THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 22, Issue of June 3, pp. 21588–21593, 2005

Printed in U.S.A.

Regulation of *N*-Methyl-D-aspartate Receptors by Calpain in Cortical Neurons*

Received for publication, February 11, 2005, and in revised form, March 23, 2005
Published, JBC Papers in Press, March 24, 2005, DOI 10.1074/jbc.M501603200

Hai-Yan Wu‡§¶, Eunice Y. Yuen§‖, Yun-Fei Lu, Masayuki Matsushita‡, Hideki Matsui‡**, Zhen Yan‖, and Kazuhito Tomizawa‡ ‡‡**

*From the ‡Department of Physiology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan, ‖Department of Physiology and Biophysics, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214, and **Protein Therapy, New Techno-Venture Oriented R&D, Japan Science and Technology Corporation, Okayama 700-8558, Japan*

The *N*-methyl-D-aspartate (NMDA) receptor is a cation channel highly permeable to calcium and plays critical roles in governing normal and pathologic functions in neurons. Calcium entry through NMDA receptors (NMDARs) can lead to the activation of the Ca^{2+}-dependent protease, calpain. Here we investigated the involvement of calpain in regulation of NMDAR channel function. After prolonged (5-min) treatment with NMDA or glutamate, the whole-cell NMDAR-mediated current was significantly reduced in both acutely dissociated and cultured cortical pyramidal neurons. The down-regulation of NMDAR current was blocked by bath application of selective calpain inhibitors. Intracellular injection of a specific calpain inhibitory peptide also eliminated the down-regulation of NMDAR current induced by prolonged NMDA treatment. In contrast, dynamin inhibitory peptide had no effect on the depression of NMDAR current, suggesting the lack of involvement of dynamin/clathrin-mediated NMDAR internalization in this process. Immunoblotting analysis showed that the NR2A and NR2B subunits of NMDARs were markedly degraded in cultured cortical neurons treated with glutamate, and the degradation of NR2 subunits was prevented by calpain inhibitors. Taken together, our results suggest that prolonged activation of NMDARs in neurons activates calpain, and activated calpain in turn down-regulates the function of NMDARs, which provides a neuroprotective mechanism against NMDAR overstimulation accompanying ischemia and stroke.

N-Methyl-D-aspartate receptors (NMDARs)[1] have been implicated in development, plasticity, learning, and memory (1). However, overstimulation of NMDARs can also induce an excessive influx of Ca^{2+}, resulting in the initiation and propagation of seizures and the neurodegeneration that accompanies ischemia and stroke (2, 3). Therefore, the modulation of NMDAR activity is of fundamental importance to the physiological functions of neurons (1). Mounting evidence has shown that the function of NMDARs in neurons is often regulated by reversible phosphorylation/dephosphorylation reactions (4–6). It remains unclear whether NMDAR function is also regulated by proteolytic processing.

Stimulation of NMDARs, which are highly Ca^{2+}-permeable, can lead to the activation of the Ca^{2+}-dependent protease, calpain (7, 8). Calpain regulates numerous downstream targets and is highly involved in the pathogenesis of several diseases, such as brain injury, Alzheimer disease, and focal or global cerebral ischemia (9–13). Biochemical studies have shown that NR2A and NR2B subunits are substrates of calpain (6, 14, 15). Calpain truncates the recombinant NR2 subunits at C-terminal regions *in vitro* (6, 15) and cleaves the heteromeric NR2A subunit in transfected cell lines (16). Recent studies also show that the NR2 subunits are cleaved at their C termini by calpain in neurons (14, 17). Although it has been suggested that post-translational modification of NMDARs by calpain may be involved in regulation of NMDAR levels (16), the physiological consequence of calpain-induced proteolysis of NMDARs in neurons is still unclear.

To investigate whether calpain activation in neurons directly alters NMDAR channel function, we recorded whole-cell NMDAR-mediated current under calpain-activated conditions in acutely isolated and cultured cortical neurons. The present results showed that activation of calpain by prolonged glutamate or NMDA application caused down-regulation of NMDAR function, which was due to calpain-dependent degradation of NR2 subunits. This study presents direct evidence showing that calpain cleavage represents a negative feedback mechanism to down-regulate NMDAR function.

EXPERIMENTAL PROCEDURES

Neuronal Culture—Primary cultures of rat cortical neurons were prepared as described previously (18). Briefly, the cortex was dissected on ice in Hanks' balanced salt solution (Sigma) and dissociated with 0.25% trypsin in Hanks' balanced salt solution. Neurons were seeded to a density of $2.7 \times 10^3/mm^2$ on dishes coated with poly-D-lysine (100 mg/ml). After 24 h, Neurobasal growth medium (Invitrogen) containing 10% fetal bovine serum was replaced with Neurobasal medium containing B27 supplement (Invitrogen) instead of serum. The cultures were maintained in a humidified 5% CO_2 incubator at 37 °C for 10–14 days.

