Proteolysis of the N-Methyl-d-Aspartate Receptor by Calpain in Situ

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ABSTRACT

N-Methyl-d-aspartate (NMDA) receptors are calcium-permeable glutamate receptors that play putative roles in learning, memory, and excitotoxicity. NMDA receptor-mediated calcium entry can activate the calcium-dependent protease calpain, leading to substrate degradation. The major NMDA receptor 2 (NR2) subunits of the receptor are in vitro substrates for calpain at selected sites in the C-terminal region. In the present study, we assessed the ability of calpain-mediated proteolysis to modulate the NR1a/2A subtype in a heterologous expression system. Human embryonic kidney (HEK293t) cells, which endogenously express calpain, were cotransfected with NR1a/2A subunits and NR2A subunit constructs lacking the final 420 amino acids. These co-agonists led to calpain activation as measured by succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosyl-aminomethyl coumarin (Suc-LLVY-AMC). Calpain activation also resulted in the degradation of NR2A and decreased binding of 125I-MK-801 (125I-dizocilpine) to NR1a/2A receptors. No stable N-terminal fragment of the NMDA receptor was formed after calpain activation, suggesting calpain regulation of NMDA receptor levels in ways distinct from that previously observed with in vitro cleavage. NR2 subunit constructs lacking the final 420 amino acids were not degraded by calpain. Agonist-stimulated NR1a/2A-transfected cells also had decreased calcium uptake and produced lower changes in agonist-stimulated intracellular calcium compared with cells cotransfected with calpastatin. Calpastatin had no effect on either calcium uptake or intracellular calcium levels when the NR2A subunit lacked the final 420 amino acids. These studies demonstrate that NR2A is a substrate for calpain in situ and that this proteolytic event can modulate NMDA receptor levels.

NMDA receptors are ionotropic glutamate receptors that play important roles in learning and memory as well as many neurological disorders (Lynch and Guttmann, 2001). These receptors exist as heteromultimers composed of subunits from two separate protein families (termed NR1 and NR2). The NR1 subunit family consists of eight splice variants (NR1a–h), whereas the NR2 family is composed of four members (NR2A–D) made from separate genes (Nakanishi, 1992). Functional NMDA receptors usually require members from each family and probably exist as a tetramer or a pentamer in vivo (Lynch et al., 1995; Hawkins et al., 1999).

Each subunit of the NMDA receptor contains an extracellular N terminus followed by four transmembrane domains. The second domain forms an intramembrane loop, whereas the C terminus is intracellular and may link the receptor to calcium-activated intracellular signaling pathway systems (Niethammer et al., 1996). The interactions of the receptor with signal transduction systems also may be modulated by the association of the NMDA receptor C-terminal tail with anchoring proteins or other cytoskeletal elements (Bi et al., 1998a, Wechsler and Teichberg, 1998). These interactions can lead to subtype-specific modulation of the receptor. For example, yotiao and protein kinase C modulate NMDA receptors in subunit-specific manners based on the properties of the C-terminal region (Grant et al., 1998; Lin et al., 1998). The importance of the C-terminal region in proper NMDA receptor function is further exemplified by the findings of Sprengel et al. (1998), which demonstrated that gene-targeted mice lacking the C-terminal tail of the NR2 subunit exhibited properties similar to mice with a complete absence of an NR2 subunit. This occurs even though receptors lacking the C-terminal region of NR2A or NR2C are electrophysio-
ologically similar to wild-type receptors (Sprengel et al., 1998). This finding suggests that post-translational or activity-dependent processing of the C-terminal tail plays a critical role in the modulation of NMDA receptor activity, localization, or function.

One means of post-translational modification of the C terminus is proteolytic processing by calpain. Calpain, most commonly activated in brain by calcium entry through NMDA receptors (Adamec et al., 1998), regulates numerous enzymes and membrane-associated proteins, including cytoskeletal components, integral membrane proteins, and receptors (Johnson and Guttmann, 1997). Calpain activity is inhibited by the protein calpastatin, an endogenous and selective inhibitor of calpain (Johnson and Guttmann, 1997).

