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Activation of Calpain I Converts Excitotoxic Neuron Death into a Caspase-independent Cell Death*

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Glutamate receptor overactivation contributes to neuron death after stroke, trauma, and epileptic seizures. Exposure of cultured rat hippocampal neurons to the selective glutamate receptor agonist *N*-methyl-D-aspartate (300 μM, 5 min) or to the apoptosis-inducing protein kinase inhibitor staurosporine (300 nM) induced a delayed neuron death. In both cases, neuron death was preceded by the mitochondrial release of the pro-apoptotic factor cytochrome *c*. Unlike staurosporine, the *N*-methyl-D-aspartate-induced release of cytochrome *c* did not lead to significant activation of caspase-3, the main caspase involved in the execution of neuronal apoptosis. In contrast, activation of the Ca^{2+}-activated neutral protease calpain I was readily detectable after the exposure to *N*-methyl-D-aspartate. In a neuronal cell-free apoptosis system, calpain I prevented the ability of cytochrome *c* to activate the caspase cascade by inhibiting the processing of procaspase-3 and -9 into their active subunits. In the hippocampal neuron cultures, the inhibition of calpain activity restored caspase-3-like protease activity after an exposure to *N*-methyl-D-aspartate. Our data demonstrate the existence of signal transduction pathways that prevent the entry of cells into a caspase-dependent cell death program after the mitochondrial release of cytochrome *c*.

Cell death caused by pathophysiological overactivation of glutamate receptors (excitotoxicity) has been implicated in neurodegeneration after stroke, cerebral trauma, and epileptic seizures (1). Activation of Ca^{2+}-permeable *N*-methyl-D-aspartate (NMDA)[1] or α-amino-3-hydroxy-5-isoxazole-4-propionic acid receptors and the subsequent neuronal Ca^{2+} overloading have been shown to mediate glutamate-induced neuron death (1). Mitochondria take up large amounts of Ca^{2+} during glutamate receptor overactivation (2). Recent studies provided evidence that this mitochondrial Ca^{2+} uptake is required to trigger excitotoxic neuron death (3–5). Excessive mitochondrial Ca^{2+} uptake has been shown to deenergize mitochondria and to trigger the mitochondrial production of reactive oxygen species (4, 6–9). After an intense or prolonged period of glutamate receptor overactivation, the disturbance of mitochondrial energetics is irreversible and induces excitotoxic necrosis (8).

Mitochondrial Ca^{2+} overloading, however, has also been shown to cause the release of the pro-apoptotic factor cytochrome *c* (10). Cytochrome *c* is able to activate a family of cysteine proteases, the caspases, by binding to the cytosolic protein apoptotic protease-activating factor-1 (10, 12). The activation of the caspase cascade starts with caspase-9, the upstream caspase in the mitochondrial apoptosis pathway (13). This activates caspase-3, which is the major executioner caspase in neurons (14, 15) and is also responsible for the activation of caspase-2, -6, -7, and -8 (16). The caspase cascade is essential for many biochemical and morphological changes occurring during apoptosis. However, sufficient ATP levels are required for the activation of the caspase cascade (17). It has therefore been proposed that the ability of mitochondria to recover their energetics after a toxic glutamate receptor overactivation decides whether the neuron is going to die by apoptosis or necrosis (8).

On the other hand, a variety of processes are activated during glutamate receptor overactivation that could also influence the mode of cell death chosen. An increased nitric oxide production has been shown to contribute to excitotoxic neuron death (18). Nitric oxide induces oxidative stress (18) but may also inhibit apoptotic processes by nitrosylation and inactivation of caspases (19, 20). The activation of the Ca^{2+}-activated neutral cysteine protease calpain I also occurs early in excitotoxic neuron death (21, 22). Like the caspases, calpain I cleaves a variety of cytoskeletal proteins, enzymes, and transcription factors and could interfere with the proteolytic activity of the caspases (23).

The present study was undertaken to investigate the role of the mitochondrial apoptosis pathway in excitotoxic neuron death. We show that calpains but not caspases are the predominantly activated proteases during excitotoxic neuron death. Furthermore, we demonstrate that calpains inhibit the ability of NMDA to initiate an apoptotic cell death program after the mitochondrial release of cytochrome *c*.

EXPERIMENTAL PROCEDURES

Materials—NMDA was purchased from Sigma. Caspase substrates and inhibitors were from Bachem (Heidelberg, Germany), and staurosporine (STS) was from Alexis (Grünstetten, Germany). Dizocilpine and tetrodotoxin were purchased from Biotrend (Cologne, Germany), and

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; CI-1, calpain inhibitor I; Me_2SO, dimethyl sulfoxide; HBS, Hepes-buffered saline; Z-IETD-AFC, benzyloxylcarbonyl-Ile-Glu-Thr-Asp-7-amido-4-(trifluoromethyl)coumarin; R-123, rhodamine-123; STS, staurosporine; Z-VDVAD-AFC, benzyloxylcarbonyl-Val-Asp-Val-Ala-Asp-7-amido-4-(trifluoromethyl)coumarin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PIPES, 1,4-piperazinediethanesulfonic acid.

calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal) was from Calbiochem. Leupeptin and the other protease inhibitors were purchased from Roche Molecular Biochemicals. All other chemicals came in analytical grade purity from Merck.

Cell Culture—Cultured hippocampal neurons were prepared from neonatal (P1) Fischer 344 rats as described (24). Dissociated hippocampal neurons were plated at a density of 2×10^5 cells/cm^2 onto poly-L-lysine-coated 24-well plates (Nunc, Wiesbaden, Germany). For immunofluorescence analysis and confocal laser scanning experiments, neurons were plated onto poly-L-lysine-coated glass coverslips that were mounted onto 35-mm Petri dishes (Willco Wells B.V., Amsterdam, The Netherlands) or 8-chamber culture slides (Becton Dickinson, Meylan Cedex, France). Cells were maintained in minimum Eagle's medium supplemented with 10% NU-serum, 2% B-27 supplement (50× concentrate), 2 mM L-glutamine, 20 mM D-glucose, 26.2 mM sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Experiments were performed on 14–16-day-old cultures. Animal care followed official governmental guide lines. Human SH-SY5Y neuroblastoma cells were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.).