Acute Dissociation Procedure—Cortical neurons from 3–5-week-old rats were acutely dissociated as described previously (19). Rats were

* This work was supported by funds from the Industrial Technology Research Grant Program in 2002 from the New Energy and Industrial Technology Development Organization of Japan (to K. T.); a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (to K. T. and H. M.); and National Institutes of Health Grant MH63128 and National Science Foundation Grant IBN-0117026 (to Z. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

¶ Present address: Division of Neurology, Children's Hospital of Philadelphia, Philadelphia, PA 19102.

‡‡ To whom correspondence should be addressed: Dept. of Physiology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata, Okayama 700-8558, Japan. Tel.: 81-86-235-7109; Fax: 81-86-235–7111; E-mail: tomikt@md.okayama-u.ac.jp.

[1] The abbreviations used are: NMDAR, *N*-methyl-D-aspartate receptor; NMDA, *N*-methyl-D-aspartate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; CS, calpastatin; GABA, γ-aminobutyric acid; CDI, Ca^{2+}-dependent inactivation; ALLN, acetyl-L-leucyl-L-leucyl-L-norleucinal; ANOVA, analysis of variance.

anesthetized and decapitated following the regulations of the Animal Care Committee of the State University of New York at Buffalo. Brain slices (400 μm) were incubated for 1–6 h at room temperature in $NaHCO_3$-buffered saline while bubbling with 95% O_2, 5% CO_2. The frontal cortical regions were then dissected and digested in an oxygenated chamber containing papain (0.4 mg/ml; Sigma) for 30–40 min at room temperature. The tissue was rinsed and bathed in low Ca^{2+}, HEPES-buffered saline for 10 min and then mechanically dissociated with a graded series of fire-polished Pasteur pipettes. Dissociated cells were dispersed into a 35-mm Lux Petri dish positioned on the stage of an inverted microscope.

Whole-cell Recordings—Experiments were performed in the standard whole-cell voltage-clamp mode using acutely dissociated and cultured cortical pyramidal neurons (20). The external solution contained 127 mM NaCl, 20 mM CsCl, 10 mM HEPES, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 0.02 mM glycine, 12 mM glucose, and 0.001 mM tetrodotoxin, pH 7.3–7.4, 300–305 mosM. The pipette solution contained 180 mM *N*-methyl-D-glucamine, 4 mM $MgCl_2$, 40 mM HEPES, 0.1 mM BAPTA, 12 mM phosphocreatine, 2 mM Na_2ATP, and 0.2 mM Na_2GTP, pH 7.2–7.3, 265–270 mosM.

Recordings were made with an Axon Instruments (Union City, CA) 200B patch clamp amplifier that was controlled and monitored with an IBM personal computer running pCLAMP (version 8) with a DigiData 1320 series interface (Axon Instruments). Electrode resistances were typically 2–4 megohms in the bath. After seal rupture, series resistance (4–10 megohms) was compensated (70–90%) and periodically monitored. The holding potential was −60 mV in all experiments. NMDA-evoked current was recorded with NMDA (100 μM) application for 2 s every 30 s. For recording of the $GABA_A$ receptor-mediated current, cell membrane potential was held at 0 mV, and GABA (50 μM) was applied for 2 s every 30 s (19). For prolonged NMDA or glutamate treatment, NMDA (100 μM) or glutamate (500 μM) was continuously applied for 5 min or sequentially applied in a pattern of 20 s on, 20 s off for 5 min in the presence of 2 mM external Ca^{2+}. Peak NMDAR current was detected before and after the prolonged NMDA receptor stimulation to generate a plot as a function of time and drug application. Drugs were delivered using a gravity-fed "sewer pipe" system with an array of capillaries positioned 150 μm from the cell under recording. Solution switches were carried out using the SF-77B fast-step solution stimulus delivery device (Warner Instrument Co., Hamden, CT).

Data were analyzed using AxoGraph (Axon Instruments), Kaleidagraph (Albeck Software, Reading, PA), and Statview (Abacus Concepts, Berkeley, CA). Results are presented as the mean ± S.E. to compare the percentage modulation of NMDAR current by prolonged NMDA or glutamate treatment in the absence or presence of various drug applications.

Western Blotting Analysis—Western blotting analysis was performed as described previously (21). Briefly, the polyclonal antibodies against NR2A (NR2A-specific, C-terminal, amino acids 1253–1391) and NR2B (NR2B-specific, C-terminal, amino acids 984–1104) were from Sigma. The polyclonal antibody against NR1 (N-terminal, amino acids 259–278) was purchased from Upstate (Lake Placid, NY), the monoclonal anti-actin antibodies were from Sigma, and the monoclonal antibodies against non-erythroid α-spectrin were from Chemicon (Temecula, CA). After incubation with the appropriate secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich), positive bands were visualized using an enhanced chemiluminescence detection system (ECL; Amersham Biosciences). To evaluate quantitative expression, the scanned digital images of each NMDAR subunit were quantified using National Institutes of Health Image software.