The C-terminal region of the NR2 subunit is a substrate for calpain (Bi et al., 1998b,c; Guttmann et al., 2001). Although NR2A appears to be a substrate in vitro or with prolonged exposure to glutamatergic agonists, it is not clear that physiological activation of calpain results in NR2A cleavage or alters NMDA receptor activity. The cleavages of the receptor subunit in vitro occur in the C-terminal regions with all sites in the NR2A subunit on the C-terminal side of amino acid 1051. Although two specific sites of cleavage in vitro occur at amino acids 1279 and 1330 of NR2A, proteolysis at these sites does not inherently alter NMDA receptor activity as receptor subunits truncated to these exact sites retain basic electrophysiological properties (Guttmann et al., 2001).

In the present study we sought to examine whether results from an in situ model system of transfected cells would also demonstrate cleavage of the NMDA receptor by calpain and whether such cleavage alters physiological properties of the NMDA receptor.

Materials and Methods

Materials

Glutamate, glycine, ketamine, aprotenin, pepstatin, phenylmethanesulfonyl fluoride, and anti-actin were from Sigma-Aldrich (St. Louis, MO); Dulbecco’s modified Eagle’s medium, horse serum, penicillin/streptomycin, and glutamine were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Suc-LLVY-AMC was from Bachem (Bubendorf, Switzerland); MK-801 was from Sigma/RBI (Natick, MA). 125I-MK-801 and 45CaCl2 were from PerkinElmer Life Sciences (Boston, MA), and HEK293t cells were from the American Type Culture Collection (Manassas, VA). Antibodies to the C-terminal portions of NR2A (AB1548) and NR1a (AB1516), with epitopes at amino acids 1445 to 1464 and 909 to 938, respectively, were from Chemicon International (Temecula, CA). An N-terminal antibody (amino acids 25–130 of NR2C) that cross-reacts with NR2A (A-6475) was from Molecular Probes (Eugene, OR). Porcine calpain I was purchased from Calbiochem (Eugene, OR). PKC inhibitor G-400 was a gift from Dr. John Elce (Queen’s University, Kingston, ON, Canada), and rabbit calpastatin cDNA was a gift from Dr. Masatoshi Maki (Nagoya University, Nagoya, Japan).

Methods

Transfection of HEK293t Cells

HEK293t cells were grown on tissue culture-grade dishes (Corning brand; Corning Glassworks, Corning, NY) in RPMI media containing 5% horse serum and 5% fetal bovine serum supplemented with 2 mM glutamine and 100 units/ml penicillin/streptomycin and placed in a 5% CO2 incubator at 37°C. Transfection of HEK293t cells with cDNA was accomplished by calcium phosphate precipitation as previously described (Grant et al., 1998). Twenty-four hours after transfection, the medium was changed and treatments were added. Calcium uptake, calpain activation, and calcium imaging were routinely performed at this point. Ketamine (500 μM) was added to the media during transfection to prevent NMDA receptor activation as previously described (Grant et al., 1997). Using HEK293t cells, the transfection efficiency is ~70%. As previously shown, individual HEK293t cells express all of the proteins that the cDNAs encode regardless of the number of different cDNA plasmids that are transfected (Grant et al., 1998).

Calpain Activity Assay with Suc-LLVY-AMC

Twenty-four hours after transfection, cells were rinsed with serum-free medium and the medium was replaced with serum-free media containing 100 μM glutamate, 100 μM glycine in the presence or absence of 100 μM MK-801 in addition to 80 μM Suc-LLVY-AMC. Cells were then replaced in a 5% CO2 incubator at 37°C. After a 40-min incubation, the plates were read in a Victor fluorescence plate reader (PerkinElmer Wallac, Turku, Finland) at wavelength settings of 390 nm and 460 nm for excitation and emission, respectively. Previous studies have demonstrated that this assay is linear with cell number, and the activity measured is representative of calpain activity observed with protein substrates (Johnson and Guttmann, 1997; Guttman and Johnson, 1998).