Induction of Excitotoxic and Apoptotic Neuronal Injury and Quantification of Cell Death and Apoptotic Nuclei—For the induction of excitotoxic neuronal injury, cultures were washed in Hepes-buffered saline (HBS) (144 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM D-glucose; 320 mosM; pH 7.4) and were then exposed for 5 min to Mg^{2+}-free HBS supplemented with 300 μM NMDA, 0.5 μM tetrodotoxin, and 100 nM glycine (25). Control cultures were exposed to Mg^{2+}-free HBS alone. After the exposure, cells were washed and returned to the original culture medium. Apoptosis was induced by the addition of the protein kinase inhibitor STS (300 nM) into the culture medium (24). The respective controls received the vehicle dimethyl sulfoxide (Me_2SO). Cell death was determined at the indicated time points using a trypan blue exclusion assay (0.5% in HBS, 5 min). Uptake of trypan blue identifies membrane leakage, the end point of neuronal degeneration that also occurs after neuronal apoptosis *in vitro* (secondary necrosis). A total number of 400–500 neurons were counted in several randomized subfields of each culture. Cell counts were performed by two investigators without knowledge of the respective treatments, and the counts were meaned for further statistical analysis. Chromatin fragmentation was visualized with the DNA-binding fluorescent dye, Hoechst 33258. Cells were fixed with paraformaldehyde, permeabilized, and exposed to 1 μg/ml Hoechst 33258 (Sigma) in phosphate-buffered saline for 15 min. Nuclei were observed with a 40× oil immersion objective using an Eclipse TE300 inverted microscope equipped with an epifluorescence unit and the appropriate filter sets (Nikon, Düsseldorf, Germany). Since chromatin condensation is a reversible process, only fragmented nuclei were considered apoptotic. The percentage of apoptotic nuclei was quantified as described above.

Rhodamine-123-based Estimation of Mitochondrial Membrane Potential—Rhodamine-123 (R-123) is a cationic, lipophilic probe that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential (26). An R-123 stock was prepared at a concentration of 1 mg/ml in Me_2SO and stored at −20 °C. Working stocks of 30 μM were made up fresh in distilled water. For the estimation of mitochondrial membrane potential after the NMDA exposure, cells were exposed to NMDA or Mg^{2+}-free HBS (sham-exposed controls) for 5 min and returned to the original culture medium. After 2 h, cells were loaded with 30 nM R-123 for 15 min in culture medium, and the fluorescence was acquired with the dye present in the extracellular solution for the entire course of data collection. R-123 fluorescence was quantified using an inverted Olympus IX70 microscope attached to a confocal laser scanning unit equipped with a 488-nm argon laser and a 20× fluorescence objective (Fluoview; Olympus, Hamburg, Germany). The regions of interest were monitored and focused by eye and then scanned once. Data were analyzed using Metmorph software (Universal Imaging, West Chester, PA). Fluorescence data are given as the ratio between the average pixel intensity of the mitochondria-rich regions and the nucleus to compensate for background differences and unequal R-123 loading (27).

Assessment of Caspase Activity—After the respective treatments, the culture medium was aspirated, cells washed three times with HBS, and lysed in 200 μl of lysis buffer (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5% CHAPS. Fifty μl of this extract was added to 150 μl of reaction buffer (25 mM Hepes, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM dithiothreitol, pH 7.5). The reaction buffer was supplemented with 10 μM acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC), a fluorogenic substrate preferentially cleaved by caspase-3, -7, and -8 but also caspase-1, -6, -9, and -10 (28). Fluorogenic substrates with higher specificity for caspase-2 (benzyloxylcarbonyl-Val-Asp-Val-Ala-Asp-7-amido-4-(trifluoromethyl)coumarin, Z-VDVAD-AFC) or caspase-6 (benzyloxylcarbonyl-Ile-Glu-Thr-Asp-7-amido-4-(trifluoromethyl)coumarin, Z-IETD-AFC) were also used. Production of fluorescent AMC or AFC was monitored over 60 min using a fluorescent plate reader (HTS 7000, Perkin-Elmer) (excitation 380 nm and emission 460 nm). Fluorescence of blanks containing no cellular extracts were subtracted from the values. Protein content was determined using the Pierce Coomassie Plus Protein Assay reagent (KMF, Cologne, Germany), and the caspase activity is expressed as change in fluorescent units per h per μg of protein.

SDS-PAGE and Western Blotting—Cells were rinsed with ice-cold phosphate-buffered saline and lysed in Tris-buffered saline containing sodium dodecyl sulfate, glycerin, and the above protease inhibitors. Protein content was determined using the Pierce BCA Micro Protein Assay kit, and samples were supplemented with 2-mercaptoethanol and denatured at 95 °C for 5 min. An equal amount of protein (10–20 μg) was separated with 5–15% SDS-PAGE and blotted to nitrocellulose membranes (Protean BA 85; Schleicher & Schuell). Nonspecific binding was blocked by incubation in Tris-buffered saline containing bovine serum albumin, non-fat dry milk, and 0.05% Tween 20 for 1 h at room temperature. The blots were then incubated overnight at 4 °C in blocking buffer containing the primary antibody. Antibodies used were a rabbit polyclonal anti-caspase-3 antibody (H-277; Santa Cruz Biotechnology, Heidelberg, Germany) raised against full-length human caspase-3 diluted 1:1,000, a rabbit polyclonal anti-active caspase-3 antibody (MF397) raised against p17/p12 x-ray crystallographic grade recombinant caspase-3 diluted 1:1,000 (29), a rabbit polyclonal anti-caspase-9 antibody (68086E; PharMingen, Becton Dickinson, Hamburg, Germany) raised against recombinant, full-length human caspase-9 diluted 1:1,000, a mouse monoclonal anti-nonerythroid α-spectrin antibody (MAB1622; Chemicon International, Hofheim, Germany) diluted 1:5,000, a rabbit polyclonal antibody specific for the calpain-proteolyzed 150-kDa α-spectrin fragment diluted 1:400 (30), a mouse monoclonal anti-β-actin antibody (clone AC-15; Sigma) diluted 1:200, or a mouse monoclonal anti-α-tubulin antibody (clone DM 1A; Sigma) diluted 1:250. Afterward, membranes were washed and incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate (1:5,000–1:20,000; Promega, Mannheim, Germany). Antibody-conjugated peroxidase activity was visualized using the SuperSignal chemiluminescence reagent (Pierce).

