Excitotoxicity Affects Membrane Potential and Calmodulin Kinase II Activity in Cultured Rat Cortical Neurons

Severn B. Churn, PhD; Sompong Sombati, PhD; William C. Taft, PhD; and Robert J. DeLorenzo, MD, PhD, MPH

Background and Purpose: Glutamate-induced excitotoxicity has been implicated as a causative factor for selective neuronal loss in ischemia and hypoxia. Toxic exposure of neurons to glutamate results in an extended neuronal depolarization that precedes delayed neuronal death. Because both delayed neuronal death and extended neuronal depolarization are dependent on calcium, we examined the effect of glutamate exposure on extended neuronal depolarization and calcium/calmodulin-dependent protein kinase II (CaM kinase II) activity.

Methods: Three-week-old cortical cell cultures from embryonic rats were exposed to 500 μM glutamate and 10 μM glycine or to control medium for 10 minutes. Cells were examined for neuronal toxicity, electrophysiology, and biochemical alterations. In one set of experiments, whole-cell current clamp recording was performed throughout the experiment. In a parallel experiment, cortical cultures were allowed to recover from glutamate exposure for 1 hour, at which time the cells were homogenized and CaM kinase II activity was assayed using standard techniques.

Results: Excitotoxic exposure to glutamate resulted in extended neuronal depolarization, which remained after removal of the glutamate. Glutamate exposure also resulted in delayed neuronal death, which was preceded by significant inhibition of CaM kinase II activity. The excitotoxic inhibition of CaM kinase II correlated with neuronal loss, was N-methyl-D-aspartate receptor-mediated, and was not due to autophosphorylation of the enzyme.

Conclusions: Glutamate-induced delayed neuronal toxicity correlates with extended neuronal depolarization and inhibition of CaM kinase II activity. Because inhibition of CaM kinase II activity significantly preceded the histological loss of neurons, the data suggest that modulation of CaM kinase II activity may be involved in the cascade of events resulting in loss of calcium homeostasis and delayed neuronal death. (Stroke 1993;24:271-278)

KEY WORDS • N-methyl-D-aspartate • MK-801 • neuronal death

Electrotoxic effects of excitatory amino acids have been implicated in various neurological disorders and have been used for excitotoxic models of delayed neuronal death.1-3 Exposure of neurons to excitotoxic levels of glutamate results in osmotic lysis4,5 (acute toxicity) or a delayed calcium-dependent neuronal death.6-11 Glutamate-induced delayed neuronal excitotoxicity has been shown to be dependent on stimulation of the N-methyl-D-aspartate (NMDA) sub-

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pear phase-bright and exclude vital dyes. Another important characteristic of END is that its expression is dependent on both the presence of extracellular calcium and the activation of NMDA-subtype glutamate receptors.12

Because delayed neuronal death and the development of END following toxic glutamate exposure are calcium-dependent, we examined the effect of excitotoxic glutamate exposure on an intracellular calcium-regulated system. Calcium/calmodulin-dependent protein kinase II (CaM kinase II) is a neuronally enriched, calcium-regulated enzyme that is sensitive to the effects of excitotoxic paradigms such as seizure activity14-17 and ischemia18-23 in many animal models. Inhibition of CaM kinase II activity is an early and long-lasting phenomenon following ischemia.19-21,23 In addition, ischemia-induced inhibition is not due to proteolytic destruction of the enzyme but is most likely due to a posttranslational modification of the protein.18-22 Therefore, we examined whether there is a correlation between inhibition of CaM kinase II activity and the development of END in the same model of excitotoxicity.

Materials and Methods

Cortical Cell Culture

Primary cortical neuron cultures were prepared from E-16 embryonic rats as described previously.3,24 Neurons were grown in 25-mm2 flasks at a density of 1.5 million cells/flask for biochemical assays and on 35-mm Petri dishes for physiology recording. Neurons were fed twice weekly with growth medium containing minimum essential medium, 5% horse serum, 25% glial-conditioned medium, and N3 nutrient supplement. The cultures were maintained in a 95% humidified atmosphere, 5% CO2 at 37°C. Three-week-old cultures from several animals were used for experimentation. For histological analysis, viable neurons were quantified by previously established techniques.3,24 Neurons were identified by phase-bright soma with intact processes. Nonviable neurons were determined by standard criteria, which included loss of phase-brightness and fragmented processes. In some experiments, trypan blue–exclusion methods were performed and found to give similar results for neuronal toxicity (data not shown). Differences between treatment groups were determined by one-way analysis of variance (ANOVA) computed using GRAPHPAD software (San Diego, Calif.). Cell counts are expressed as percent of viable neurons originally present.

