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Calpain Activation and Na/Ca Exchanger Degradation Occur Downstream of Calcium Deregulation in Hippocampal Neurons Exposed to Excitotoxic Glutamate

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Abstract

Delayed calcium deregulation (DCD) plays an essential role in glutamate excitotoxicity, a major detrimental factor in stroke, traumatic brain injury, and various neurodegenerations. In the present study, we examined the role of calpain activation and Na⁺/Ca²⁺ exchanger (NCX) degradation in DCD and excitotoxic cell death in cultured hippocampal neurons. Exposure of neurons to glutamate caused DCD accompanied by secondary mitochondrial depolarization. Activation of calpain was evidenced by detecting NCX isoform 3 (NCX3) degradation products. Degradation of NCX isoform 1 (NCX1) was below the detection limit of western blotting. Degradation of NCX3 was detected only after an hour of incubation with glutamate, while DCD occurred on average within 15 minutes after glutamate application. Calpeptin, an inhibitor of calpain, significantly attenuated NCX3 degradation but failed to inhibit DCD and excitotoxic neuronal death. Calpain inhibitors I, III, and VI also failed to influence DCD and glutamate-induced neuronal death. On the other hand, MK801, an inhibitor of the NMDA-subtype of glutamate receptors, added shortly after the initial glutamate-induced jump in cytosolic Ca²⁺, completely prevented DCD and activation of calpain and strongly protected neurons against excitotoxicity. Taken together, our results suggest that, in glutamate-treated hippocampal neurons, the initial increase in cytosolic Ca²⁺ that precedes DCD is insufficient for sustained calpain activation, which most likely occurs downstream of DCD.

Keywords

neuron; glutamate; calcium; excitotoxicity; calpain; calpeptin

INTRODUCTION

A prolonged exposure of neurons to glutamate causes overstimulation of glutamate receptors and produces a massive Ca²⁺ influx and significant elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]_c) (Tymianski et al., 1993b). In neurons exposed to excitotoxic glutamate, the changes in [Ca²⁺]_c occur in three steps. Exposure to glutamate causes (i) an initial jump in [Ca²⁺]_c followed by (ii) its transient decrease to a lower but still elevated level. After some delay, this decrease in [Ca²⁺]_c is followed by (iii) a larger, sustained

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elevation of $[Ca^{2+}]_c$ that can persist while glutamate is present and even after the removal of glutamate (Nicholls and Budd, 2000). The secondary increase in $[Ca^{2+}]_c$, or “*delayed calcium deregulation*” (DCD) (Nicholls and Budd, 1998; Tymianski et al., 1993a), appears to be essential for neuronal death, and therefore is considered a hallmark of glutamate excitotoxicity (Budd and Nicholls, 1996; Manev et al., 1989; Thayer and Miller, 1990; Tymianski et al., 1993b). Stabilizing $[Ca^{2+}]_c$ by chelating excessive cytosolic Ca^{2+} with BAPTA increased the survival rate of neurons exposed to excitotoxic glutamate (Tymianski et al., 1993c; Tymianski et al., 1994). Thus, DCD represents a key step in excitotoxicity. However, the precise molecular mechanisms leading to DCD are still not completely understood.

An elevation of cytosolic Ca^{2+} in neurons exposed to glutamate may cause activation of calpain, a cytosolic Ca^{2+} -dependent protease (Adamec et al., 1998; Siman et al., 1989). In the cell, calpain has different substrates including elements of cytoskeleton, such as α -spectrin, and components of Ca^{2+} transport machinery, such as glutamate/NMDA receptors (NMDAR), glutamate/AMPA receptors, and the Ca^{2+} -ATPase of the plasma membrane (Pottorf et al., 2006; Wang, 2000; Wu et al., 2005; Xu et al., 2007; Yuen et al., 2007). While early studies with cultured hippocampal neurons failed to establish an essential role of calpain 1 in excitotoxic cell death (Adamec et al., 1998; Manev et al., 1991), more recent studies reveal that the activation of calpain and proteolytic degradation of Na^+/Ca^{2+} exchanger isoform 3 (NCX3) contribute to DCD in cerebellar granule neurons and lead to subsequent neuronal death (Bano et al., 2005).

There are two groups of plasmalemmal Na^+/Ca^{2+} exchangers (NCX) in the central nervous system, K^+ -dependent and K^+ -independent (Blaustein and Lederer, 1999). There are three major isoforms of K^+ -independent NCX in the brain: NCX1, NCX2, and NCX3 (Li et al., 1994; Nicoll et al., 1990; Nicoll et al., 1996). In cultured hippocampal and cortical neurons, NCX1 is a dominant isoform, whereas NCX3 is less expressed in these neurons (Kiedrowski et al., 2004; Sakaue et al., 2000). NCX2 is predominantly expressed in glial cells (Thurneysen et al., 2002a; Thurneysen et al., 2002b), although other investigators also found neuronal localization of NCX2 (Minelli et al., 2007). In cultured cerebellar granule neurons, all three isoforms are detected, with NCX3 being a dominant isoform (Kiedrowski et al., 2004). Only NCX3 appeared to be a substrate for glutamate-triggered, calpain-mediated proteolytic degradation in cerebellar granule neurons (Bano et al., 2005). Application of the membrane-permeable peptide calpeptin, an exogenous inhibitor of calpain, as well as overexpression of calpastatin, a potent, endogenous inhibitor of calpain, significantly diminished proteolytic degradation of NCX3 in cerebellar granule neurons. In addition, overexpression of calpastatin suppressed glutamate-induced DCD in cerebellar granule neurons and reduced excitotoxic neuronal death (Bano et al., 2005).