Immunofluorescence Microscopy—After exposure to NMDA or STS, hippocampal cultures were washed, fixed with 4% paraformaldehyde, and permeabilized. Unspecific binding was blocked, and neuron cultures were incubated with primary antibodies for 2 h at room temperature in blocking buffer. The following primary antibodies were used: a mouse monoclonal anti-cytochrome *c* antibody (6H2.B4; PharMingen, Becton Dickinson) at a concentration of 10 μg/ml, a rabbit polyclonal antibody specific for postautolytic calpain I (large subunit) diluted 1:200 (31), the rabbit polyclonal antibody specific for the calpain-proteolyzed 150-kDa α-spectrin fragment diluted 1:200, and a rabbit polyclonal antibody specific for caspase-cleaved actin (fractin) diluted 1:200 (32). After washing, biotin-conjugated anti-mouse or anti-rabbit IgG (1:1,000, Vector Laboratories, Burlingame, CA) was added for 1 h, followed by a streptavidin-Oregon Green or -Texas Red conjugate (1 μg/ml, 20 min; Molecular Probes, Leiden, The Netherlands). As a negative control, cultures were incubated with the secondary antibody only. Fluorescence was observed using the Eclipse TE300 inverted microscope. Digital images of equal exposure were acquired with a digital camera (SPOT-2; Diagnostic Instruments, Sterling Heights, MI) using Metamorph software.

Neuronal Cell-free Apoptosis System—Cytoplasmic extracts were prepared from SH-SY5Y neuroblastoma cells as described (33). Protein content was determined using the Coomassie Plus Protein Assay reagent. For activation of apoptosis, 50 μg of protein extract was diluted in 25 μl of reaction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 1 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0, 1 mM phenylmethylsulfonyl fluoride), and cytochrome *c* (500 nM; purified from horse heart, Sigma), dATP (1 mM; Sigma), calpain I (0.8 milliunits/μl; purified from porcine erythrocytes; Calbiochem), calpain inhibitor I (10 μM) or leupeptin (10 μM) were added as indicated. Controls received the vehicle. Caspase activity was determined fluorimetrically over 90 min using the fluorigenic substrate Ac-DEVD-AMC (10 μM).

Statistics—Data are given as means ± S.E. For statistical comparison, *t* test or one-way analysis of variance followed by Tukey's test were

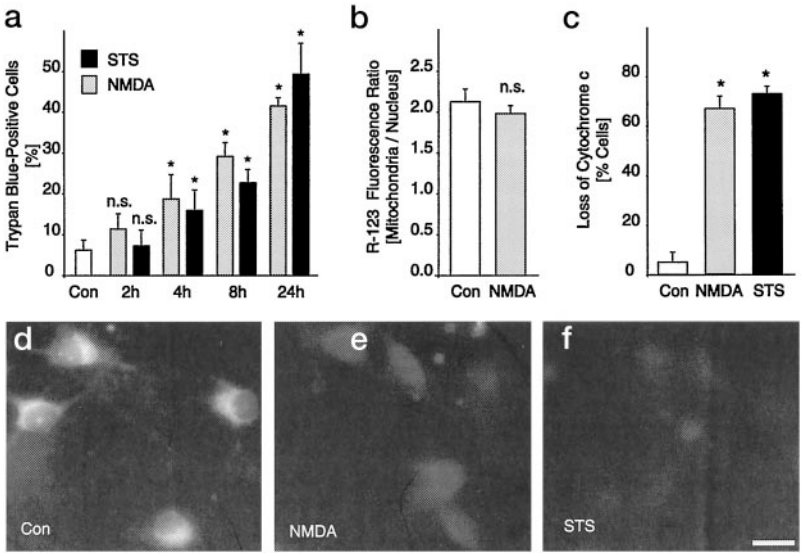

FIG. 1. **Delayed neuron death, recovery of mitochondrial membrane potential, and mitochondrial cytochrome *c* release after exposure of cultured rat hippocampal neurons to NMDA or STS.** *a,* time course of neuronal degeneration. Cultures were exposed for 5 min to NMDA (300 μM), washed, and returned to the original culture medium or were exposed to STS (300 nM). After the indicated period of time, cell death was quantified by trypan blue exclusion. Data are means ± S.E. from $n = 4$ to 16 cultures per time point. *, different from controls (*Con*) (analysis of variance and Tukey test; $p < 0.05$). *n.s.* not statistically significant. *b*, estimation of mitochondrial potential after the NMDA exposure. Live confocal microscopy imaging of R-123 uptake was performed in hippocampal neurons 2 h after a 5-min exposure to NMDA (300 μM) or Mg^{2+}-free HBS (sham-exposed controls). Histograms show the average R-123 fluorescence ratio between the mitochondria-rich regions and the nucleus. Data are means ± S.E. from $n = 119$ neurons per treatment. *c,* quantification of neurons that exhibited a loss of mitochondrial cytochrome *c* immunofluorescence 8 h after exposure to NMDA or 8 h after onset of the STS exposure. Data are means ± S.E. from $n = 9$ to 12 cultures in two experiments. Different from sham-exposed controls: *, $p < 0.05$. *d–f*, detection of cytochrome *c* in hippocampal neurons by immunofluorescence microscopy. *d*, sham-exposed control culture showing a high level of cytochrome *c* immunofluorescence excluding the nucleus. *e,* loss of cytochrome *c* after the 5-min NMDA exposure. *f*, STS-treated hippocampal neurons also exhibit a significant reduction of cytochrome *c* immunofluorescence. *Scale bar,* 10 μm.

employed. *p* values smaller than 0.05 were considered to be statistically significant.

RESULTS

Mitochondrial Cytochrome c Release Precedes Delayed Hippocampal Neuron Death—To investigate the involvement of apoptotic processes in excitotoxic neuron death, we established a model in which a transient, 5-min exposure to the selective glutamate receptor agonist NMDA (300 μM) induced a delayed cell death in primary cultures of rat hippocampal neurons (Fig. 1*a*). The time course of neuronal degeneration was comparable to that during a continuous exposure to the apoptosis-inducing agent STS (300 nM). In both cases, the first significant increase in percentage of trypan blue-positive cells was 4 h after onset of the stimulus. After 24 h, the degree of neuronal degeneration reached 41.5 ± 2.2% in cultures exposed for 5 min to NMDA ($n = 16$ cultures in four separate experiments) and 49.3 ± 7.5% in cultures exposed to STS (n = eight cultures in two separate experiments). The percentages showed no significant differences at all time points investigated.