RESULTS

Prolonged NMDA or Glutamate Stimulation Induces Sustained Down-regulation of NMDAR Current in Both Acutely Isolated and Cultured Cortical Neurons—To study the impact of prolonged NMDA receptor stimulation on NMDA receptor function, we recorded whole-cell NMDAR-mediated current in acutely isolated cortical neurons. NMDAR current was evoked by brief applications of NMDA (100 μM, 2 s) every 30 s in a Mg^{2+}-free solution (20, 22). As shown in Fig. 1*A*, the NMDAR current amplitude was stable throughout the recording period. However, if a prolonged application of NMDA (100 μM, 5 min) was given to the cell, the subsequently recorded NMDAR current was markedly reduced in amplitude (Fig. 1*B*, 38.4 ± 5.0%; $n = 8$). Similar results were obtained with cultured cortical neurons (37.2 ± 3.2%; $n = 4$). To examine whether the NMDA-induced current reduction was reversible, we performed long recordings of the whole-cell NMDAR current (every 20 min for 100 min after prolonged NMDA application). As shown in Fig. 1, *C* and *D*, following prolonged NMDA treatment (100 μM, 5 min), NMDA current stayed at the depressed level persistently (for >100 min), with no progressive decline or recovery. Prolonged treatment with glutamate (500 μM, 5 min) also caused a profound reduction of the NMDAR current amplitude (Fig. 1*E*, 39.1 ± 3.6%; $n = 3$). Blocking the opening of NMDAR channels with 2-amino-5-phosphonopentanoic acid (APV) (25 μM) during prolonged NMDA treatment prevented the suppression of NMDAR current (Fig. 1*F*, 5.3 ± 2.9%; $n = 4$). These results indicate that prolonged NMDA receptor stimulation causes a sustained and irreversible down-regulation of NMDAR current in both acutely dissociated and cultured cortical neurons.

It has been found that during a few seconds of NMDA application, the Ca^{2+} influx causes the NMDAR-mediated whole-cell current to inactivate from its peak to a lower steady-state level, a process called Ca^{2+}-dependent inactivation (CDI) (23–26). CDI can be quantitated by using the ratio of the steady-state current (I_{ss}) measured at the end of a 2-s NMDA pulse to the peak current (I_p) (26). In this study, we found that although the amplitude of NMDAR current (both I_p and I_{ss}) was substantially reduced following prolonged NMDAR stimulation, the CDI (the ratio of I_{ss} to I_p) was not significantly altered (CDI measured at 10 min after prolonged NMDA treatment: 0.31 ± 0.05, $n = 8$; CDI measured at the same time but without prolonged NMDA treatment: 0.34 ± 0.02, $n = 4$).

The Reduction of NMDAR Current by Prolonged NMDA Treatment Is due to Calpain Activation—We then investigated the mechanism underlying the down-regulation of NMDAR current by prolonged NMDA treatment. We first examined the Ca^{2+} dependence using intracellular injection of a high concentration (10 mM) of the Ca^{2+} chelator, BAPTA. As shown in Fig. 2*A*, prolonged NMDA treatment failed to suppress NMDAR current in the cell dialyzed with a high concentration of BAPTA, suggesting the requirement of elevation of intracellular Ca^{2+} in this process.

Activation of NMDA receptors triggers a Ca^{2+} influx, which could activate many Ca^{2+}-dependent enzymes, such as calpain (8), calcineurin (27–29), and Ca^{2+}/calmodulin-dependent protein kinase II (30). We hypothesize that prolonged NMDA receptor stimulation may induce the accumulation of excessive intracellular Ca^{2+} and activate calpain, which in turn cleaves functional NMDARs and suppresses NMDAR current. To test this hypothesis, we examined the consequences of inhibiting calpain activity.

