, 47.5 ± 3.6%; FK506 + Glu.: 12 h, 44 ± 5.7%; 24 h, 52.34 ± 7.1; *, p < 0.01). There are no differences between the effect of calpain inhibitor and calcineurin inhibitor. Glutamate dose-dependently evoked neuronal apoptosis starting from a concentration of 50 μM and peaking at 1000 μM and both calpain inhibitors and calcineurin inhibitor inhibited the apoptosis induced by various concentrations (50–1000 μM) of glutamate even when glutamate was applied at the highest concentration of 1000 μM (Fig. 7A, b). We also examined the effect of the calpain inhibitors on glutamate-induced TUNEL-positive neurons, which reflected degenerative neurons. Both inhibitors significantly inhibited the glutamate-induced neuronal degeneration (Fig. 7C; glutamate, 78.3 ± 4.6%; ALLM + Glu., 32.4 ± 3.9%; 11R-CS + Glu., 30.5 ± 3.9%; FK506 + Glu., 39.6 ± 8.2; *, p < 0.005, **, p < 0.001).

Correspondingly, KA treatment (500 μM) markedly induced neuronal cell death. Both calpain inhibitors and calcineurin inhibitor significantly decreased the numbers of both TUNEL-positive neurons and apoptotic neurons after KA treatment (data not shown). Taken together, these results suggest that calpain plays an important role in glutamate- and KA-induced neuroexcitotoxicity, and both its inhibitors and FK506 offer potential neuroprotection in primary cultured neurons.

Calpain-mediated Cleavage of CnA in the Hippocampus of KA-treated FVB/N Mice and the Protective Effect of ALLM on the Cleavage of CnA and Neuronal Degeneration in Vivo—We next investigated whether calpain-dependent truncation of CnA could be observed during excitotoxic neurodegeneration in vivo. Systemic administration of KA has been considered an important model of excitotoxic neurodegeneration (28, 39–41). In particular, severe neuronal loss is observed in the hippocam-

![Fig. 5. Cleavage amino acid sites of rat CnA by calpain (A) and schematic diagrams of full-length CnA and calpain-dependent truncated forms of CnA identified by MS representing the amino acid sequence (B). Arrows in A represent the cleavage sites identified by MS in the present study. Pink, CnB binding domain; yellow, calmodulin binding domain; red, autoinhibitory domain.](http://www.jbc.org/)

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Fig. 6. Calcineurin activity of each truncated CnA in 293 cells (A) and the effect of overexpression of the truncated CnAs on the caspase activity and TUNEL staining in hippocampal neurons (B–D). A, a, reporter plasmid (1.0 μg pNFAT-AT-Lu), 1.0 μg of pcDNA-wild-type CnB, and 1.0 μg of each pcDNA CnA; full-length (Full), 57-kDa truncation (57 kDa), 48-kDa truncation (48 kDa), and 45-kDa (45 kDa) were transfected into HEK 293 cells. As a control, 1 μg of pcDNA vector was transfected instead of the transfection of each CnA cDNA (Mock). After 24 h, these cells were treated with 2 μM A23187 (Ca²⁺ ionophore (+)) or new medium (Ca²⁺ ionophore (−)) was added as a control. The cell extracts were assayed for luciferase activity after standardization. Data are mean ± S.E. (n = 5 each). *, p < 0.001; **, p < 0.01 (Scheffe’s post hoc analysis). A, b, the expression of CnA in 293 cells transfected with Full and each truncated CnA cDNA. Cell lysates in each CnA transfected cells were used for Western blotting analysis using CnA antibody. In the full and 57-kDa overexpressing cells, additional bands were observed. The additional bands were cleaved fragments cleaved between CnA and myc-His tag because both anti-myc and anti-His antibodies did not recognize the additional bands (data not shown), and the molecular mass of small fragments was less than 3 kDa. B, double staining for active-caspase-3 (red) and TUNEL (green) in each of the adenovirus-infected primary cultured hippocampal neurons. The neurons were fixed with 4% paraformaldehyde and stained 24 h after the virus infection. FK506 (1 μM) was added with virus infection at the same time. Scale bar, 50 μm. C and D, quantitative analysis of the number of active-caspase-3- (C) and TUNEL- (D) positive staining neurons among each of the virus-infected and FK506-treated cells. Representative graphs are shown for experiments where at least 8 randomly chosen fields were scored. *, p < 0.005; **, p < 0.01 (Scheffe’s post hoc analysis).
Systemic administration of KA (20 mg/kg) induced neuronal degeneration in both CA1 and CA3 regions of FVB/N mouse hippocampus 5 days after KA injection (Fig. 8A). We used this model to investigate whether calpain cleaved CnA during neuronal degeneration in vivo and whether ALLM inhibited KA-induced neuronal degeneration and CnA truncation in vivo. ALLM injection inhibited KA-induced neuronal degeneration in the mouse hippocampus (Fig. 8A).