We have previously demonstrated that mitochondria remained polarized during STS-induced apoptosis of cultured rat hippocampal neurons (24). Glutamate receptor overactivation has been suggested to induce apoptosis or necrosis, depending on the recovery of mitochondrial function (8). To investigate whether mitochondrial membrane potential had recovered after the exposure to NMDA, the uptake of the voltage-sensitive probe R-123 was determined in individual hippocampal neurons by confocal laser scanning microscopy 2 h after termination of the NMDA exposure. Excitotoxic neuron death was preceded by a recovery of mitochondrial membrane potential, as uptake of R-123 was indistinguishable from that of sham-exposed control cultures (Fig. 1*b*).

The key trigger for the mitochondrial activation of apoptosis is the release of cytochrome *c* from the mitochondrial intercristal space into the cytosol (11). Cytochrome *c* release was investigated in cultured rat hippocampal neurons after the exposure to NMDA and STS by immunofluorescence microscopy using a monoclonal antibody specific for native cytochrome *c* (Fig. 1, *c–f*). In control cultures, cytochrome *c* immunoreactivity was distributed in the cytoplasm in a punctate pattern excluding the nucleus (Fig. 1*d*). Two h after cells were exposed to NMDA, cytochrome *c* immunoreactivity was largely intact. After 8 h, mitochondrial cytochrome *c* immunofluorescence decreased significantly (Fig. 1*e*). Hippocampal neurons exposed to 300 nM STS also exhibited a decrease in their cytochrome *c* immunofluorescence (Fig. 1*f*).

Mitochondrial Release of Cytochrome c Does Not Lead to a Significant Activation of the Caspase Cascade after Glutamate Receptor Overactivation—Despite the release of cytochrome *c*, recovery of mitochondrial membrane potential, and delayed neuronal degeneration, cell death induced by the NMDA exposure occurred without a significant activation of the caspase cascade (Fig. 2). We determined the cleavage of fluorigenic caspase substrates in cytosolic extracts prepared from NMDA-exposed hippocampal neuron cultures (Fig. 2*a*). Cellular extracts from sham-exposed control cultures showed a negligible cleavage of Ac-DEVD-AMC, a substrate for caspase-3, -7 and -8, as well as caspase-1, -6, -9, and -10 (28). The exposure to STS led to a significant increase in caspase-3-like protease activity after 6 h of treatment. In contrast, Ac-DEVD-AMC cleavage of NMDA-exposed neuron cultures was not significantly different from base-line activity of sham-exposed controls at all time points investigated. Evidence against a prominent activation of caspase-3 after glutamate receptor overactivation was also obtained from Western blot experiments. During apoptosis, caspase-3 is proteolytically activated by cleavage of its 32-kDa precursor, procaspase-3, into active subunits. We observed a pronounced decrease in the 32-kDa

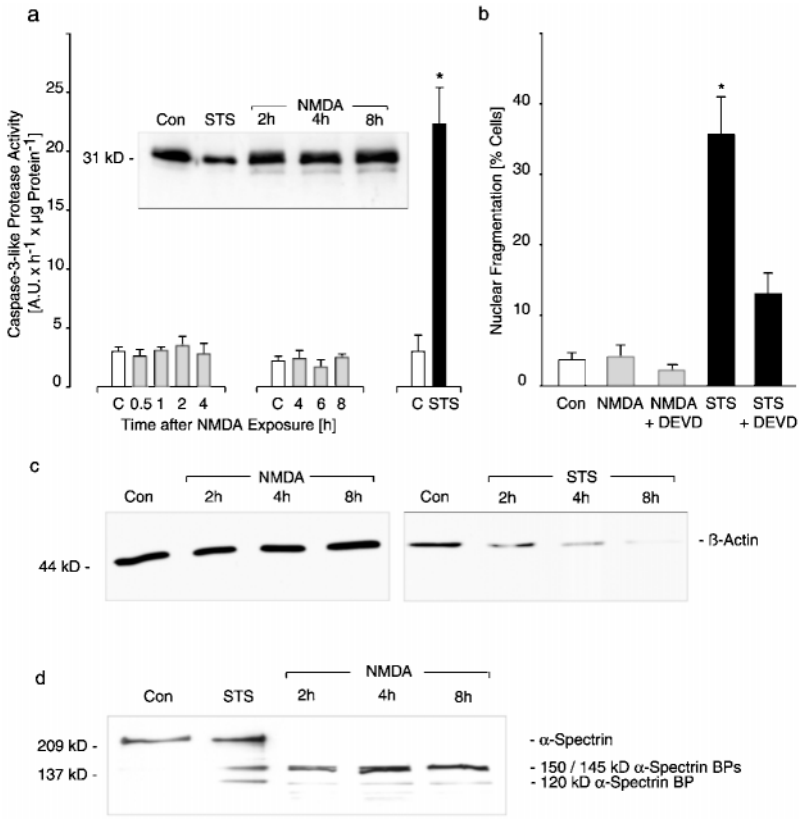

FIG. 2. **Glutamate receptor overactivation does not trigger a prominent activation of the caspase cascade in rat hippocampal neurons.** *a*, detection of caspase-3-like protease activity. Cytosolic protein extracts were prepared at different time points after exposure to 300 μM NMDA for 5 min. Caspase-3-like protease activity was measured by cleavage of the fluorigenic substrate Ac-DEVD-AMC (10 μM). As a positive control, cultures were treated for 6 h with STS (300 nM). Data are means ± S.E. from $n = 12$ to 24 cultures in 2–4 separate experiments per treatment. Different from respective controls (*C*): *, $p < 0.05$. *Inset*, immunoblot analysis of procaspase-3 in cytosolic extracts after an exposure to NMDA or STS (6 h). The experiment was performed in triplicate, with similar results. *b,* quantification of nuclear fragmentation visualized by staining chromatin with Hoechst 33258. Neurons were pretreated with the caspase-3-like inhibitor Ac-DEVD-CHO (DEVD) (10 μM) or the vehicle for 1 h and stained 20 h after exposure to NMDA or STS. Data are means ± S.E. from $n = 12$ to 24 cultures in 2–4 separate experiments per treatment. Different from respective controls: *, $p < 0.05$. *c*, cleavage of β-actin during NMDA neurotoxicity and STS-induced neuronal apoptosis. *d*, cleavage of α-spectrin during excitotoxicity and apoptosis. Cultures were treated with 300 nM STS or exposed to NMDA for 5 min. Cytosolic protein extracts were prepared after 6 h (STS) or 2, 4, and 8 h after the NMDA exposure. Note the appearance of the 120-kDa caspase-3-specific breakdown product (*BP*) in the STS-treated cultures. In contrast, 150- and 145-kDa breakdown products accumulate predominantly in the NMDA-exposed neuron cultures. The experiment was performed in duplicate with comparable results. Locations of molecular mass markers are provided on the *left* side of the figures.