In contrast to cerebellar granule cells, hippocampal neurons are derived from the brain region, which is much more vulnerable to cell death following ischemic stroke than the cerebellum, and therefore these neurons seem to be more relevant in studies of excitotoxicity and stroke (Nicholls, 2004). In the present study, we examined the role of calpain activation and NCX degradation in DCD and cell death of cultured hippocampal neurons exposed to excitotoxic glutamate. The results obtained suggest that, in cultured hippocampal neurons, calpain activation and NCX degradation are not involved in the development of DCD and most likely take place downstream of DCD.

Experimental procedures

Materials

Glutamate, glycine, EGTA, and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Sigma (St. Louis, MO). Fura-2FF AM was bought from Teflabs (Austin, TX), and Rhodamine-123 (FluoroPure™ grade) was purchased from Invitrogen (Carlsbad, CA). MK801 and calpain inhibitors were from Calbiochem (San Diego, CA).

Cell culturing

Primary cultures of hippocampal neurons were prepared from postnatal day 1 rat pups according to Institutional Animal Care and Use Committee approved protocol and previously described procedures (Dubinsky, 1993). We used entire hippocampi dissected from the brains of rat pups as a starting material. For toxicity experiments, western blotting and fluorescence measurements, neurons were plated on glass-bottomed Petri dishes coated with poly-D-lysine without preplated glia as previously described (Dubinsky, 1993). For all platings, 35 µg/ml uridine plus 15 µg/ml 5-fluoro-2'-deoxyuridine were added 24 hours after plating to inhibit proliferation of non-neuronal cells. Neuronal cultures were maintained in a 5% CO₂ atmosphere at 37°C in Minimum Essential Medium with Earle's salts (Invitrogen) supplemented with 10% NuSerum (BD Bioscience, Bedford, MA), 27 mM glucose, and 26 mM NaHCO₃ (Dubinsky et al., 1995). The plating density of cultured neurons was ~10⁵ cells/cm². In our hands, hippocampal neurons did not require growth medium change over two weeks of culturing *in vitro*.

Fluorescence imaging of cultured neurons

Neurons (12–14 DIV) were co-loaded at 37°C with 2.6µM Fura-2FF-AM and 1.7µM Rhodamine 123 (Rh123). Then, neurons were rinsed twice with a standard bath solution containing 139 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 10 mM NaHEPES, pH 7.4, 5 mM glucose, and 65 mM sucrose. The ion composition of the bath solution is close to those used previously in studies of DCD and excitotoxicity (Dubinsky et al., 1995; Kushnareva et al., 2005; Wang and Thayer, 1996; White and Reynolds, 1996). Sucrose was used to maintain osmolarity similar to that in the growth medium (340 mosm). Osmolarity of the bath solution was measured with an osmometer Osmette II™ (Precision Systems Inc., Natick, MA). Fluorescence imaging was performed with an inverted microscope Nikon Eclipse TE2000-U using Nikon objective Plan Fluor 20× 0.45 NA and a back-thinned EM-CCD camera Hamamatsu C9100-12 (Hamamatsu Photonic Systems, Bridgewater, NJ) controlled by Simple PCI software 6.1 (Compix Inc., Sewickley, PA) or using objective Super Fluor 20× 0.75 NA and a Photometrics cooled CCD camera CoolSNAP_{HQ} (Roper Scientific, Tucson, AZ) controlled by MetaFluor 6.3 software (Molecular Devices, Downingtown, PA). The excitation light was delivered by a Lambda-LS system (Sutter Instruments, Novato, CA). The excitation light at 480 nm was attenuated by a neutral density filter to 10%. The excitation filters (340±5, 380±7, and 480±20) were controlled by a Lambda 10-2 optical filter changer (Sutter Instruments, Novato, CA). Fluorescence was recorded through a 505 nm dichroic mirror at 535±25 nm. To minimize photobleaching and phototoxicity, the images were taken every 15 seconds during the time-course of the experiment using the minimal exposure time that provided acceptable quality of images. The experiments were performed in the bath solution without perfusion. After 3 minutes of fluorescence recording in the standard bath solution, 100µM glutamate plus 10µM glycine were applied to neurons. At the end of the experiment, glutamate and Ca²⁺ were removed by rinsing cells three times with the bath solution without glutamate and Ca²⁺. 1µM FCCP was applied to neurons to depolarize neuronal mitochondria and to release accumulated Ca²⁺. The changes in [Ca²⁺]_c were monitored by following Fura-2FF fluorescence ratio F₃₄₀/F₃₈₀,

calculated after subtracting the background from both channels. The Fura-2FF results were not converted in μM of Ca^{2+} because it was shown previously that the calibration of Fura-2-related dyes cannot be accurately performed without measuring intracellular dye concentration (Dineley et al., 2002). The changes in mitochondrial membrane potential ($\Delta\psi$) were monitored by following changes in fluorescence of Rh123 (excitation at 480 nm, emission at 535 nm) expressed as F/F_0 . The Rh123 fluorescence traces were also constructed after subtracting the background.