Analysis of NMDA Receptor Subunit Degradation by Calpain in Situ

Twenty-four hours after transfection, cells were rinsed with serum-free media and the media were replaced with serum-free media containing 100 μM glutamate, 100 μM glycine in the presence or absence of 100 μM MK-801. Cells were then replaced in the incubator for 30 min. After incubation, the cells were rinsed once with PBS and scraped into 1× Laemmli stop buffer without bromophenol blue, EGTA, or dithiothreitol (DTT). Samples were heated to 100°C for 5 min and briefly sonicated. Protein concentrations were determined using the biechinonic acid assay (Pierce Chemical Co., Rockford, IL). Bromophenol blue and DTT were then added, and the samples were stored at −20°C until used. For assessment of the blockade of calpain, cells were cotransfected with calpastatin, a specific inhibitor of calpain. This inhibitor shows fewer toxic actions than do synthetic calpain inhibitors and their vehicles (reviewed in Johnson and Guttmann, 1997).

Western Blotting

For HEK293t cellular homogenates, 40 to 50 μg of total protein was loaded on a 7% polyacrylamide gel. After SDS gel electrophoresis, proteins were transferred to nitrocellulose, blocked with 3% bovine serum albumin, and incubated with primary antibodies to NR2A, NR1a, actin, or calpain. Blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies to NR2A, NR1a, actin, or calpain. Blots were then washed with PBS and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence (Pierce). Each blot was quantitated using imaging densitometry and analyzed using NIH Image software (National Institutes of Health, Bethesda, MD). Statistical differences were determined by analysis of variance.

Calcium Uptake Assay

Six-well plates of transfected HEK-293t cells were washed two times with HEPES-buffered saline solution (HBSS) without CaCl2, and then 2 × 10^6 cpm of 45Ca in HBSS were added to each well along with 100 μM glutamate and 100 μM glycine in the presence or absence of 100 μM MK-801. Plates were incubated at room temperature for 10 min and then washed once with HBSS containing 2 mM CaCl2. Cells were then harvested by addition of 500 μl of 0.05% trypsin for 5 min. Twenty milliliters of scintillation cocktail were then added, and the radioactivity was quantified using a Beckman (model LS 5000TD) scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Statistical difference was determined by two-sample t test.

125I-MK-801 Binding

Cell membranes were prepared as previously described, using preparations that remove MK-801 and other NMDA receptor antagonists (Lynch et al., 1995). Briefly, to remove MK-801, the membrane fractions were homogenized in assay buffer (20 mM HEPES, pH 7.5, 100 μM glutamate, 100 μM glycine, and 300 μM MgCl2) and incubated at 32°C for 30 min. Homogenates were then centrifuged, and the pellet was resuspended in assay buffer. This process was repeated two more times. Membrane suspensions
were then assayed in saturating glycine (100 μM) and glutamate (100 μM), spermidine (100 μM), 100 μM MgCl₂, and 300 μM 125I-MK-801 (Lynch et al., 1994). Membranes were harvested (Brandel Harvester) onto polyethyleneimine-coated glass-fiber filters (Schleicher & Schuell, Keene, NH), and the radioactivity was quantified using a Beckman (model 5500B) gamma counter. Using this protocol, binding to NR1a/2A combinations was observed, whereas no binding to NR1a or NR2A was detected (Lynch et al., 1994). Statistical differences were determined by two-sample t test.

**Calcium Imaging.** Twenty-four hours after transfection, cells were rinsed twice with HBSS. The medium was then replaced with HBSS containing 2 μM Fura-2 acetoxymethyl ester and returned to the incubator. After a 30-min incubation, cells were rinsed twice with HBSS and placed on the stage of a Nikon Eclipse TE300 microscope (Nikon, Melville, NY). Images of cells were obtained and analyzed using the Metafluor imaging system (Universal Imaging, Downingtown, PA). Calibrations were done as previously described (Guttmann and Johnson, 1998; Lynch et al., 2001). Prior to agonist application, images were obtained for several minutes to establish a stable baseline calcium measurement. Agonists (glutamate and glycine) were then applied, and images were obtained at 1-s intervals. Peak calcium concentrations were typically observed in less than 30 s.