As shown in Fig. 2*B*, bath application of the calpain inhibitor ALLN (20 μM) had no effect on basal NMDAR current but completely abolished the down-regulation of NMDAR current by prolonged NMDA treatment. To further confirm the involvement of calpain, we applied a peptide (namely, CS peptide) derived from the endogenous calpain inhibitor calpastatin. The CS peptide contains the functional sequence of TIPPKYR that is critical for inhibition of calpain activity (31, 32). To make the CS peptide membrane-permeable, we tethered it with 11 polyarginine, namely, 11R-CS. Our previous studies showed that the 11R-CS peptide is a potent, membrane-permeable, and specific inhibitor of calpain (28, 29). As demonstrated in Fig. 2*C*, 11R-CS (2 μM) itself had no effect on basal NMDAR current, but in the presence of 11R-CS, prolonged application of NMDA failed to reduce the NMDAR current. Consistently, dialysis with the CS peptide (5 μM) also prevented down-regu-

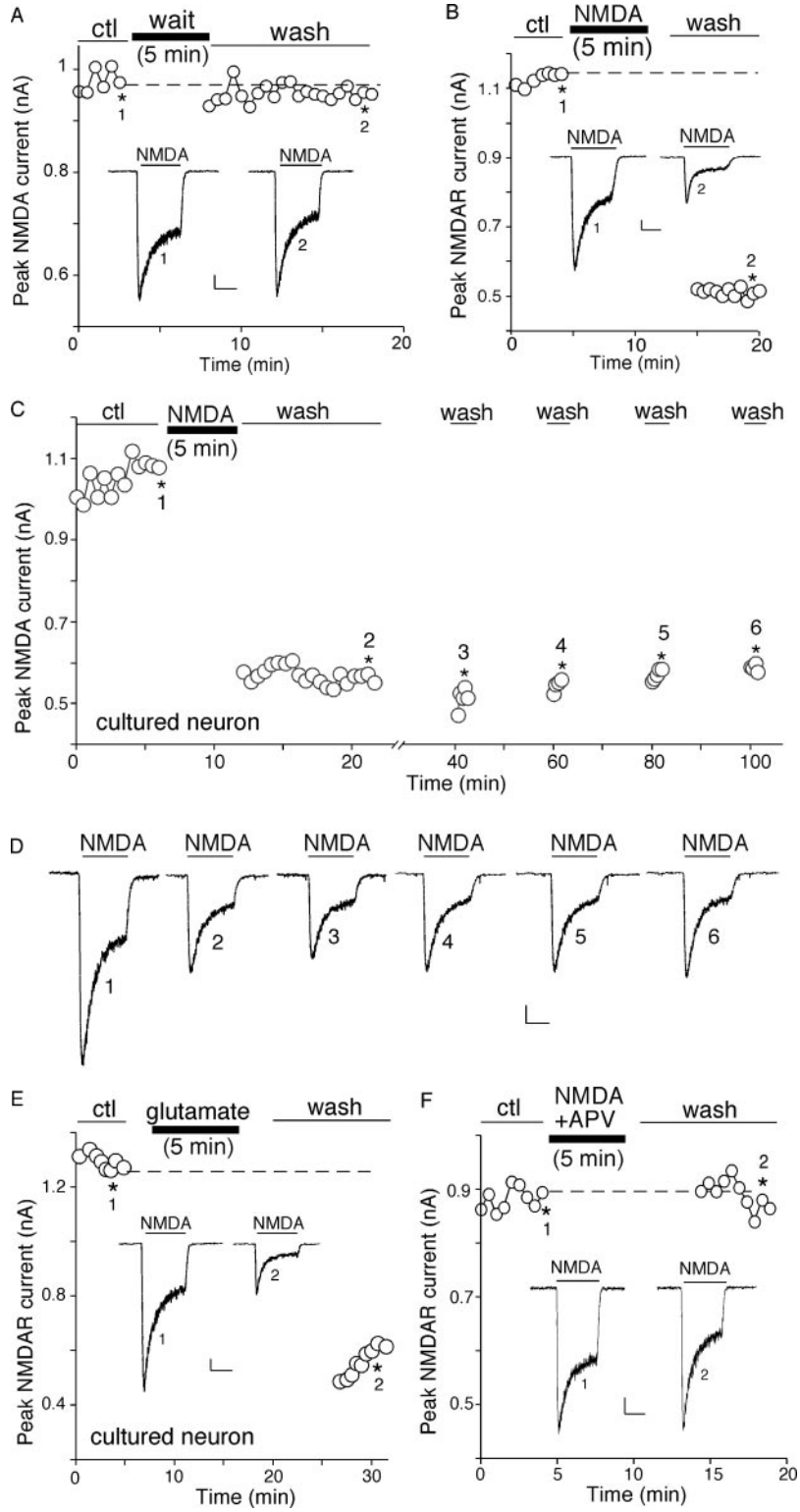

FIG. 1. **Prolonged NMDA or glutamate treatment causes sustained down-regulation of NMDAR current in cortical neurons.** *A* and *B*, plot of peak NMDA-evoked current (I_p) in dissociated cortical neurons. Stable I_p was achieved throughout the recording (*A*). Following a prolonged NMDA treatment (100 μM, 5 min), I_p was markedly reduced (*B*). *C*, plot of long-recorded I_p in a cultured cortical neuron showing that a sustained reduction of NMDAR current was obtained after prolonged NMDA treatment (100 μM, 5 min). *D*, representative traces taken from the recordings in *C* at the indicated times. *E*, plot of I_p showing that prolonged glutamate (500 μM, 5 min) application also down-regulated NMDAR current. *F*, plot of I_p showing that the NMDAR channel blocker APV (25 μM) prevented down-regulation of the NMDAR current. *Insets* in *A*, *B*, *E*, and *F*, representative traces taken from the recordings at the indicated times. *Scale bars*, 0.1 nA, 1 s.

lation of the NMDAR current by prolonged NMDA treatment (Fig. 2*D*). In the presence of the control peptide, 11R (5 μM), the suppression of NMDAR current by prolonged NMDA treatment remained intact (Fig. 2*E*).