Western blotting

Cultured hippocampal neurons were solubilized with a solution containing 50 mM Tris-HCl, pH 7.35, 2 mM EDTA, 5 mM dithiothreitol, 1% Nonidet P-40, and supplemented with a Proteinase Inhibitor Cocktail (Roche, Indianapolis, IN). Aliquots of this solution were mixed with NuPAGE[®] LDS sample buffer (Invitrogen, Carlsbad, CA), supplemented with a 1× NuPAGE[®] reducing agent (Invitrogen), and incubated at 70°C for 15 minutes. 3–8% Tris-Acetate gels for α -spectrin and 7% Tris-Acetate gels (Invitrogen) for NCXs were used in electrophoresis (5 μg protein/lane for α -spectrin and 5–50 μg protein/lane for NCX1 and NCX3). After electrophoresis, proteins were transferred to Hybond[™]-ECL[™] nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Blots were incubated for an hour at room temperature with one of the following primary antibodies: mouse monoclonal antibody against α -spectrin (Biomol International, Plymouth Meeting, PA) at 1:2000 dilution with 5% non-fat dry milk, 0.15% Triton X-100 in phosphate-buffered saline (PBS), pH 7.2, mouse monoclonal anti-NCX1 antibody (NCX1 mAb R3F1) and rabbit polyclonal antibody against NCX3 (NCX3 pAb 95209), generated in the laboratory of Dr. Kenneth Philipson (UCLA), both at 1:1000 dilution. The same blots were re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with rabbit polyclonal anti-GAPDH antibody (Abcam, Cambridge, MA) to ensure equal loading. Blots were developed using goat anti-mouse or goat anti-rabbit IgG (1:20000) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and Supersignal West Pico chemiluminescent reagents (Pierce, Rockford, IL). Molecular weight marker HiMark[™] Pre-Stained Standards (15 μl) (Invitrogen) were used to determine molecular weights of the bands. Western blots were quantified using Quantity One[®] software (Bio-Rad Laboratories, Hercules, CA), and densitometry data were expressed in arbitrary units (a.u.) for the corresponding bands.

Glutamate toxicity

The growth medium from cultured hippocampal neurons (12–14 DIV) was removed and replaced with Minimum Essential Medium (Invitrogen) without serum supplemented with 27 mM glucose, 35 mM sucrose, 100 μM glutamine, and 10 μM glycine (Brustovetsky et al., 2004). Cultures were exposed to various concentrations of glutamate (3–1000 μM) for 10 minutes with or without MK801. MK801 (20 μM) was added to neurons 90 seconds after glutamate. Then, glutamate was removed by rinsing cells three times with the Earle's Balanced Salt Solution (EBSS, Sigma), returned in incubator and cell death was evaluated 24 hours later using the Trypan Blue exclusion method in a blind manner (Dubinsky and Rothman, 1991). In addition, we evaluated neuronal death with or without MK801 (20 μM) following exposure to 100 μM glutamate (plus 10 μM glycine) by assessing propidium iodide nuclear staining (Li et al., 2009). In these experiments, calcein staining was used to visualize viable cells. Calcein staining was performed with 1 μM calcein-AM (Invitrogen). Alternatively, cells were exposed to 30 or 100 μM glutamate plus 10 μM glycine with or without calpain inhibitors. In these experiments, neurons were pre-incubated with calpain inhibitors for 30 minutes prior to glutamate exposure. Calpain inhibitors were present in the bath solution during glutamate application as well as during the 24 hours after glutamate removal. After 24 hours, cell death was evaluated in a blind manner using the Trypan Blue

exclusion method (Dubinsky and Rothman, 1991). In addition, we evaluated neuronal death with or without calpeptin (20 μ M) following exposure to 100 μ M glutamate (plus 10 μ M glycine) by assessing propidium iodide nuclear staining (Li et al., 2009).

Statistics

Statistical analysis of the experimental results consisted of one-way ANOVA followed by Bonferroni's *post hoc* test (GraphPad Prism® 4.0, GraphPad Software Inc., San Diego, CA). The data represent mean $\pm$ S.E.M. of at least three separate, independent experiments.

Results

Calpain activation and NCX degradation

NCX1 and NCX3 are the major isoforms of NCX responsible for Ca²⁺ extrusion from neurons (Kiedrowski et al., 2004; Sakaue et al., 2000). In the present study, we performed western blot analysis to determine the time-course and the extent of NCX1 and NCX3 degradation in the experiments with cultured hippocampal neurons exposed to glutamate. With monoclonal anti-NCX1 antibody we did not detect NCX1 degradation products (Fig. 1). Larger protein loading (50 μ g protein per lane) did not reveal NCX1 degradation bands (not shown). Therefore, in our experiments, degradation of NCX1 could be judged only by evaluating a change in the amount of intact NCX1. Exposure of cultured hippocampal neurons to 100 μ M glutamate (plus 10 μ M glycine) for 20 or 60 minutes failed to cause a decrease in intensity of the NCX1 band, suggesting that most of NCX1 remained intact (Fig. 1).