**Cell Toxicity Assay.** Toxicity assays were performed by modifications of our previously described techniques using cotransfection with GFP as a surrogate marker of cell viability (Anegawa et al., 2000). HEK293t cells were cotransfected with NMDA receptor combinations, calpastatin or vector control, and GFP. Twenty-four hours later, cells were rinsed twice with HBSS and the media were replaced with HBSS containing 100 μM glutamate and 100 μM glycine. Selected plates had 100 μM MK-801 included throughout the transfection to provide a control for non-receptor-mediated cell death. Zero, 4, or 8 h after agonist addition, the medium was aspirated and the cells were collected in 1× Laemmli stop buffer without bromphenol blue, EGTa, or DTT. Samples were heated to 100°C for 5 min and briefly sonicated. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Bromphenol blue and DTT were then added, and the samples were stored at −20°C until use. To quantify cell death, samples were separated by SDS-PAGE and immunoblotted for the presence of intact GFP. Assays were performed in triplicate, and results were normalized to the mean cell viability at the beginning of agonist application. Statistical differences were determined by analysis of variance.

**cDNA Constructs.** The shortened NR2A subunit NR2A1051 was constructed as previously described (Grant et al., 1998). This construct does maintain the epitope tag required for immunoreactivity to antibody AB1548, consisting of the last six amino acids of the NR2A subunit, and has normal electrophysiological and ligand-binding properties (Grant et al., 1998; Guttmann et al., 2001).

### Results

**Identification of Calpain in HEK293t Cells and Selective Activation of Calpain by NMDA Receptors.** To determine whether calpain I was present within HEK293t cells, 40 μg of total cellular homogenate was separated by SDS-PAGE and immunoblotted with a monoclonal antibody directed at calpain I (a generous gift of Dr. John Elce). An 80-kDa band was detected, indicating that calpain is present in this cell line (Fig. 1A).

The ability of HEK293t cells transfected with the NMDA receptor combination NR1a/2A to activate calpain in response to glutamate and glycine treatment was evaluated using the fluorescent calpain substrate Suc-LLVY-AMC, which has been utilized in situ in similar paradigms (Fig. 1B). To examine calpain activity in HEK293t cells, cells were transfected with NMDA receptor subunits. HEK293t cells were cotransfected with NR1a/2A and the specific calpain inhibitor calpastatin since the increase in fluorescence was not significantly different from that observed in untransfected cells treated with agonists. Some increase in fluorescence was observed in both untransfected cells and cells cotransfected with calpastatin, likely due to the ability of other proteases to cleave Suc-LLVY-AMC (Guttmann et al., 1997). However, because the increase in fluorescence in NR1a/2A-transfected cells was inhibited by calpastatin, the proteolysis stimulated by agonist must result from calpain activation. In contrast, activation of endogenous adenosine receptors with ATP, which activates a variety of calcium-dependent processes (Short and Taylor, 2000), did not stimulate calpain activity as measured by cleavage of the substrate Suc-LLVY-AMC (Fig. 1B, inset) in ATP-treated cells. This selective activation of calpain by NMDA receptors matches the patterns observed in neurons (Adamec et al., 1998).

**In Situ Proteolysis of NR2A in NR1a/2A-Transfected HEK293t Cells.** Since endogenous calpain was activated by NMDA receptor agonists, we tested the ability of calpain to cleave NMDA receptor subunits. HEK293t cells were cotransfected with NR1a/2A in the presence or absence of calpastatin and analyzed by SDS-PAGE and immunoblotting.