The effect of prolonged NMDA treatment on NMDAR current in the absence or presence of various inhibitors is summarized in Fig. 2*F*. Prolonged NMDA treatment (5 min, 100 μM) induced a marked reduction of NMDA current in dissociated or cultured cortical pyramidal neurons (38.0 $\pm$ 5.5%; n = 12). This effect was significantly attenuated when the intracellular Ca^{2+} was chelated by a high concentration of BAPTA (5.2 $\pm$ 2.8%; n = 4). This effect was also blocked when endogenous calpain was inhibited by external application of ALLN (8.5 $\pm$ 1.3%; n = 4) or 11R-CS peptide (5.6 $\pm$ 5.9%; n = 5), as well as by internal dialysis with the CS peptide (4.3 $\pm$ 2.1%; n = 7). However, the current-reducing effect was not affected in neurons dialyzed with the 11R control peptide (33.8 $\pm$ 3.1%; n = 4). Taken together, these results indicate that the reduction of NMDAR current induced by prolonged NMDA treatment is specifically mediated by endogenous calpain activation.

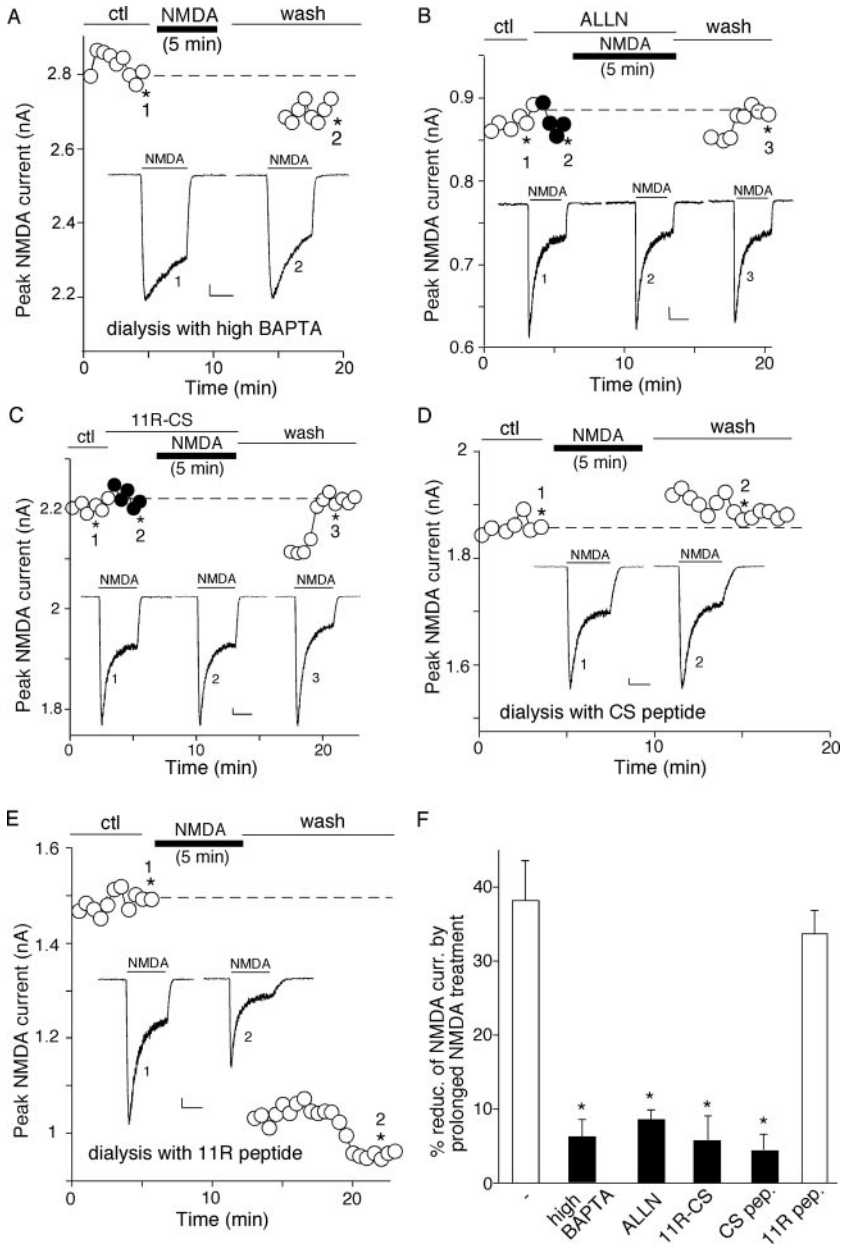

FIG. 2. **Prolonged NMDA treatment reduces NMDAR current through a mechanism involving the activation of calpain.** *A*, plot of I_p showing that dialysis with a high concentration of the Ca^{2+} chelator BAPTA (10 mM) blocked the effect of prolonged NMDA application on NMDAR current. *B* and *C*, plot of I_p showing that bath application of the selective calpain inhibitor ALLN (20 μM; *B*) or the specific and membrane-permeable calpain inhibitor 11R-CS (2 μM; *C*) markedly attenuated the prolonged NMDA-induced reduction of NMDAR current. *D* and *E*, plot of I_p showing that dialysis with the CS peptide (5 μM; *D*), but not the 11R control peptide (5 μM; *E*), eliminated the effect of prolonged NMDA application on NMDAR current. *Insets* in *A*–*E*, representative current traces (at the indicated times). *Scale bars*, 0.1 nA, 1 s. *F*, cumulative data (mean ± S.E.) summarizing the percentage reduction of NMDAR current by prolonged NMDA treatment in the absence or presence of various agents. *, $p < 0.01$, ANOVA.