Similarly to NCX1, treatment of hippocampal neurons with 100 μ M glutamate (plus 10 μ M glycine) for 20 minutes, an hour, or two hours failed to cause a decrease in intensity of the NCX3 band (Fig. 2A,C). However, with larger protein loading (50 μ g protein per lane) anti-NCX3 antibody could detect NCX3 degradation products (Fig. 2B,D). Yet, NCX3 degradation products could be definitely detected only after an hour of incubating neurons with 100 μ M glutamate (plus 10 μ M glycine), while after 20 minutes of glutamate exposure the level of NCX3 degradation products was statistically insignificant. Of note, the amount of NCX3 degradation product appeared to be miniscule in regard to the amount of intact NCX3 (Fig. 2B). Calpeptin (20 μ M), a membrane-permeable calpain inhibitor, attenuated generation of NCX3 degradation products (Fig. 2B,D) attributing NCX3 degradation to calpain activity.

Inhibitors of calpain failed to prevent DCD

In our study, we hypothesized that calpeptin, by inhibiting calpain and preventing NCX3 degradation, might suppress DCD in hippocampal neurons exposed to glutamate. In addition to calpeptin, we tested three other potent calpain inhibitors in the calcium imaging experiments. In our experiments with cultured hippocampal neurons, prolonged exposure to glutamate caused elevation in [Ca²⁺]_c as judged by an increase in Fura-2FF F₃₄₀/F₃₈₀ fluorescence ratio (Fig. 3A,D) accompanied by mitochondrial depolarization (data not shown). The increase in [Ca²⁺]_c occurred in three steps, including (i) an initial fast rise in [Ca²⁺]_c followed by (ii) a transient decrease and (iii) a secondary sustained elevation in [Ca²⁺]_c. The latter we, like other investigators (Nicholls and Budd, 2000), defined as DCD. To provide a statistical analysis of the data obtained in different experiments, we introduced a quantitative parameter: *the time from the beginning of glutamate exposure to completion of the DCD* (*t*_{DCD}) (Fig. 3A). Recently, a similar approach was used to analyze secondary mitochondrial depolarization in cultured neurons exposed to glutamate (Vergun et al., 2003). To find *t*_{DCD} we determine x value of the interception point of two lines using Origin 7.5 software (OriginLab Corp., Northampton, MA): a horizontal, straight line corresponding to

the maximal value of Fura-2FF ratio $F_{340/380}$ and a line derived by linear regression approximating the uprising phase in Fura-2FF ratio $F_{340/380}$ signal. Here and in other similar Figures, thin grey traces show signals from individual neurons from the same dish, while thick black traces show averaged signals (mean $\pm$ SEM) for Fura-2FF fluorescence ratio F_{340}/F_{380} . We did not routinely convert the Fura-2FF F_{340}/F_{380} ratio in μM of Ca^{2+} because it was previously shown that the calibration of Fura-2-related dyes cannot be accurately performed without measuring intracellular dye concentration (Dineley et al., 2002). However, using the method described by Grynkiewicz et al. (Grynkiewicz et al., 1985), we made evaluations of $[\text{Ca}^{2+}]_c$ based on the assumption that Fura-2FF $K_d(\text{Ca}^{2+})$ is $5.5\mu\text{M}$ (information provided by the manufacturer), and intracellular dye concentration does not significantly affect $[\text{Ca}^{2+}]_c$ calculation. In our experiments, $100\mu\text{M}$ glutamate (plus $10\mu\text{M}$ glycine) produced the initial glutamate-induced rise in $[\text{Ca}^{2+}]_c$ up to $3\text{--}4\mu\text{M}$ and DCD with sustained increase in $[\text{Ca}^{2+}]_c$ up to $8\text{--}12\mu\text{M}$.

Our experiments demonstrated that neither calpeptin ($\text{IC}_{50}=52\text{ nM}$ for calpain 1 and 34 nM for calpain 2, Fig. 3A–C) nor calpain inhibitor-III (CI-III or MDL-28170, $\text{IC}_{50}=8\text{ nM}$ for both calpain 1 and 2, Fig. 3D–E; here and further, IC_{50} determined with purified calpain enzymes *in vitro*) protected neurons against DCD. The secondary, sustained mitochondrial depolarization that occurred simultaneous to DCD also was not deferred by calpain inhibitors (data not shown). Calpain inhibitor-I (CI-I or ALLN, $\text{IC}_{50}=190\text{ nM}$ for calpain 1 and 220 nM for calpain 2) and calpain inhibitor-VI (CI-VI, $\text{IC}_{50}=7.5\text{ nM}$ for calpain 1 and 78 nM for calpain 2) were also ineffective in protecting against DCD (Supplementary Figs. 1 and 2). Of note, in the experiments shown in these Figures, we used concentrations of calpain inhibitors significantly higher than their IC_{50} . Lower ($0.1\mu\text{M}$) and higher ($50\mu\text{M}$) concentrations of the inhibitors (calpeptin, CI-I, CI-III, and CI-VI) also did not protect against DCD (data not shown). Interestingly, calpeptin and CI-III statistically significantly shortened the time to DCD. The mechanisms of this deleterious effect are not yet clear.