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**Fig. 1.** Identification and agonist activation of calpain I in NMDA receptor-transfected HEK293t cells. Forty micrograms of total protein was separated by SDS-PAGE, and a representative Western blot demonstrating the presence of endogenous calpain I in HEK293t cells is shown (A). Calpain activity was measured in untransfected cells or in NR1a/2A-transfected cells that were cotransfected with calpastatin or control vector (B). Calpain was activated by the stimulation of NMDA receptors with 100 μM glutamate and 100 μM glycine for 40 min, and the amount of fluorescence was quantitated. Cells that were cotransfected with calpastatin had significantly decreased calpain activity as measured by cleavage of the substrate Suc-LLVY-AMC (*, p < 0.05, n = 3–5). No significant difference in fluorescence was observed in calpastatin- or vector-control-transfected cells incubated in the presence of 100 μM ATP (B, inset).
for NR1a, NR2A, and actin (Fig. 2A). Agonist stimulation of NR1a/2A-transfected cells for 30 min decreased NR2A immunoreactivity to a C-terminal antibody by 45% (Fig. 2B) compared with cells that were treated with the NMDA receptor antagonist MK-801 in addition to glutamate and glycine. There was no significant decrease in NR2A immunoreactivity in agonist-stimulated cells that were cotransfected with calpastatin, identifying the role of calpain activity in the cleavage of NR2A. NR1a subunit levels were not altered following NMDA receptor activation, and no change in the amount of the poor calpain substrate actin was observed. Interestingly, the immunoreactivity at the beginning of agonist addition was slightly increased in NR1/2A-transfected cells that were cotransfected with calpastatin, compared with vector controls (although statistical significance was not reached), suggesting that basal calpain activity may play a role in turnover of this NMDA receptor combination (see inset Fig. 2B). These data indicate that activation of calpain by NMDA receptor stimulation leads to selective cleavage of the NR2A subunit.

We also examined whether in situ proteolysis decreased the level of 125I-MK-801 binding to assembled NMDA receptors. 125I-MK-801 binding serves as a marker of assembled receptors because binding of labeled channel-blocking agents such as 125I-MK-801 is not observed in individual subunits (e.g., NR1a or NR2A) alone (Lynch et al., 1994; Lynch et al., 1995). 125I-MK-801 binding of agonist-treated NR1a/2A/vector-transfected cells was decreased by 20% compared with cells that were protected with MK-801 during agonist exposure (Fig. 2C). This decrease following agonist treatment was not observed in NR1a/2A-transfected cells that were cotransfected with calpastatin, demonstrating that calpain activity

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**Fig. 2.** Effects of NMDA receptor activation on the calpain-mediated degradation of NMDA receptor subunits. Shown are representative Western blots of NR1a, NR2A, and actin from HEK293t cells cotransfected with NR1a, NR2A, and calpastatin or control vector treated with 100 μM glutamate and 100 μM glycine in the presence or absence of 100 μM MK-801 for 0 or 30 min (A). NMDA receptor activation resulted in significant degradation of NR2A (C-terminal AB1548; *, p < 0.05, n = 5–7) but not NR1a or actin (B). Quantitated data are expressed in terms of percentage of NR2A immunoreactivity in transfected cells in the presence of glutamate and glycine plus 100 μM MK-801. Agonist-stimulated degradation was inhibited by cotransfection of calpastatin. No proteolysis of NR2A was observed when NMDA receptor activity was blocked by addition of MK-801 (100 μM). The presence of calpastatin slightly increased the amount of NR2A present at 0 min compared with control vector (see inset). The change in 125I-MK-801 binding after 30 min of agonist stimulation is shown in panel C. Data are expressed in terms of 125I-MK-801 binding of the transfection combination incubated with agonist in the presence of 100 μM MK-801. 125I-MK-801 binding was decreased by 20% in NR1a/2A-transfected cells cotransfected with calpastatin, whereas no difference was observed for calpastatin-cotransfected cells (*, p < 0.05, n = 4). No detectable NR2A C-terminal (AB1548)- or N-terminal (A-6475)-immunoreactive fragments were observed subsequent to calpain activation as shown in D (blots were overexposed in attempts to determine the presence of stable products; thus, decreases in intact NR2A are underestimated in this panel), even though the intact NR2A subunit was significantly degraded (compare at arrow) after 30 min of agonist treatment (+) compared with control (−). Arrowheads indicate position of molecular weight markers in kilodaltons.
Calpain Proteolysis of NR2A