precursor protein during STS treatment (Fig. 2*a, inset*). In contrast, no significant decrease in the 32-kDa proenzyme was detected after the NMDA exposure. Moreover, we could not detect a significant increase in the p17 caspase-3 subunit above the level seen in sham-exposed controls using an antibody raised against active caspase-3 (see below, Fig. 5*b*). NMDA exposure also did not lead to significant cleavage of Z-VDVAD-AFC, a substrate cleaved by caspase-2 and -3, or Z-IETD-AFC, a substrate cleaved by caspase-6 and -8. In contrast, cleavage of both substrates was detectable after the exposure to STS (data not shown).

A biochemical event in apoptosis that has been shown to require caspase-3 activity is chromatin fragmentation (34–36). Although NMDA-induced cell death was accompanied by nuclear condensation, chromatin fragmentation was not increased above the level seen in sham-exposed control cultures (Fig. 2*b*). In contrast, the exposure to STS induced a prominent chromatin fragmentation that could be significantly reduced by a treatment with the caspase-3-like protease inhibitor, Ac-DEVD-CHO (10 μM). The lack of a significant activation of executioner caspases after NMDA exposure was also confirmed by immunoblot experiments using antibodies against caspase substrates. β-Actin is a major cytoskeletal protein cleaved by caspases during neuronal apoptosis (32, 37). Although the exposure to STS induced a prominent degradation of β-actin, no degradation was observed after the NMDA exposure (Fig. 2*c*). Moreover, the caspase-cleaved β-actin 32-kDa fragment could be detected in STS-treated neurons, but only a limited accumulation was observed in the NMDA-exposed neuron cultures (see below, Fig. 5, *c, d,* and *f*). Similar results were obtained with the caspase substrates, Bcl-x_L and Akt (data not shown). Nonerythroid α-spectrin is cleaved by caspases into two breakdown products of 150 and 120 kDa (38, 39). The accumulation of both cleavage products was clearly detectable after the exposure of hippocampal neurons to STS (Fig. 2*d*). In contrast, the exposure to NMDA mostly led to a cleavage of α-spectrin into breakdown products of 150 and 145 kDa, a pattern characteristic for the activation of calpains (38).

Activation of Calpain I Is Detectable Early after NMDA Exposure and Contributes to Excitotoxic Neuron Death—The cleavage pattern of α-spectrin during excitotoxic neuron death suggested that calpains, but not caspases, were the predominantly activated proteases after glutamate receptor overactivation. To confirm the activation of calpains after the NMDA exposure, we performed an immunofluorescence analysis using a polyclonal antibody specific for the postautolytic, large subunit of calpain I. Control neurons showed a low basal immunoreactivity for active calpain I in their somata and processes

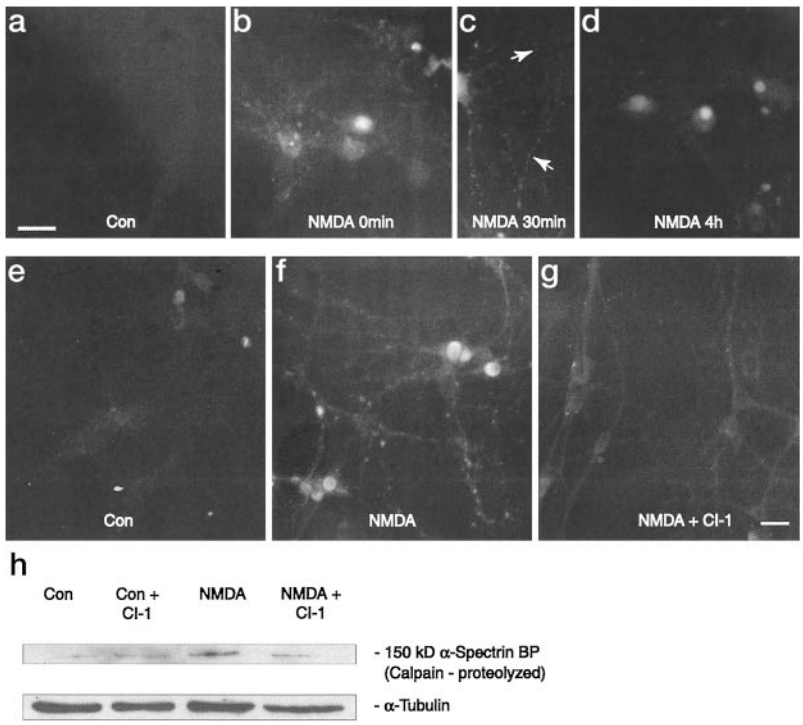

FIG. 3. **Activation of calpain after exposure of cultured rat hippocampal neurons to NMDA.** *A–d,* activation of calpain I detected by immunofluorescence analysis using an antibody specific for postautolyzed, active calpain I. *a*, sham-exposed control neurons showing a low level of active calpain I immunoreactivity. *b,* culture fixed immediately after the 5-min NMDA exposure. Calpain I becomes prominent in the somata and neurites. *c*, calpain immunoreactivity in neurites is frequently observed in a beaded pattern (*arrows*, 30 min after NMDA exposure). *d,* active calpain I immunoreactivity is still present 4 h after the NMDA exposure. *Scale bar* = 25 μm. *e–g,* immunofluorescence microscopy analysis of the accumulation of the 150-kDa calpain-generated α-spectrin breakdown product. The α-spectrin breakdown product is strongly detectable 2 h after an NMDA exposure in the cell soma as well as in the neurites. Whereas it is much less detectable in hippocampal neurons after NMDA exposure under a 1-h pretreatment with calpain inhibitor I (*CI-1*). *Scale bar* = 25 μm *h*. *Upper panel,* detection of the calpain-specific 150-kDa α-spectrin breakdown product by Western blot analysis. Cultures were exposed to NMDA or Mg^{2+}-free HBS (*Con*) for 5 min and pretreated with calpain inhibitor I (1 μM) or vehicle for 1 h. Cytosolic protein extracts were prepared 8 h after termination of the NMDA exposure. *Lower panel,* expression of α-tubulin after reprobing with a monoclonal anti-α-tubulin antibody.