Inhibitors of calpain failed to protect neurons against glutamate-triggered cell death

The failure of calpain inhibitors to antagonize DCD in neurons exposed to glutamate suggested that these inhibitors might be not effective in protecting neurons against excitotoxic neuronal death. To assess neuronal viability, we employed the Trypan Blue exclusion method (Dubinsky and Rothman, 1991). None of the four calpain inhibitors tested in this study (calpeptin and CI-III, Fig. 4; CI-I, and CI-VI, Supplementary Fig. 3) increased the survival rate of neurons exposed to excitotoxic glutamate. A similar result was obtained with propidium iodide (PI) nuclear staining used to assess neuronal death (Fig. 5). Glutamate exposure ($100\mu\text{M}$ Glu plus $10\mu\text{M}$ glycine, 10 minutes) significantly increased PI nuclear staining 24 hours later indicating permeabilization of the plasma membrane of affected neurons. Calpeptin ($20\mu\text{M}$) failed to protect neurons against glutamate toxicity (Fig. 5). Thus, either efficacy of calpain inhibitors was not sufficient to completely inhibit calpain and thus protect neurons, or other calpain-independent deleterious mechanisms might contribute to neuronal death following exposure of neurons to excitotoxic glutamate.

MK801 added shortly after glutamate prevented DCD and calpain activation

In the next experiments, we addressed the question whether the initial glutamate-induced increase in $[\text{Ca}^{2+}]_c$ could be sufficient for sustained activation of calpain and cell death in cultured hippocampal neurons. An exposure of cultured hippocampal neurons to glutamate produced typical changes in $[\text{Ca}^{2+}]_c$ culminating in DCD (Fig. 6A). In these experiments, we simultaneously monitored changes in mitochondrial membrane potential to monitor mitochondrial depolarization that occurred parallel to the increase in $[\text{Ca}^{2+}]_c$ (Fig. 6A, a lower part). Application of $100\mu\text{M}$ glutamate (plus $10\mu\text{M}$ glycine) produced a rapid but transient increase in $[\text{Ca}^{2+}]_c$ and initial mitochondrial depolarization (Fig. 6A). Blockade of

NMDAR with MK801, applied 90 s after glutamate application, completely prevented DCD and secondary mitochondrial depolarization (Fig. 6B). To assess the extent of calpain activation in these experiments, we followed formation of the degradation products of α -spectrin, an abundant component of cytoskeleton and a typical substrate for calpain (Sorimachi and Suzuki, 2001). Exposure of neurons to glutamate caused a distinct accumulation of α -spectrin degradation products manifested as the appearance of 150/145 kDa bands (Fig. 6C). Degradation of α -spectrin occurred in a time-dependent manner, and the products of α -spectrin degradation could be detected as early as 20 minutes after glutamate application. In untreated cells, the level of α -spectrin degradation products was below the detection limit of western blotting (not shown). The blockade of NMDAR with MK801 applied 90 s after glutamate and suppression of DCD prevented activation of calpain and formation of α -spectrin degradation products (Fig. 6C,D).

The MK801-imposed NMDAR blockade, suppression of DCD, and prevention of calpain activation were accompanied by an increased survival rate of neurons over the wide range of glutamate concentrations tested in our experiments (Fig. 6E). A similar result was obtained with propidium iodide (PI) nuclear staining used to assess neuronal death (Fig. 7). Exposure of neurons to glutamate (100 μ M, plus 10 μ M glycine) for 10 minutes significantly increased the number of cells with PI nuclear staining 24 hours after glutamate removal. MK801 (20 μ M) added 90 s after glutamate protected neurons against glutamate toxicity (Fig. 7). These results confirmed previous observations that MK801, added shortly after glutamate, protected neurons against excitotoxic cell death (Brustovetsky et al., 2004) and emphasized an essential role of sustained elevation in $[Ca^{2+}]_c$ in glutamate excitotoxicity. Taken together, the results obtained with MK801 strongly suggest that the transient increase in $[Ca^{2+}]_c$ preceding DCD is not sufficient to activate calpain and cause neuronal death.

Discussion

Overstimulation of glutamate receptors and the increase in $[Ca^{2+}]_c$ that occurs in acute ischemic neurodegeneration result in activation of calpain (Bartus et al., 1995), a Ca^{2+} -dependent protease that degrades vitally important proteins in the neuron resulting in neuronal death (Goll et al., 2003). It has been shown that activation of calpain 1 takes place in traumatic spinal cord injury and significantly contributes to degradation of cytoskeletal proteins (Springer et al., 1997). Focal cerebral ischemia also resulted in calpain activation, which could be inhibited by NMDAR antagonists (Minger et al., 1998). Glutamate application to cultured cerebellar granule neurons resulted in calpain activation within 30 minutes (Bano et al., 2005). This calpain activation led to subsequent NCX3 degradation that occurred upstream of DCD. The temporal relationship between calpain activation and DCD in hippocampal neurons exposed to excitotoxic glutamate up until now has remained unknown. In the present study, we provide evidence that calpain activation in glutamate-treated cultured hippocampal neurons most likely takes place downstream of DCD, and therefore calpain activation represents the consequence of DCD rather than its cause. However, a few points have to be kept in mind. The material for western blotting analysis of calpain activation is taken from a population of neurons while the timing of DCD is measured at the single cell level. Thus, it is possible that calpain could be activated in certain individual cells prior to DCD, but due to the asynchronous timing of DCD, this calpain activation could be below the western blotting threshold for detection of NCX3 degradation products at the cell population level.