The cleavage of CnA clearly appeared 1 h after KA injection in the mouse hippocampus, but was not detected in the hippocampus of control mice (Fig. 8B). To explore calpain activity in the mouse hippocampus after KA administration, we examined the cleavage of non-erythroid α-spectrin. KA injection induced the cleavage of α-spectrin into the characteristic 145-kDa and 150-kDa fragments 1 h after the injection, confirming that calpain was activated in the hippocampus after KA injection. Furthermore, both truncations of CnA and α-spectrin were completely blocked by pretreatment with ALLM, indicating that the generation of truncated CnA is mediated by calpain in the hippocampus of KA-treated mice.
We further examined the time-dependent changes of CnA cleavage in the mouse hippocampus after KA injection in vivo. One cleavage product of CnA was observed 1, 3, and 6 h after KA injection (red arrowhead, Fig. 8C). Two more cleavage products of CnA with apparent molecular weights of 48 and 45 kDa were observed 12 and 24 h after KA injection, respectively (arrowheads, Fig. 8C). To determine the precise molecular weights of these truncated CnA in vivo, we compared the molecular weights of calpain-dependent truncations of CnA in vitro, in cultured neurons and in vivo. The molecular masses of the three cleaved products of CnA which appeared in the hippocampus after KA injection in vivo were consistent with those of the calpain-dependent truncations in primary cultured neurons (Fig. 8D). Pretreatment with ALLM also inhibited the cleavage of CnA observed at 12 and 24 h after KA injection in vivo (data not shown). These results suggest that calpain is activated after KA injection and the active enzyme cleaves CnA in vivo.

**DISCUSSION**

The results in the present study provide five important findings as follows. 1) Calpain directly cleaved CnA to three truncations during glutamate and KA neuroexcitotoxicity in vitro and in vivo, and mass-spectrometry identified these cleavage sites. 2) There was a close relationship in the time-dependent changes between calpain activation and truncation of CnA in neurons. 3) The 45-kDa and 48-kDa truncations among the three calpain-dependent cleaved CnAs were constitutively active forms. 4) Overexpression of the 45-kDa or 48-kDa CnA truncations induced neuronal cell death in hippocampal neurons without glutamate exposure. 5) Calpain inhibitors blocked not only CnA cleavage but also glutamate- and KA-induced excitotoxic neuronal cell death in vitro and in vivo.

**A Crucial Intrinsic Cascade of Neuronal Cell Death: Cross-talk between the Calpain-mediated Signal Pathway and Calcineurin-mediated Signal Pathway upon Excitotoxic Neuronal Cell Death**—Identifying the key mediators of neuronal cell death and elucidating the signal cascades involved are of great importance. Calcineurin and calpain are two such intracellular biochemical molecules. It has been reported (10, 36, 37, 42) that excessive Ca\(^{2+}\) entry into the cytosol activates calcineurin activity and the activated calcineurin induces apoptosis by transcription dependent and transcription-independent pathways.
shown that calpain is involved in these calcineurin-mediated cell signals.

In addition to its reversible activation by Ca\(^{2+}\)/calmodulin, previous in vitro studies have shown that calcineurin is able to be irreversibly activated by limited proteolysis with calpain, trypsin, and chymotrypsin C (23, 29, 46–48). The limited proteolysis of calcineurin changes the enzyme to the constitutively active form, which no longer requires Ca\(^{2+}\) and calmodulin for full activity, and this activation of calcineurin is irreversible (47). These in vitro proteolysis studies suggest that calpain-dependent calcineurin activation may remain in cells even though intracellular Ca\(^{2+}\) decreases after a transient increase. However, it has been unclear whether this irreversible activation of calcineurin actually occurs in cells. In the present study, we provide evidence for the first time that during glutamate and KA neuroexcitotoxicity, CnA is proteolytically activated by calpain and this activation is irreversible because of truncation of the autoinhibitory domain and calmodulin-binding domain (Fig. 5B). The activity of calpain-dependent cleaved CnA was significantly higher than that of wild-type CnA. In accordance with this, a previous in vitro study (40) has shown that the activity of the fully proteolyzed calcineurin is 1.4–1.6-fold higher than the activity of the native enzyme in the presence of Ca\(^{2+}\)/calmodulin. The previous and present studies suggest that calpain-dependent regulation of calcineurin activity may occur only under pathological conditions such as neuroexcitotoxicity, whereas under physiological conditions, the phosphatase activity is regulated by Ca\(^{2+}\)/calmodulin in a reversible manner (Fig. 9B).

Bidirectional Ca\(^{2+}\)/Calmodulin-independent Regulation of Calcineurin Activity under Pathological Conditions—A recent study (18) has shown that calpain-dependent cleavage of cain/cabin1 activates calcineurin activity through inhibition of the interaction with CnA. Because cain/cabin1 binds to the CnB-binding domain of CnA, the protein may also inhibit the activity of calpain-dependent truncated CnA such as 48-kDa and 45-kDa CnAs. Moreover, even though calpain cleaves cain/cabin1, full-length calcineurin still requires binding with Ca\(^{2+}\)/calmodulin for the full activity. However, if calpain cleaves both CnA and cain/cabin1, calcineurin may have Ca\(^{2+}\)/calmodulin-independent full activity. In the present study, cain/cabin1 was also cleaved in glutamate/KA treated neurons and ALLM inhibited the cleavage. These results suggest that calpain may bidirectionally activate calcineurin activity through CnA cleavage itself and cain/cabin1 cleavage during neuronal degeneration, resulting in severe neuronal cell death.

In this study, we established a molecular link between calpain and calcineurin, thereby demonstrating a new mechanism for proteolytical regulation of calcineurin by calpain in response to certain pathological states (Fig. 9B). Thus, it is possible that calpain can control cell death through proteolysis of CnA and activation of the calcineurin-mediated downstream cell death cascade (Fig. 9A). The identification of CnA as a cell death substrate of calpain will provide not only insights into the molecular basis of how Ca\(^{2+}\)-mediated molecules execute death commitment after neuroexcitotoxicity, but also potential approaches for therapeutic intervention targeted at inhibiting calpain function in Ca\(^{2+}\)-mediated neurodegenerative disorders.

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