The Reduction of NMDAR Current by Prolonged NMDA Treatment Does Not Involve Dynamin-mediated Endocytosis of NMDA Receptors—An alternative mechanism that may underlie the down-regulation of NMDAR current by prolonged NMDA treatment is a decrease of functional surface NMDA receptors mediated by clathrin/dynamin-dependent endocytosis (33, 34). To evaluate this potential mechanism, we tested the effect of prolonged NMDA application on NMDAR current in cells dialyzed with the dynamin inhibitory peptide QVPSRPNRAP, which competitively blocks binding of dynamin to amphiphysin (35) and thus prevents endocytosis when administered intracellularly (36, 37). The effectiveness of the dynamin inhibitory peptide in blocking clathrin-dependent endocytosis was confirmed by its ability to prevent the internalization of $GABA_A$ receptors and up-regulate GABA type A receptor-mediated current (Fig. 3*A*, 18.5 ± 3.0%; $n = 7$), consistent with previous results (37). However, when NMDAR endocytosis was inhibited by the dynamin inhibitory peptide (50 μM), prolonged NMDA treatment still induced a potent suppression of NMDAR current (Fig. 3, *B* and *C*, 32.0 ± 2.9%; $n = 5$). This result suggests that the clathrin/dynamin-dependent NMDAR endocytosis is not involved in the down-regulation of NMDAR current induced by prolonged NMDA treatment.

Calpain Induces Degradation of the NR2 Subunits in Glutamate-treated Neuronal Cultures—Our previous studies showed that calpain is activated during the application of excessive glutamate in cultured cortical neurons (28, 29). To provide more direct evidence for the regulation of NMDA receptors by calpain, we examined the protein expression levels of NMDAR subunits in cultured cortical neurons following prolonged glutamate treatment. As shown in Fig. 4*A*, the NMDAR subunits NR1, NR2A, and NR2B were normally expressed in cultured cortical neurons. NR2C was undetectable (data not shown), consistent with the lack of NR2C in some areas of the brain such as the hippocampus and cortex (17, 38). After a 15-min application of glutamate (500 μM), a markedly reduced protein level of NR2A and NR2B was detected. The NR2A or NR2B antibody detects the Western blot band with a relative molecular mass of 180 kDa, which is the predicted size of full-length NR2A or NR2B, suggesting that both antibodies recognize the non-cleaved full-length NR2A and NR2B subunits remaining in neurons after prolonged glutamate treatment. The loss of