Recently, it was shown that calpain activation in cerebellar granule cells leads to degradation of NCX3 (Bano et al., 2005), one of the major NCX isoforms in neurons, particularly in cerebellar granule neurons (Kiedrowski et al., 2004). The authors proposed that NCX3 degradation and a failure to extrude cytosolic Ca^{2+} are accountable for DCD in

this type of neurons. In neurons, NCX has the highest transport capacity for Ca^{2+} extrusion and therefore plays a key role in maintenance of calcium homeostasis (Carafoli et al., 2001). In the forward mode, NCX catalyzes an extrusion of 1 Ca^{2+} in exchange for 3 Na^+ , contributing significantly to maintaining a low level of cytosolic Ca^{2+} (Blaustein and Lederer, 1999). However, in neurons exposed to glutamate, collapse of the Na^+ gradient and depolarization of the plasma membrane (Mayer and Westbrook, 1987; Tsuzuki et al., 1994; Yamaguchi and Ohmori, 1990) might cause a reversal of NCX (Kiedrowski et al., 1994) leading to Ca^{2+} influx into the cell. Therefore, the outcome of NCX inactivation due to proteolytic degradation seems not so obvious.

Calpain activation in neurons exposed to glutamate or NMDA has been investigated in numerous studies. Vanderklish et al. (2000) performed a FRET measurements with a fusion protein containing enhanced yellow and enhanced cyan fluorescent proteins linked by peptide (Vanderklish et al., 2000). This peptide included the μ -calpain cleavage site from α -spectrin that allowed detecting an early activation of calpain in dendritic spines of hippocampal neurons exposed to 100 μM glutamate or NMDA. In a study by Lankiewicz et al. (2000), exposure of cultured hippocampal neurons to NMDA for 5 minutes resulted in calpain 1 activation as judged by subtle α -spectrin degradation (Lankiewicz et al., 2000). The calpain-mediated NCX degradation was not evaluated in either of these studies. However, the authors found that calpain inhibitor 1 protected hippocampal neurons against excitotoxic cell death, and this protection was much stronger if calpain inhibitor 1 was added one hour after NMDA exposure. The authors concluded that the early activation of calpain could be neuroprotective (Lankiewicz et al., 2000). Very recently, Bevers et al. (Bevers et al., 2009) reported neuroprotection achieved by downregulating calpain 2 with shRNA in a primary hippocampal neuron model of NMDA-mediated excitotoxicity while downregulating calpain 1 was not neuroprotective.

The major difference between calpain 1 and 2 is the Ca^{2+} concentration required for activation. In *in vitro* experiments, the activation of calpain 2 requires 400–800 μM Ca^{2+} , whereas calpain 1 activation requires 3–50 μM Ca^{2+} (Bevers et al., 2009; Goll et al., 2003). In our experiments, 100 μM glutamate (plus 10 μM glycine) produced a sustained increase in $[\text{Ca}^{2+}]_c$ up to 8–12 μM which might be sufficient to activate calpain 1 but not calpain 2. Nevertheless, in study by Bevers et al. (Bevers et al., 2009), downregulating calpain 1 was not protective. One possible explanation is that at early stages of excitotoxicity, activation of calpain 1 does not inflict severe damage to the neuron. It is possible that Bevers et al. (Bevers et al., 2009) dealt with the late consequences of NMDAR activation when the plasma membrane essentially lost its barrier properties and $[\text{Ca}^{2+}]_c$ rose to the levels sufficient to activate calpain 2. The authors also reported neuroprotection produced by 1 μM MDL-28170 (Calpain Inhibitor-III) in the experiments with cultured hippocampal neurons treated with 200 μM NMDA (Bevers et al., 2009). In our experiments with glutamate-treated hippocampal neurons, we did not observe neuroprotection with MDL-28170 (CI-III).

Activation of calpain could be just one of multiple pathways leading to neuronal injury and cell death. While the deleterious effect of calpain activation in neuronal ischemia is well documented (Pottorf et al., 2006; Wu et al., 2005; Xu et al., 2007; Yuen et al., 2007), other factors such as activation of poly(ADP-ribose) polymerase-1 (PARP-1) and energy deficit in the cell (Eliasson et al., 1997; Moroni, 2008) or activation of Ca^{2+} -dependent phospholipase A_2 (cPLA $_2$) and degradation of membrane phospholipids (Farooqui et al., 1997) might also contribute to the demise of neurons exposed to excitotoxic glutamate. In the early paper by Manev et al. (1991), stimulation of glutamate receptors in cerebellar granule neurons led to activation of calpain and degradation of its substrate α -spectrin (Manev et al., 1991). In this study, an inhibition of calpain strongly diminished α -spectrin break-down but did not significantly influence cell death. Consistent with this, Manev et al. (1991) proposed that

other enzymes such as lipases, xanthine oxidase, phosphatases, and other proteases might contribute to glutamate excitotoxicity, but individually they are not essential for the initial pathological event, and correspondingly the inhibition of only one of the mechanisms contributing to excitotoxicity would not be sufficient for significant neuroprotection (Manev et al., 1991).