Fig. 3. Effects of NR2A1051 deletion mutant on calpain cleavage of NMDA receptor subunits. Agonist treatment of cells cotransfected with either wild-type NR1a/2A or NR1a/2A1051 mutant both resulted in increased calpain activity as measured by Suc-LLVY-AMC fluorescence (A). Data represent adjusted values for calpain-dependent increase in fluorescence after background subtraction of agonist stimulation in calpastatin-cotransfected cells for each experiment (n = 3). Representative immunoblot (n = 3) of NR2A1051 before and after 30-min agonist stimulation of NR1a/2A1051 cotransfection with vector control is shown in B. No breakdown of NR2A1051 was observed in contrast to wild-type (see Fig. 1, A or D) as detectable by the N-terminal antibody A-6475.

leads to decreased levels of $^{125}$I-MK-801 binding to assembled receptors, and further suggesting that calpain may play a role in NMDA receptor turnover and processing.

Since the epitope of NR2A necessary for immunoreactivity is located in the last 20 amino acids of the C terminus, the absence of a relatively large immunoreactive fragment (Fig. 2D) suggests that the calpain cleavage sites are located near this epitope in the C-terminal region of NR2A, consistent with previous data from in vitro experiments (Guttmann et al., 2001) and the intracellular localization of calpain. We therefore sought to identify whether a stable N-terminal product of calpain was produced using an antibody to the N terminus of NR2A. Activation of NR1a/2A-transfected cells with glutamate and glycine decreased immunoreactivity using this antibody, but no stable N-terminal product was detectable (see Fig. 2D). This strongly suggests that in situ activation of calpain by the NMDA receptor leads directly or indirectly to more complete degradation of the NMDA receptor.

These results contrast with previous demonstrations of the stability of the N-terminal two-thirds of the NMDA receptor to in vitro digestion by calpain (Bi et al., 1998a; Guttmann et al., 2001), and under postmortem conditions (Wang et al., 2000). This could be explained if the initial cleavages by calpain allow complete receptor degradation by other cellular processes. To assess this, we investigated whether a truncated form of NR2A (NR2A1051), which is not a substrate for calpain in vitro (Guttmann et al., 2001), was also degraded in a receptor-activated manner. Whereas activation of the mutant NR1a/2A1051 receptor resulted in activation of calpain as measured by increased Suc-LLVY-AMC fluorescence (Fig. 3A), activation of the receptor did not decrease immunoreactivity for this NR2A1051 mutant (Fig. 3B), and inclusion of calpastatin had no effect on NR2A1051 protein levels (data not shown). This shows that the crucial structural determinants for in situ degradation of the NR2 subunit by calpain localize to the C-terminal region of the receptor and that other processing systems are likely involved subsequent to calpain-mediated proteolysis.

Effects of Calpastatin Cotransfection on $^{45}$Ca Uptake, Intracellular Free Calcium, and Cell Death. To determine whether calpain-mediated proteolysis alters NMDA receptor function, the effects of calpain inhibition on NMDA receptor-mediated calcium influx and agonist-induced intracellular calcium transients were studied. Like $^{125}$I-MK-801 binding, these assays serve as markers of physiologically active receptors, because channel activity in this heterologous expression system is only present when a receptor contains both NR1 and NR2 subunits (Grant et al., 1997). Calcium uptake from the media in NR1a/2A cells cotransfected with calpastatin was increased over NR1a/2A/vector-transfected cells by 50% (Fig. 4A) during agonist stimulation for 10 min. Similarly, agonist-induced intracellular calcium responses of NR1a/2A-transfected cells were significantly potentiated by cotransfection with calpastatin, suggesting that calpain activation functionally limits NMDA receptor activity. To verify that the physiological effects of calpastatin are directed at the same structural region of the receptor as calpain cleavage, the effects of calpastatin on agonist-induced calcium uptake and intracellular calcium level changes in NR1a/2A1051-transfected cells were examined. The presence of calpastatin did not significantly increase either calcium uptake (see Fig. 4A) or intracellular calcium changes (see Fig. 4B) in NR1a/NR2A1051 receptors. Although these truncated receptors may lack regulatory sites of NMDA receptor control, the correlation of preserved receptor levels with physiological properties of these truncated constructs further supports the possibility that the effects of calpain on calcium uptake and intracellular calcium levels occur through the structural region regulating calpain-mediated degradation of the receptor, the last 420 amino acids of NR2A (Grant et al., 2001).