Electrophysiology Methods

 Cultures were removed from the incubator, and the culture medium was gradually changed to a recording solution containing (in mM) 145 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 d-glucose, 10 Na-HEPES, and 0.01 glycine (pH 7.3), adjusted to 325 mosm with sucrose. The cultures were then transferred to the stage of an inverted phase-contrast microscope (Diaphot, Nikon, Tokyo, Japan), and continuously perfused with recording solution. Fire-polished patch electrodes (2–5 MΩ; fill solution 140 mM K+-gluconate, 1 mM MgCl2, and 10 mM K-HEPES, pH 7.2) were used for whole-cell current clamp recording at room temperature.20 Data were recorded both on-line using a chart recorder (Dash II, Astro-Med, Inc., West Warwick, R.I.; frequency response of DC–500 Hz) and on videotape using a PCM device (Instrutech, Mineola, N.Y.) for later analysis. Recording solutions containing 500 μM glutamate and 10 μM glycine were applied by microperfusion through a multibarrel Teflon concentration clamp apparatus27,28 for 10 minutes. Solution changes were accomplished by moving the perfusing system laterally, with each barrel flowing at a rate of 0.5 ml/hr.12

Calcium/Calmodulin-Dependent Protein Kinase II Assays

Under sterile conditions, the culture medium was replaced with recording solution containing (in mM) 145 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 d-glucose, 10 Na-HEPES, and 0.01 glycine (pH 7.3), adjusted to 325 mosm with sucrose containing 500 μM glutamate and 10 μM glycine for 10 minutes. The cultures were washed five times with culture medium, and the neurons were allowed to recover for 1 hour. Where specified, 20 μM MK-801 (dizocilpine, Merck Sharp & Dohme, West Point, Pa.), a noncompetitive glutamate antagonist, was included in the glutamate/glycine mixture.

For quantification of CaM kinase II activity, cortical neurons were washed twice with recording solution at 37°C. The wash solution was replaced with ice-cold homogenization buffer (100 mM piperezine-N,N′-bis [2-ethanesulfonic acid] [PIPES] [pH 6.9], 1 mM ethylendiaminetetraacetic acid, 2 mM [ethylene-bis(oxyethylenenitrilo)tetracetic acid, and 0.3 mM phenylmethylsulfonylf humble) and the cells were scraped from the culture dish surface. The suspension was transferred into glass homogenizers (Kontes, Vineland, N.J.) and disrupted as described.19-22 Homogenates were normalized for protein concentration and studied for endogenous calcium-dependent protein phosphorylation. Standard phosphorylation reaction solutions contained 41 μg protein, 10 mM MgCl2, 7 μM γ-[32P]ATP, 10 mM PIPES (pH 7.4), 0.5 μM CaCl2, and 1 μM calmodulin. Standard reactions were performed in a shaking water bath at 30°C. Reactions were initiated by the addition of calcium, continued for 1 minute, and terminated by the addition of 5% sodium dodecyl sulfate solution. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and protein bands were visualized as described previously.19-22 Stained gels were dried and exposed to x-ray film (XRP-1, Kodak, Rochester, N.Y.) for autoradiography. The autoradiograph was then used as a template for excising radioactive phosphoproteins for quantification in a liquid scintillation spectrometer (model LS 2800, Beckman Instruments, Carlsbad, Calif.) with a counting efficiency of 80%.22 Inhibition of CaM kinase II activity was analyzed by Student’s t test (GRAPHPAD).