(Fig. 3*a*). In cultures fixed immediately after termination of the 5-min NMDA exposure, active calpain I could be detected in the neuronal somata and along neurites of individual hippocampal neurons (Fig. 3*b*). Notably, calpain I immunoreactivity in neurites was frequently observed in a beaded fashion (Fig. 3*c*). This is consistent with previous findings that localized disruptions of ionic homeostasis in neurites are among the earliest manifestations of Ca^{2+}-mediated, excitotoxic neuron death (22, 25, 40). Active calpain I could be detected up to 4 h after the NMDA exposure (Fig. 3*d*). The activation of calpain after the NMDA exposure was also confirmed using a polyclonal antibody that specifically recognizes the calpain-cleaved, 150-kDa α-spectrin breakdown product, by immunofluorescence analysis (Fig. 3, *e* and *f*) and by Western blot analysis (Fig. 3*h*). Moreover, the accumulation of the calpain-cleaved 150-kDa α-spectrin breakdown product was significantly reduced in cultures pretreated for 1 h with the calpain inhibitor I (1 μM) (Fig. 3, *g* and *h*).

We next determined whether the activation of calpain was involved in the execution of excitotoxic neuron death. Treatment with calpain inhibitor I (1 μM) protected the hippocampal neurons against excitotoxic neuron death (Table I). Interestingly, however, the calpain inhibitor was significantly more effective when administered 1 h after, rather than 1 h before, the NMDA exposure. This suggested that the early activation of calpain had a neuroprotective function.

Calpain I Inhibits Cytochrome c-induced Activation of the Caspase Cascade—Our observation that calpain I was activated after NMDA receptor overactivation and previous reports (38) showing that calpains were activated in apoptotic cell death prompted us to investigate the interaction between these two proteolytic systems. NMDA-induced neuron death was accompanied by the mitochondrial release of cytochrome *c* (Fig. 1). To investigate the effect of calpain I on the ability of cytochrome *c* to activate the caspase cascade, we used a neuronal cell-free apoptosis system (11, 33). Addition of purified horse heart cytochrome *c* and dATP to cytosolic extracts from human SH-SY5Y neuroblastoma cells induced a caspase-3-like protease activity (Fig. 4*a*). In contrast, yeast cytochrome *c* plus dATP or dATP alone had no effect (data not shown). Simultaneous

TABLE I
Time dependence of the protective effect of calpain inhibitor I on NMDA neurotoxicity

Cultured rat hippocampal neurons were exposed for 5 min to NMDA (300 μM), washed, and returned to the original culture medium. Controls were exposed to Mg^{2+}-free HBS for 5 min. Cultures were treated with calpain inhibitor I (CI-1; 1 μM) or vehicle as indicated. After 24 h, cell death was quantified by trypan blue exclusion. Data are means ± S.E. from *n* cultures in two separate experiments per treatment.

Treatment	Trypan blue-positive cells	*n*
	%	
Controls	4.6 ± 1.4	6
Controls + CI-1, 1 h pre	11.5 ± 2.2	6
Controls + CI-1, 2 h post	10.0 ± 1.4	6
NMDA	42.0 ± 1.9	6
NMDA + CI-1, 1 h pre	32.1 ± 3.8	6
NMDA + CI-1, 0 h post	17.0 ± 4.3[a]	6
NMDA + CI-1, 1 h post	10.5 ± 2.0[a]	6
NMDA + CI-1, 2 h post	39.7 ± 1.6	6

[a] Different from NMDA-exposed controls (analysis of variance and Tukey test; $p < 0.05$).

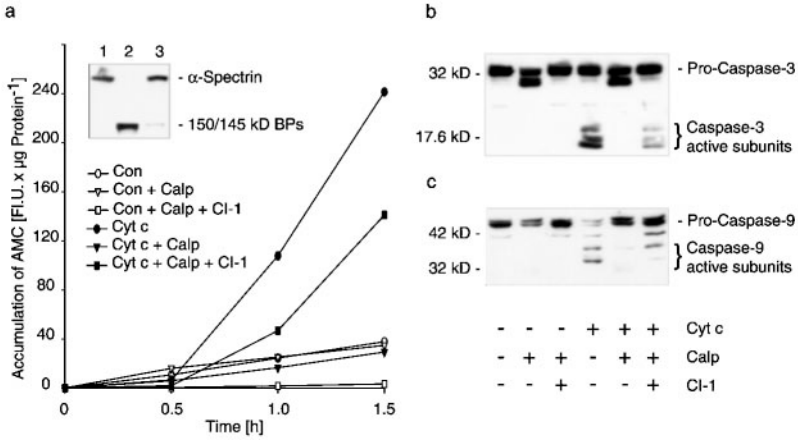

FIG. 4. **Calpain I inhibition of the caspase cascade.** *a,* calpain I inhibits cytochrome *c*-induced caspase activation in cytosolic extracts from human neuroblastoma SH-SY5Y cells. Extracts were normalized for protein content and incubated at 30 °C with or without 0.5 μM cytochrome *c* plus 1 mM dATP (*Cyt c*). Extracts were treated with calpain I (*Calp*) (0.8 milliunits/μl), calpain inhibitor I (*CI-1*) (10 μM), or vehicle as indicated. Ac-DEVD-AMC cleavage was measured using a fluorescent plate reader. Experiment was performed in triplicate with comparable results. *Inset,* cleavage of α-spectrin during the same experiment. *Lane 1,* control cytosol. *Lane 2,* treatment with calpain I for 90 min. *Lane 3,* simultaneous treatment with calpain I and the inhibitor for 90 min. *b* and *c,* calpain I inhibits cytochrome *c*-induced activation of procaspase-3 and procaspase-9. Cytosolic extracts from SH-SY5Y cells treated as in *a* were subjected to SDS-PAGE and immunoblot analysis using antibodies to caspase-3 and -9. Note the absence of active caspase subunits in extracts treated simultaneously with cytochrome *c* and calpain I. Locations of molecular mass markers are provided on the *left side* of the figures.

addition of horse heart cytochrome *c* and purified calpain I (0.8 milliunits/μl) prevented the ability of cytochrome *c* to trigger a caspase-3-like protease activity in this system (Fig. 4*a*). At the same concentration, calpain I led to a complete cleavage of full-length α-spectrin (Fig. 4*a, inset*). A simultaneous treatment with calpain inhibitor I (10 μM) inhibited the cleavage of full-length α-spectrin in the cytosolic extracts and also reversed the inhibitory effect of calpain I on the Ac-DEVD-AMC cleavage. Another calpain inhibitor, leupeptin (10 μM), also inhibited the effect of calpain I (data not shown).