In a recent paper, Araujo et al. (2007) described experiments in which stimulation of AMPA receptors in hippocampal neurons resulted in the reversal of NCX and calpain activation followed by proteolysis of NCX3 (Araujo et al., 2007). In this study, similar to our experiments, the proteolysis of NCX3 was detected only after an hour following exposure to kainate plus cyclothiazide, a selective blocker of AMPA receptor desensitization (Partin et al., 1993). Silencing NCX3 with siRNA attenuated Ca^{2+} uptake by neurons induced by kainate plus cyclothiazide (Araujo et al., 2007). From this viewpoint, proteolytic cleavage and inactivation of the reverse NCX3 by activated calpain could attenuate Ca^{2+} influx and thus could be beneficial for neurons.

In recent studies with an *in vitro* hypoxia model, NCX3 was found to be essential for the maintenance of calcium homeostasis and survival of BHK cells transfected with NCX3 (Secondo et al., 2007b; Secondo et al., 2007a). Consistent with this, calpain-mediated degradation and inactivation of NCX3 was found to be a pivotal factor leading to collapse of calcium homeostasis and cell death in cerebellar granule neurons exposed to excitotoxic glutamate (Bano et al., 2005). However, in our experiments with hippocampal neurons, a subtle increase in NCX3 degradation products was observed only after an hour of incubation with glutamate, while DCD occurred within 15 minutes following glutamate application. Importantly, calpeptin strongly inhibited NCX3 degradation in our experiments. However, neither calpeptin nor other calpain inhibitors protected against DCD. Overall, this suggested that in hippocampal neurons calpain activation and NCX3 degradation most likely occurred downstream of DCD.

This conclusion is further supported by the fact that MK801, a selective NMDAR inhibitor, added shortly after the initial jump in $[\text{Ca}^{2+}]_i$ prevented calpain activation and significantly increased neuronal survival rate. MK801 was also found to strongly attenuate calpain activation in cultured hippocampal neurons exposed to 3-nitropropionic acid (Nasr et al., 2003; Pang et al., 2003). In addition, our results obtained with MK801 suggest a pivotal role for NMDAR in the development of DCD. Later on, DCD manifested in the sustained elevation of $[\text{Ca}^{2+}]_i$ could activate calpain and cause degradation of vital proteins contributing to neuronal injury and eventual cell death.

Supplementary Material

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Figure 1. Western blot analysis of proteolytic degradation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoform 1 (NCX1) in cultured hippocampal neurons exposed to glutamate

In A, neurons were incubated without or with $100\mu\text{M}$ glutamate plus $10\mu\text{M}$ glycine (+Glu) for 20 minutes or an hour as indicated. The same blots were re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading. In B, the columns show the densitometry data normalized by GAPDH loading control, averaged from three independent experiments (mean $\pm$ SEM) and expressed in arbitrary units (a.u.) for the corresponding bands.

Figure 2. Western blot analysis of proteolytic degradation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoform 3 (NCX3) in cultured hippocampal neurons exposed to glutamate

In A and B, neurons were incubated without or with 100 μM glutamate plus 10 μM glycine (+Glu) for 20 minutes, 1 hour, or 2 hours. A 55 kDa band represented a major degradation product of NCX3 (Bano et al., 2005). In A and B, protein loading was 5 or 50 μg per lane respectively. Where indicated, neurons were pre-treated with 20 μM calpeptin for 30 minutes prior to glutamate application. In addition, calpeptin (20 μM) was present in the bath solution during incubation with glutamate. The same blots were re-probed for GAPDH to ensure equal loading. In C and D, the columns show the densitometry data normalized by GAPDH loading control, averaged from three independent experiments (mean $\pm$ SEM) with low (5 μg per lane) and high (50 μg per lane) protein loading and expressed in arbitrary units (a.u.) for the corresponding bands obtained without and with calpeptin. * p <0.05, ** p <0.01 comparing intensity of NCX3 degradation product bands without glutamate treatment and after one hour or two hours of incubation with glutamate respectively; # p <0.05, ## p <0.01 comparing intensity of NCX3 degradation product bands obtained without or with 20 μM calpeptin after 1 or 2 hours of glutamate treatment respectively.