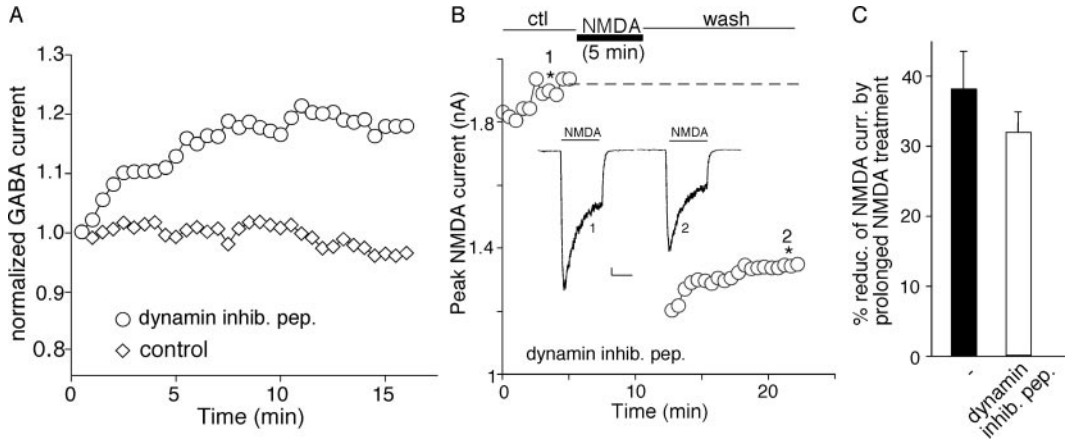

FIG. 3. **Suppression of the NMDAR current by prolonged NMDA treatment does not involve dynamin-dependent endocytosis of NMDA receptors.** *A*, plot of peak GABA-evoked current (normalized) in cells dialyzed with or without the dynamin inhibitory peptide (10 μM). *B*, plot of peak NMDA-evoked current showing that internal application of the dynamin inhibitory peptide (50 μM) failed to affect down-regulation of the NMDAR current by prolonged NMDA treatment. *C*, cumulative data (mean ± S.E.) summarizing the percentage reduction of NMDAR current by prolonged NMDA treatment in the absence or presence of the dynamin inhibitory peptide.

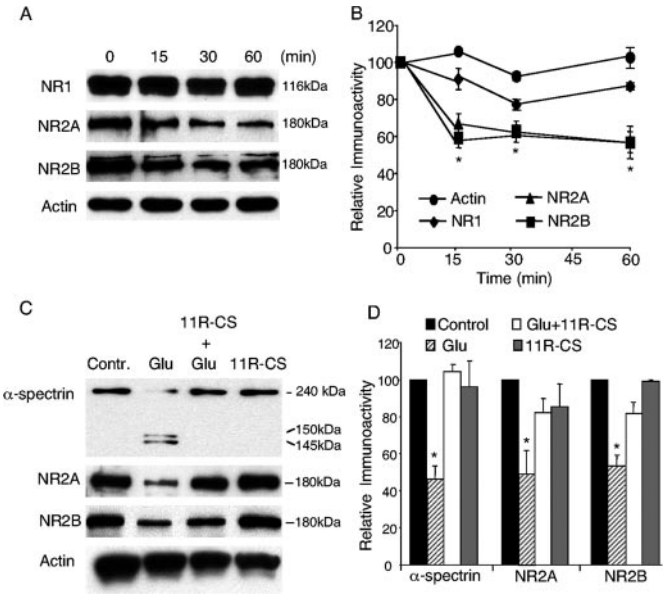

FIG. 4. **Calpain induces degradation of NR2A and NR2B subunits in glutamate-treated neuronal cultures.** *A*, immunoblotting of various proteins in lysates of cultured cortical neurons (14 days *in vitro*). After being exposed to prolonged glutamate treatment (500 μM, 15 min), neurons were washed and returned to the original culture medium. Neurons were collected at each indicated time point, and an equal amount of protein was analyzed by Western blot analysis using antibodies against NR1, NR2A, NR2B, and actin. *B*, quantitative analysis of the expression level of NR1, NR2A, and NR2B at different time points after prolonged glutamate treatment. For evaluation of quantitative expression, the scanned digital images of each NMDAR subunit were quantified using National Institutes of Health Image software. The ratio of the expressions was normalized to the 0 min time point to derive the percentage of original values. Data are the means ± S.E. from five experiments. *, $p < 0.01$, ANOVA. *C*, immunoblotting of various proteins in lysates of cultured cortical neurons (14 days *in vitro*). Neurons were incubated with or without 11R-CS and then treated with or without glutamate (500 μM, 15 min). Neurons were collected 30 min after glutamate treatment, and an equal amount of protein was analyzed by Western blot analysis using antibodies against α-spectrin, NR2A, NR2B, and actin. *D*, quantitative analysis of the expression level of α-spectrin, NR2A, and NR2B under different treatments. The expression levels were quantified by scanning densitometry using National Institutes of Health Image software. The expression level of each control was normalized to 100%, and the expression levels of α-spectrin, NR2A, and NR2B after various treatments were calculated as the percentages of corresponding control values. Data are the means ± S.E. from seven experiments. *, $p < 0.01$, ANOVA.

full-length NR2A and NR2B proteins could be detected 15 min after glutamate treatment and gradually increased thereafter. Semiquantitative assays did not show any significant change in the NR1 level or actin level under the same experimental conditions, which is consistent with a previous study showing that the NR1 subunit (detected with a C-terminal NR1 antibody) was not degraded during glutamate excitotoxicity in cultured hippocampal neurons (17). The expression level of NR1, NR2A, and NR2B at different time points after prolonged glutamate treatment is summarized in Fig. 4*B*. Both NR2A and NR2B expression showed a 25–50% decrease within 1 h after glutamate treatment (500 μM, 15 min).