Western blot experiments confirmed the ability of calpain I to inhibit cytochrome *c*-induced activation of the caspase cascade (Fig. 4, *b* and *c*). Calpain I inhibited the processing of procaspase-3 and procaspase-9 into their active subunits p17 (caspase-3), as well as p37 and p35 (caspase-9). Interestingly, we observed that procaspase-3 was partially cleaved by calpain I into a product of approximately 30 kDa (see also Fig. 2*a, inset*). In contrast, little degradation of procaspase-9 was observed.

Inhibition of Calpain Activity Restores a Caspase-3-like Protease Activity after Glutamate Receptor Overactivation—To investigate the interaction between calpains and caspases in intact neurons, we treated the hippocampal neuron cultures with calpain inhibitor I 1 h prior to the NMDA exposure. As shown above, this treatment inhibited the NMDA-induced activation of calpain (Fig. 3, *e–h*). In agreement with the findings obtained in the cell-free apoptosis system, pretreatment with calpain inhibitor I (1 μM) restored a caspase-3-like protease activity in the hippocampal neuron cultures (Fig. 5*a*) and led to the appearance of the active p17 subunit of caspase-3 on Western blots (Fig. 5*b*). In addition, caspase-specific cleavage products could be detected in the calpain inhibitor-treated, NMDA-exposed neuron cultures, such as the 32-kDa caspase-cleaved β-actin fragment (fractin) (Fig. 5, *c–f*).

DISCUSSION

The present study provides several important findings that improve our understanding of the biochemical events leading to excitotoxic neuron death. First, a transient exposure of rat hippocampal neurons to the glutamate receptor agonist NMDA was able to induce a delayed excitotoxic neuron death that was preceded by the mitochondrial release of cytochrome *c*. Second, unlike STS-induced neuronal apoptosis, mitochondrial cytochrome *c* release did not lead to a significant activation of the major neuronal executioner caspase, caspase-3. In contrast, activation of calpain I was readily detectable after the exposure to NMDA. Third, calpain I inhibited the ability of cytochrome *c* to activate the caspase cascade in a cell-free apoptosis system. Finally, inhibition of calpain activity restored a caspase-3-like protease activity in the hippocampal neuron cultures after the exposure to NMDA. Thus, the present study demonstrates the existence of signal transduction pathways that inhibit the entry of cells into a caspase-dependent cell death after the mitochondrial release of cytochrome *c*.

In agreement with previous reports (41–43), we found little evidence for prominent activation of executioner caspases after glutamate receptor overactivation (Figs. 2 and 5). Of note, only a very limited activation of caspase-3 was observed under conditions that should actually favor apoptosis, such as delayed neuron death (Fig. 1*a*), mitochondrial membrane potential recovery after the NMDA exposure (Fig. 1*b*), and mitochondrial cytochrome *c* release (Fig. 1, *c–e*). The lack of significant caspase-3 activation was confirmed by caspase substrate cleavage assays, Western blot analysis, immunofluorescence analysis, as well as morphological observations. In the mitochondrial apoptosis pathway, caspase-3 activation is required for the downstream activation of caspase-6, as well as caspase-2, -8, and -10 (16). It is unlikely that these caspases were activated in the absence of a functional caspase-3. In support of this mechanism, NMDA-exposed neuron cultures showed no significant cleavage activity using a broad spectrum of caspase substrates (DEVD, VDVAD, and IETD substrates). We cannot exclude that certain executioner caspases were activated in a subpopulation of the hippocampal neurons below the level of detection. In other settings, however, the activation of glutamate receptors has been shown to actually protect against apoptotic neurodegeneration (44), whereas administration of NMDA receptor antagonists induced apoptotic neuron death (44, 45).

Excitotoxic neuron death exhibits some of the morphological changes that accompany neuronal apoptosis, such as chromatin condensation, internucleosomal DNA fragmentation, and cell shrinkage (8, 25, 46, 47). Internucleosomal DNA fragmentation has also been shown to occur in neuronal necrosis (47)