Because NMDA receptor activity is linked with various types of excitotoxic cell death, we also sought to determine whether calpain significantly affected cell death produced by NMDA receptors in this model system (Anegawa et al., 1995, 2000). Cells cotransfected with calpastatin in addition to NR1a/2A/GFP died at an accelerated rate compared with those transfected with NR1a/2A/GFP and vector control (Fig. 5) as determined by the amount of GFP immunoreactivity remaining after either 4 or 8 h of agonist stimulation. This is consistent with the increased intracellular calcium rise observed in calpastatin-cotransfected cells (see Fig. 4, A and B) and prior results linking increased extent of NMDA receptor-mediated cell death with increasing rises in intracellular calcium (Anegawa et al., 1995, 2000). These results suggest
that calpain can slow the rate of NMDA receptor-mediated cell death in HEK293t cells.

**Discussion**

The present study demonstrates that the NR2A subunit is a selective in situ substrate for calpain in a cell culture expression system and that calpain-mediated proteolysis in the C terminus results in NMDA receptor degradation and reduced activity. The effects of calpain are mediated by the final 420 amino acids of the NR2A subunit inasmuch as receptor constructs truncated to amino acid 1051 are not cleaved by calpain, consistent with in vitro results (Guttmann et al., 2001). However, in contrast to in vitro studies, the N-terminal portion of the receptor is not stable following calpain activation, suggesting that in this in situ system, the N-terminal products of calpain cleavage are further degraded by other mechanisms. Taken together, these data suggest that calpain-mediated cleavage can be the controlling event for stability of the NMDA receptor and that calpain processing in the C-terminal region of the NMDA receptor may modulate NMDA receptor function in vivo.

This interpretation provides an understanding of paradoxical components of previous studies examining calpain-mediated cleavage of the NMDA receptor. Although selective cleavage of the NR2A subunit in the C-terminal region (an intracellular domain which is not required for activity) (Sprengel et al., 1998) has been shown, stably cleaved forms of the NMDA receptor have not been readily found in neurons (Dingledine et al., 1999). In addition, some studies suggest that a loss of NMDA receptor activity is produced by calpain cleavage in situ (Bi et al., 2000), even though cleavage occurred in a region that is nonessential for activity. The present results suggest that calpain cleavage may act as a
trigger for NMDA receptor processing by other intracellular proteases. This hypothesis is consistent with the findings of Wang et al. (2000), who demonstrated that NR2 subunits were rapidly degraded in post-mortem brain to fragments similar in size to those observed previously in vitro (Guttmann et al., 2001) and also retained ligand binding, although a direct role for calpain was not determined.

Protease-mediated regulation of glutamate receptors has been proposed previously in tissue plasminogen activator regulation of NMDA receptors (Nicole et al., 2001) and AMPA receptor cleavage by calpains or caspases (Glazner et al., 2000). The proposed calpain cleavage of the NMDA receptor may be important in controlling secondary modulatory systems similar to those in AMPA receptor regulation, where caspase-mediated degradation of the AMPA receptor specifically shifts cell death from necrosis to apoptosis (Glazner et al., 2000). However, the putative mechanisms that lead to further NMDA receptor degradation beyond calpain are not clear. Recent studies have demonstrated regulation of NMDA receptor levels by binding of interacting proteins such as F-actin and specific internalization motifs in the C terminus (Lan et al., 2001; Lei et al., 2001; Roche et al., 2001; Scott et al., 2001). Cleavage by calpain could alter interactions with such binding proteins, perhaps leading to degradation by other proteases or revealing internalization motifs that cause lysosomal degradation (a subject of future study).