Figure 3. Calpeptin and Calpain Inhibitor-III (CI-III or MDL-28170) failed to protect cultured hippocampal neurons against DCD

In A, B and D, E, neurons were treated with 100 μ M glutamate (Glu) plus 10 μ M glycine as indicated. Here and in other similar Figures, thin grey traces show signals from individual neurons from the same dish, while thick black traces show averaged signals (mean $\pm$ SEM) for Fura-2FF fluorescence ratio F_{340}/F_{380} . In A and D, neurons were pre-treated with a vehicle (1.5 μ l DMSO per 1.5 ml of the standard bath solution) for 30 minutes, and the vehicle was present in the bath solution during the experiment. Here and in other similar Figures, N is the number of neurons examined in each individual representative experiment. In B and E, neurons were pre-treated with 20 μ M calpeptin or 10 μ M CI-III respectively for 30 minutes prior to application of glutamate. In addition, calpeptin and CI-III were present in the bath solution as indicated. In C and F, statistical analysis of t_{DCD} obtained without

calpain inhibitors and in the presence of 5 and 20 μ M calpeptin or 1 and 10 μ M CI-III. t_{DCD} was determined by finding the time between the beginning of glutamate exposure and the intersection point of two linear graphs approximating the uprising fragment of the averaged Fura-2FF ratio F_{340}/F_{380} trace and the fragment corresponding to the elevated $[Ca^{2+}]_c$ plateau. In C, $*p<0.01$ between control without inhibitors and experiments with calpeptin. In F, $*p<0.01$ between control without inhibitors and experiments with 1 μ M CI-III; $**p<0.001$ between control without inhibitors and experiments with 10 μ M CI-III. Here and in other similar *panels* with statistical analysis, N is the total number of neurons examined in these experiments. In A, B and D, E, data are mean $\pm$ SEM, N=6 (6 independent experiments with neurons from 2 different platings). Here and in other similar experiments where indicated, the following agents were added to neurons: EGTA, 250 μ M; FCCP, 1 μ M.

Figure 4. Calpeptin and CI-III failed to improve survival rate of cultured hippocampal neurons exposed to glutamate

Neuronal death was evaluated using the Trypan Blue exclusion method (Dubinsky and Rothman, 1991). Neurons were exposed to 30 or 100 μ M glutamate (Glu) plus 10 μ M glycine for 10 minutes in the absence or presence of various concentrations of calpain inhibitors as indicated. Then, glutamate was removed and cell death was evaluated in a blind manner after 24 hours by unbiased observer counting Trypan Blue stained neurons. Where indicated, calpain inhibitors were added to neurons 30 minutes prior to glutamate application and were kept in the bath solution during and after incubation of neurons with glutamate. Data are mean $\pm$ SEM, N=3 (3 independent experiments with neurons from 3 different platings).

Figure 5. Calpeptin failed to protect cultured hippocampal neurons against glutamate toxicity Neurons were exposed to 100 μ M glutamate (Glu) plus 10 μ M glycine for 10 minutes in the absence or presence of calpeptin (20 μ M) as indicated. Then, glutamate was removed and cell death was evaluated by assessing propidium iodide (PI) nuclear staining (Li et al., 2009). In these experiments, calcein staining was used to visualize viable cells. Calpeptin was added to neurons 30 minutes prior to glutamate application and was kept in the bath solution during incubation of neurons with glutamate and after removal of glutamate. Where indicated, neurons were pre-treated with a vehicle (1.5 μ l DMSO per 1.5 ml of the standard bath solution) for 30 minutes, and the vehicle was present in the bath solution during the experiment. *Bf*, bright field.

Figure 6. MK801 added shortly after the initial rise in $[Ca^{2+}]_c$ prevented DCD, calpain activation, and significantly reduced neuronal death

In A and B, cultured hippocampal neurons (12 DIV) co-loaded with Fura-2FF and Rh123 were exposed to 100 μ M glutamate (Glu) plus 10 μ M glycine. N is the total number of neurons examined in these representative experiments. In B, MK801 (20 μ M) was added as indicated. In A and B, where indicated, the following agents were added to neurons: EGTA, 250 μ M; FCCP, 1 μ M. In C, western blot analysis of proteolytic degradation of α -spectrin in cultured hippocampal neurons exposed to glutamate with and without MK801. Neurons were incubated without or with 100 μ M glutamate plus 10 μ M glycine for 20 or 60 minutes. Where indicated, 20 μ M MK801 was added to neurons 90 s after glutamate application. The same immunoblots were re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading. In D, the columns show the densitometry data

normalized by GAPDH loading control, averaged from three independent experiments (mean±SEM) and expressed in arbitrary units (a.u.) for the corresponding bands. * $p<0.05$ in comparison with control without glutamate; ** $p<0.01$ in comparison with control without glutamate. In E, a glutamate dose-dependence of neuronal death with and without MK801. Neurons were exposed to various concentrations of glutamate plus 10μM glycine for 10 minutes with or without MK801 treatment (20μM, added 90 s after glutamate application). Cell death was evaluated by the Trypan Blue exclusion method 24 hours after glutamate removal (Dubinsky and Rothman, 1991).

Figure 7. MK801 added shortly after glutamate protected cultured hippocampal neurons against glutamate toxicity

Neurons were exposed to 100 μ M glutamate (Glu) plus 10 μ M glycine for 10 minutes in the absence or presence of MK801 (20 μ M) added 90 s after glutamate. Then, glutamate was removed and cell death was evaluated by assessing propidium iodide (PI) nuclear staining (Li et al., 2009). In these experiments, calcein staining was used to visualize viable cells. Where indicated, neurons were pre-treated with a vehicle (1.5 μ l DMSO per 1.5 ml of the standard bath solution) for 30 minutes, and the vehicle was present in the bath solution during the experiment. *Bf*, bright field.