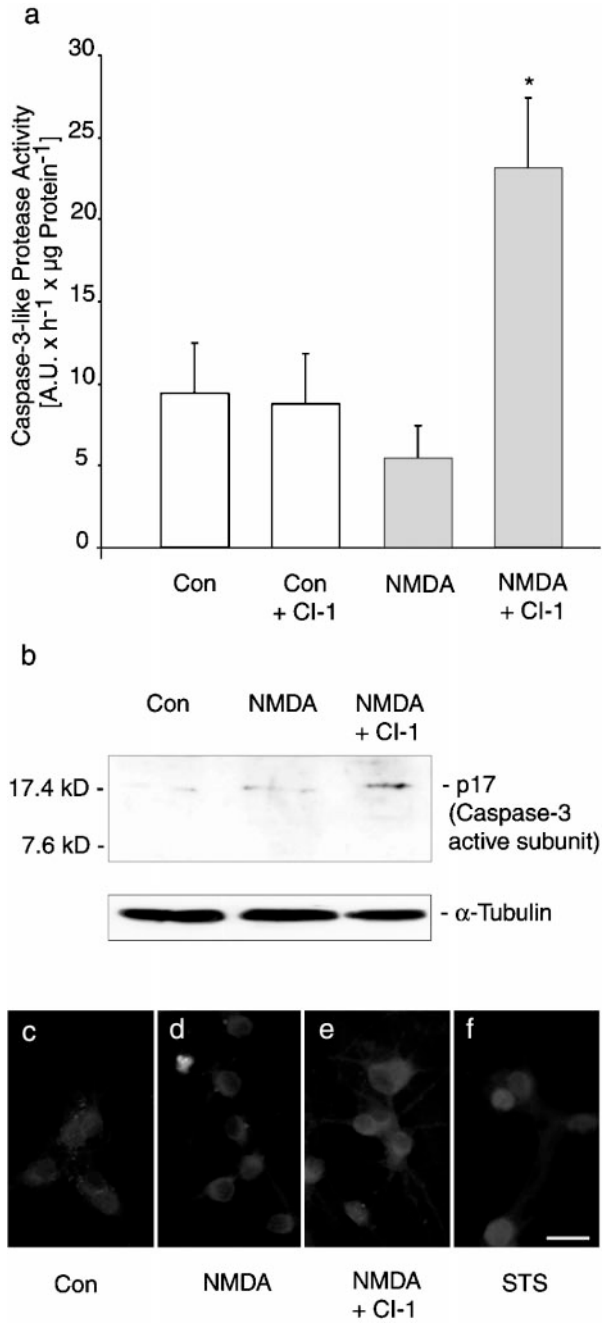

FIG. 5. **Treatment with calpain inhibitor I restores caspase activity after glutamate receptor overactivation.** *a,* cultured rat hippocampal neurons were exposed to 300 μM NMDA or Mg^{2+}-free HBS (*Con*) for 5 min. Calpain inhibitor I (*CI-1*) (1 μM) or vehicle were added to the cultures 1 h prior to the exposure and remained in the cultures after the exposure. Caspase-3-like activity was measured in cytosolic extracts prepared 8 h after the exposure. Data are means ± S.E. from $n = 6$ cultures. Difference from controls: * $p < 0.05$. Experiment was performed in duplicate with comparable results. *b, upper panel,* detection of the p17 caspase-3 subunit in the CI-1-treated neuron cultures 8 h after the exposure using an anti-active caspase-3 antibody. *Lower panel,* expression of α-tubulin after reprobing with a monoclonal anti-α-tubulin antibody. *c–f,* detection of the caspase-generated β-actin 32-kDa cleavage product (fractin) in the hippocampal neuron cultures by immunofluorescence analysis. Cultures were treated as in *a* or exposed to 300 nM STS for 8 h. Especially the NMDA plus calpain inhibitor 1 (CI-1) but also the STS-treated cells show an elevated level of the caspase-3 cleavage product. *Scale bar*, 25 μm.

and could be due to DNases that do not require caspase-3 for activation. It is conceivable that chromatin condensation and internucleosomal DNA fragmentation during excitotoxic neuron death is caused by apoptosis-inducing factor, which is a nuclease that is co-released with cytochrome *c* after a significant increase in mitochondrial permeability (48). Interestingly, activation of the protease calpain I has recently been shown to induce morphological changes in thrombocytes that resemble a caspase-mediated apoptotic cell death, including exposure of phosphatidylserine on the outer plasma membrane, microvesiculation, and cell shrinkage (49). Calpain I is strongly activated during excitotoxic neuron death (21, 22) (Fig. 3). Therefore, the apoptosis-like morphology known to occur in excitotoxic neuron death could be partially caused by the activation of calpain I, rather than by the activation of executioner caspases. Protective effects of broad spectrum caspase inhibitors have been reported in models of excitotoxic neuron death (50, 51). However, inhibitors such as benzoyloxycarbonyl-Val-Ala-Asp(*O*-methyl)-fluoromethyl ketone have been shown to be equally potent in inhibiting the activity of calpain I as well as caspase-3 (49).

The discrepancy between mitochondrial cytochrome *c* release and lack of significant activation of the caspase cascade can be explained by several factors. Nitrosylation of caspases has been shown to inhibit their activation and the execution of apoptosis (19, 20). In our hands, treatment of rat hippocampal neurons with the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (10 μM) 1 h prior to the NMDA exposure failed to restore a caspase-3-like protease activity in the hippocampal neuron cultures.[2] The present study provides strong evidence for a calpain I-driven negative regulation of the caspase cascade. In a cell-free apoptosis system, calpain I inhibited the cytochrome *c*-induced increase in caspase-3-like protease activity, as well as the processing of procaspase-9 and -3 into their active subunits. Previously, calpains have been reported to cleave endogenous or recombinant procaspase-3 into 30- and 18-kDa fragments, as well as procaspase-9 into 35- and 10-kDa fragments (49, 52). Of note, calpain-cleaved procaspase-3 could still be activated by the caspase activator granzyme B (49). In our cell-free apoptosis system, only a partial proteolysis of procaspase-3 into a 30-kDa cleavage product occurred (Fig. 4*b*). Moreover, procaspase-9 was not significantly proteolyzed (Fig. 4*c*). Under the same condition, calpain I efficiently blocked the ability of cytochrome *c* to activate the caspase cascade and led to a full cleavage of α-spectrin (Fig. 4*a*). We also observed a very limited cleavage of procaspase-3 into a 30-kDa cleavage product in the hippocampal neuron cultures after an exposure to NMDA (Fig. 2*a, inset*). It is therefore conceivable that calpain I inhibits the caspase cascade upstream of procaspase-3 or procaspase-9 processing.

The present study also demonstrates that an inhibition of calpain activity restores the ability of cytochrome *c* to activate the caspase cascade, both in the cell-free apoptosis system and in the hippocampal neuron cultures (Figs. 4 and 5). In line with these findings, NMDA has been shown to induce caspase-3-like protease activity and apoptosis in neocortical neurons when neuronal Ca^{2+} (and Na^{+}) overloading was significantly reduced (43), actually conditions that should inhibit the activation of calpain I. Conversely, our study suggests that activation of calpain may also exert some anti-apoptotic and presumably neuroprotective function. The calpain inhibitor experiments suggest that calpain I activation had a protective function in the early phase of excitotoxic neuron death but significantly contributed to the destruction of neurons in the later stage (Table I). Interestingly, previous studies using calpain inhibitors as neuroprotective agents also demonstrated U-shaped dose-response curves against excitotoxic neuron death (53).

[2] S. Lankiewicz, C. M. Luetjens, N. T. Bui, A. J. Krohn, M. Poppe, G. M. Cole, T. C. Saido, and J. H. M. Prehn, unpublished observation.

Calpain I has been shown to be activated during apoptosis (38). The activation of calpain I could represent a negative feedback loop that limits the activation of caspases once the apoptotic cascade is activated. One potential mechanism is the cleavage of the endogenous calpain inhibitor calpastatin by caspase-3-like proteases (54). Consistent with the concept that calpains play a dominant role in the suppression of apoptosis, they have been shown to cleave and inactivate other gene products involved in the initiation of apoptosis, such as p53 and Bax (55, 56). Activation of calpains may therefore inhibit the entry of cells into a caspase-dependent, apoptotic cell death program both upstream and downstream of mitochondrial cytochrome *c* release.

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