Since calpain in this system is stimulated directly by NMDA receptor activation, the present results suggest that calpain cleavage of the NR2A subunit can be a selective pathway for feedback inhibition of NMDA receptor activity, perhaps by decreasing the number of functional receptors. Prolonged stimulation of NMDA receptors results in a phenomenon known as calcium-dependent inactivation (Legendre et al., 1993) for which multiple mechanisms have been proposed. Several studies have shown that calcium-dependent inactivation does not involve calpain, because inactivation was not altered by calpain inhibitors but required a specific calmodulin-binding component of the NR1 subunit (Krupp et al., 1996; Zhang et al., 1998) and involved dissociation of the NMDA receptor from actin. Another proposed calpain-independent mechanism of NMDA receptor inactivation involves the dissociation of NMDA receptors from actin by the actin-cleaving protease gelsolin (Furukawa et al., 1997). The physiological effects observed in the present study, however, suggest that in addition to these mechanisms, there is a calpain-mediated pathway that can result in decreased NMDA receptor activity. Additionally, in contrast to other proposed mechanisms, neither the NR1 subunit nor actin is significantly degraded in the present paradigm.

The present results link calpain cleavage of NMDA receptors to models of excitotoxicity using modest-duration exposure to glutamate in which biochemical assays and physiological approaches were performed in parallel with similar results. Although synaptic modifications of NMDA receptors may occur over a shorter time period than those used in the present study, the results of calcium imaging studies suggest that calpain may modulate NMDA receptor function either at a basal level of activity or rapidly after agonist-induced activation of the receptor. In addition, the degree of NMDA receptor modification necessary for significant modification of synaptic architecture and the exact properties of calpain activation in single dendritic spines make quantitative comparison of the events in our model system with synaptic events difficult. However, direct application of the results of the present study to neuronal paradigms may help to better define the role of calpain cleavage of the NMDA receptor in synaptic modification.

Besides the NMDA receptor, other synaptic proteins are physiological substrates of calpain including neuronal nitric oxide synthase, calmodulin kinase, and other calcium channels (Dosemeci and Reese, 1995; Hell et al., 1996). Class L-type calcium channels have increased calcium permeability following calpain cleavage (Hell et al., 1996), whereas ryanodine receptor channel activity is decreased by calpain proteolysis (Shevchenko et al., 1998). In the case of NR2A, calpain cleavage appears to result primarily in decreased NMDA receptor amount. Although the C-terminal region of NR2A where calpain cleaves is not required for electrophysiological activation of the receptor, it may affect other properties to a modest degree (Sprengel et al., 1998). Thus, if neuronal stabilizing mechanisms exist, calpain cleavage might lead to intermediate NMDA receptors with novel properties as previously suggested (Guttmann et al., 2001). In addition, previous studies have shown that NR2A phosphorylation by protein kinase C inhibits calpain cleavage of the NR2 subunit (Bi et al., 1998a), protein kinase C phosphorylation may be involved in blocking calpain-mediated degradation of the AMPA receptor. Thus, some of these second messenger systems themselves may be regulated by calpain, and further amplification of the direct calpain-mediated NMDA receptor modulation may also occur.

Because both NMDA receptors and calpain have been linked to neuropathological conditions such as excitotoxicity (Lynch and Guttmann, 2001), the present findings suggest new possibilities for understanding the mechanisms of neurodegeneration and neuroprotection. Calpain inhibitors have been proposed as neuroprotective agents. The present data suggest that although calpain inhibition may block the cellular degradation in response to excitotoxic stimuli, it may indirectly potentiate cell death. Based on the current findings, antagonists to NMDA receptors, while preventing the normal activation of calpain, would decrease NMDA receptor turnover. The presence of additional NMDA receptors may lead to a delayed and more dramatic increase in intracellular calcium once the pharmacological inhibitor is removed or metabolized. Alternatively, the low molecular weight products generated by the selective calpain cleavage of the NR2A subunit may have specific roles in neuronal function or pathology, as similar calpain-generated fragments from tropolin have been shown to have in cardiac disease (Murphy et al., 2000). This is consistent with our previous finding that NMDA receptors lacking the final 420 amino acids are less toxic when transfected into cells, despite having similar physiologic properties (Anegawa et al., 2000; Guttmann et al., 2001).

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