Phosphatase Reversal Studies

Homogenates were incubated with purified phosphatases 1 and 2A (PRP-C) for 20 minutes at 30°C in buffer containing 10 mM PIPES (pH 7.4), 10 mM MgCl2, 0.5 mM dl-dithiothreitol, and 1 μM calmodulin.22,29 Phosphatase reactions were terminated by incubating the samples in 0.6 μM okadaic acid (BIOMOL Research Laboratories, Inc., Plymouth Meeting, Pa.) for 1 minute at 30°C. Following PRP-C treatment, standard CaM kinase II reactions were performed.
Results

Mixed cortical cells were plated at a density of $1.5 \times 10^6$ cells/flask (Figure 1A). In mature cultures, neurons could be identified as large, phase-bright cells with one long branching axon and several short, straight dendrites. Viable neurons appeared phase-bright with intact processes (Figures 1A and 1B). When exposed to 500 $\mu$M glutamate for 10 minutes, the cultures demonstrated a reproducible glutamate-induced delayed neuronal death (Figure 2) that could be prevented by coincubation with MK-801. Nonviable neurons exhibited fragmented processes (Figure 1C) and did not exclude the vital dye trypan blue. Cell morphology was observed at 1 and 24 hours after exposure of the cultures to control, glutamate, or glutamate plus MK-801 solutions. Excitotoxic glutamate exposure (500 $\mu$M for 10 minutes) resulted in $87.0 \pm 1.6\%$ loss of cortical neurons after 24 hours (Figure 2), confirming results reported previously. Approximately 20–30% neuronal loss after 1 hour was also observed. The acute toxic reaction to glutamate exposure agrees with previously reported acute neuronal loss and is probably sodium- or chloride-dependent. The addition of MK-801 to the incubation medium resulted in significant protection ($p<0.001$, one-way ANOVA, $n=20$) from the glutamate-induced neuronal loss after 24 hours (Figure 2). Therefore, any cell loss during the first 24 hours after glutamate exposure was due predominantly to glutamate treatment and not to an artifact of handling the cultures.

Recordings from cortical neurons after 3–4 weeks in culture revealed a resting membrane potential of about $-60$ mV ($n=10$) and overshooting spikes with mean action potential amplitudes of approximately 80 mV. Some cells responded to a brief intracellular current injection (0.5 nA, 2 seconds) with a train of spikes (Figure 3A). Short-term glutamate application (2 minutes) resulted in depolarization of the cortical neurons, but membrane potential returned to the preexposure level upon removal of the glutamate (Figure 3B). Excitotoxic application of glutamate (10 minutes) elicited a stereotypical response that consisted of a rapid initial depolarization accompanied by burst firing, followed by a sustained depolarization to a level of $-8$ to 0 mV for the duration of glutamate exposure. Upon removal of the glutamate, the neurons repolarized only partially and then remained depolarized at this level throughout the recording session (up to 2 hours) with no sign of repolarization ($n=7$ of 7, Figure 3C). The sustained depolarization did not appear to be the manifestation of cell death because the neurons retained responsiveness to glutamate application (data not shown). In addition, glutamate-treated neurons could be repolarized with current and demonstrated the capacity to fire action potentials upon rebound if repolarized with current injection 1 hour after the 10-minute glutamate application (Figure 3D).
Cortical cultures parallel to those used for cell death and electrophysiological studies were used for biochemical studies. Homogenates from cortical neuron cultures demonstrated calcium-stimulated phosphate incorporation above the magnesium-dependent (basal) phosphorylation into specific peptide bands (Figure 4). The calcium-dependent phosphorylation pattern is similar to that observed in homogenates from whole brain,15,18–21 hippocampal slices,14 and hippocampal neuron cultures.31 Calcium-dependent phosphate incorporation into the 50-, 55-, 60-, 70-, and 80-kd protein bands was observed in the presence of calcium (lanes 2 and 4), and these were consistently the most significantly calcium-stimulated phosphoproteins. Comigration with purified CaM kinase II on high-resolution gel electrophoresis had been performed previously to identify the 50- and 60-kd phosphoproteins as the alpha and beta subunits of CaM kinase II in forebrain homogenates18–22 and hippocampal neuron cultures.31 Comigration with rat CaM kinase II was used to identify the 50- and 60-kd subunits as the alpha and beta subunits of CaM kinase II in cortical cell cultures (arrows). Short-term exposure of cortical cells to glutamate did not result in significant inhibition of CaM kinase II activity (data not shown). Glutamate exposure that produced significant neuronal death after 24 hours and END in cortical cells resulted in 35% inhibition of CaM kinase II activity when assayed 1 hour after treatment (Figure 4, lane 4). The observed glutamate-induced inhibition was significant compared with control cultures \((p<0.001,\) paired Student’s \(t\) test, \(n=19\) culture dishes).

To determine whether the glutamate-induced inhibition of CaM kinase II activity was due to autophosphorylation, homogenates were treated with phosphatases shown to be active toward autophosphorylation of CaM kinase II.22–29 Homogenates obtained from both control and glutamate-treated cultures were exposed to PRP-C for 20 minutes and subsequently reacted for CaM kinase II activity as described in “Materials and Methods.” PRP-C pretreatment and subsequent kinase reactions resulted in an approximately 40–50% increase in calcium-stimulated phosphate incorporation into the 50-kd band in both control and glutamate-treated samples (data not shown). The increased incorporation of labeled phosphate was probably due to phosphatase-dependent removal of unlabeled phosphate, thus uncovering potential phosphorylation sites on the enzyme. The observed increase in labeled phosphate in PRP-C-treated tissue provided an internal control to ensure activity of the phosphatases toward CaM kinase II. PRP-C treatment did not restore CaM kinase II activity to glutamate-treated cell homogenates (Figure 5). CaM kinase II activity was inhibited 33.6±4.9% before PRP-C treatment and remained inhibited 28.7±4.7% after PRP-C treatment. Activities of PRP-C–treated homogenates obtained from glutamate-exposed cultures were significantly different from those of PRP-C–treated homogenates from control samples \((p<0.001,\) one-way ANOVA, \(n=7\)), demonstrating that the difference between control and glutamate-treated cells was not reversible by phosphatase treatment.

To further determine the mechanism by which glutamate treatment causes changes in CaM kinase II activity, cortical neuron cultures were exposed to glutamate in the presence or absence of MK-801.7,8 Concomitant exposure of cortical cells to 500 \(\mu\)M glutamate and 20 \(\mu\)M MK-801 resulted in 100% cell survival after 24 hours (Figure 2). The results are similar to previous findings in other laboratories.1,3–11 In addition, CaM kinase II activity in homogenates from cultures exposed to both glutamate and MK-801 was not significantly different from that in control tissue (Figure 6). Signifi-
cant inhibition was observed in tissue obtained from glutamate-treated cultures; however, no significant inhibition was observed in homogenates treated with both glutamate and MK-801 or with MK-801 alone. Furthermore, MK-801 treatment alone did not have a significant effect on CaM kinase II activity. Therefore, the protective effects of MK-801 are due to antagonism of the NMDA receptor and not to a nonspecific effect on CaM kinase II activity.

Discussion
Primary cultured rat cortical neurons were used to study the biochemical and physiological aspects of excitotoxic exposure to exogenous glutamate. A standardized 10-minute glutamate exposure that results in delayed neuronal death was employed.\textsuperscript{1,3–11} When monitored by whole-cell current clamp intracellular recording, this excitotoxic glutamate exposure resulted in an END that remained after removal of the glutamate. The same exposure resulted in a significant inhibition of CaM kinase II activity when measured 1 hour after glutamate exposure. In addition, coinubcation of cortical cells with glutamate and MK-801 resulted in preservation of both CaM kinase II activity and neuronal survival. The results suggest that both END and inhibition of CaM kinase II activity may be involved in the glutamate-induced neuronal excitotoxicity.

Information on membrane potentials during glutamate application provides an insight into the underlying biophysical or biochemical processes that ultimately result in delayed neuronal death. Electrophysiological results using patch electrodes from this study have allowed us to monitor membrane potential changes during and after the application of neurotoxic concentrations of glutamate. We show that neurotoxic glutamate application to cortical neurons resulted in an END that was observed long after glutamate washout and usually lasted for the duration of the recording session (up to 2 hours). A comparable effect of glutamate exposure has been observed in neonatal hippocampal cultures, and the END was elicited by NMDA receptor activation.\textsuperscript{12,13}

CaM kinase II is a major neuronal calcium-regulated effector system that is sensitive to conditions that alter neuronal excitability.\textsuperscript{14–23,32} Changes in CaM kinase II activity have been documented in alterations of neuronal excitability such as LTP,\textsuperscript{32} kindling and seizure activity,\textsuperscript{14–17} and ischemia.\textsuperscript{18–23} In addition, no significant loss of CaM kinase II subunit protein, measured by biotinylated calmodulin overlay, is observed in homogenates from ischemic animals\textsuperscript{19–21} or in purified enzyme from ischemic gerbils.\textsuperscript{22} Whole-animal data support the hypothesis that excitotoxic phenomena such as ischemia result in a posttranslational modification of the CaM kinase II enzyme that results in subsequent inhibition of the enzyme.\textsuperscript{22,23} This report demonstrates for the first time that the excitotoxic application of glutamate in vitro results in inhibition of CaM kinase II activity in cortical cell cultures.

The phosphorylation state of CaM kinase II has been shown to modulate the calcium-stimulated enzymatic activity.\textsuperscript{34,35} To determine whether glutamate-induced inhibition of CaM kinase II is due to autophosphorylation, homogenates from control and glutamate-treated
cells were incubated with phosphatases directed toward CaM kinase II. It has been shown that PRP-C is responsible for the removal of phosphates from CaM kinase II (autophosphorylation). However, phosphatase treatment of homogenates obtained from glutamate-treated cells did not reverse the glutamate-induced inhibition of CaM kinase II activity. Phosphatase treatment does not reverse the CaM kinase II inhibition resulting from magnesium-deficiency seizures in rat hippocampal slices or from global forebrain ischemia in gerbils. Therefore, the mechanism(s) by which glutamate induces inhibition of CaM kinase II activity cannot be solely due to autophosphorylation.

There are at least five glutamate receptor subtypes identified in the brain. To determine which subtype is activated to induce the inhibition of CaM kinase II activity, specific inhibitors of the NMDA-type channel were used. Coincubation of cell cultures with the NMDA receptor antagonist MK-801 resulted in complete protection from the glutamate-induced inhibition of CaM kinase II activity. In addition, it has been demonstrated that MK-801 application will prevent the development of END. These observations agree with an analogous observation that MK-801 protects against glutamate-induced neuronal death. Michaels and Rothman have shown that application of MK-801 alone significantly increases the intracellular concentration of calcium. However, the glutamate-induced increase in intracellular calcium is more than 10-fold greater than that observed for MK-801 alone. Therefore, the possibility exists that a threshold intracellular calcium concentration is necessary for pathological alteration of neuronal physiology. Future experiments will be conducted to address this possibility, which is not within the scope of the present study.

This report demonstrates that exposure of cultured neurons to excitotoxic levels of glutamate results in END and significant inhibition of CaM kinase II activity. This same treatment has been shown to result in extensive, delayed neuronal loss (80-90%), which is observed after 24 hours. It is tempting to speculate that such inhibition of CaM kinase II activity may underlie neuronal loss. In this study, MK-801 coincubation resulted in protection from both glutamate-induced loss of neuronal viability and inhibition of CaM kinase II activity. In addition, there is significant correlative evidence of inhibition of CaM kinase II activity and neuronal death in whole-animal models of ischemia and status epilepticus. The cell culture model provides a useful tool to decipher the pharmacological and cellular mechanisms whereby glutamate induces delayed neuronal death. Future studies using the long-term cell culture methods described in this report will be performed to determine the mechanisms of glutamate toxicity in cortical cells.

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Editorial Comment

The importance of protein phosphorylation in the central nervous system has become increasingly evident. This area has been the subject of biochemical investigation for many years, but studies of these reactions and signal transduction have become the major goal of many investigations. The prominence that this body of work has achieved is demonstrated by the award of the 1992 Nobel Prize for Physiology or Medicine for work in this area.

Signal transduction is probably the most important activity of the nervous system, so studies of protein phosphorylation in the brain would seem to be quite logical. When receptors on the neuronal surface are activated by a variety of stimuli, a series of reactions occur that result in modulation of intracellular calcium. The calcium acts as a second messenger to activate calcium/calmodulin-dependent protein kinase II (CaM kinase II). This kinase then phosphorylates many intra-
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