To examine whether calpain is responsible for the degradation of both the NR2A and NR2B subunits after prolonged glutamate application, we examined the protein levels of NMDA receptor subunits after excessive glutamate treatment in the presence of calpain inhibitors. As shown in Fig. 4*C*, degradation of NR2A and NR2B was clearly detected 30 min after prolonged glutamate treatment, and cleavage of NR2A and NR2B, as well as cleavage of α-spectrin, a well-defined substrate of calpain (9), was substantially blocked by the 11R-CS peptide. Treatment with the 11R-CS peptide alone (without glutamate application) caused no change in any of the proteins tested. Semiquantitative assay (Fig. 4*D*) showed that glutamate treatment (500 μM, 15 min) significantly decreased the protein level of full-length NR2A and NR2B (NR2A: 49.8 ± 10.2% of control, NR2B: 54.1 ± 4.9% of control, $n = 7$; $p < 0.01$, ANOVA, compared with control). Glutamate exposure also induced a significant loss of the full-length α-spectrin (43.9 ± 5.7% of control, $n = 7$; $p < 0.01$, ANOVA, compared with control), further confirming that calpain was activated after prolonged glutamate treatment. Pre-incubation with the calpain inhibitory peptide 11R-CS significantly reduced the glutamate-induced change in both NR2A and NR2B (NR2A: 80.3 ± 5.8% of control, NR2B: 83.1 ± 4.7% of control, $n = 7$; $p < 0.01$, ANOVA, compared with Glu). These biochemical findings suggest that prolonged glutamate treatment induces calpain-specific degradation of NR2A and NR2B subunits in cultured cortical neurons.

DISCUSSION

The present report provides the first direct evidence showing that NMDAR function in neurons is modulated by calpain following NMDAR overstimulation. Using whole-cell patch clamp recording, we found that activation of calpain by pro-

longed glutamate or NMDA treatment caused sustained suppression of the NMDAR current in cortical pyramidal neurons, and this effect was blocked specifically by calpain inhibitors. Biochemical data show that both NR2A and NR2B subunits were degraded following excessive glutamate stimulation in cultured cortical neurons, and this effect was also abolished by the specific calpain inhibitory peptide. Although dynamin-dependent NMDAR endocytosis via clathrin-coated pits participates in the regulation of NMDAR function (34), we found that dynamin-mediated NMDAR internalization is not involved in the reduction of NMDAR current induced by prolonged NMDAR stimulation in neurons. Taken together, these findings support the idea that prolonged stimulation of NMDA receptors induces the activation of calpain, which leads to the cleavage and degradation of NMDA receptors, resulting in the down-regulation of NMDAR function.

Prolonged NMDA receptor stimulation profoundly reduced both the peak (I_p) and steady-state (I_{ss}) NMDAR current, but it did not alter the CDI of NMDA receptors (measured by the ratio of I_{ss} to I_p), indicating that the calpain-mediated down-regulation of NMDA receptors is different from the previously reported CDI (23–25), which occurs via Ca^{2+}/calmodulin-dependent release of the NMDA receptor from the actin cytoskeleton (26). Because previous electrophysiological studies often used high concentrations of Ca^{2+} chelators (23, 26) and the protease inhibitor leupeptin (39) in recording pipettes, substantial elevation of intracellular Ca^{2+} and ensuing activation of calpain were prevented, which explains the lack of findings regarding calpain modulation of NMDAR current in former studies.

Increasing evidence has suggested that calpain proteolysis modifies the activities of the substrate. For example, calpain converts its substrates, protein kinase C and calcineurin, to active forms (28, 40, 41). In this study, we provide electrophysiological and biochemical evidence showing that calpain down-regulates the function of another substrate, NMDA receptors, by cleaving NR2 subunits, which leads to a significant reduction of the number of functional channels on the neuronal membrane. Consistent with this, in a heterologous expression system, calpain-mediated cleavage of NR2 subunits destroys NMDA receptors, resulting in the loss of NMDA receptors from the cell surface and thus the reduced calcium uptake (16). Our data suggest that calpain may act as an endogenous down-regulator of NMDAR activity in response to extreme physiological conditions, such as NMDAR overstimulation.

It was found previously that calpain plays a major role in cell death during ischemia and stroke (9, 10, 42), whereas our current results suggest that calpain activation may function as a negative feedback to reduce neuronal NMDAR activity and protect neurons from excitotoxicity. This paradox can be explained as follows. In addition to NMDA receptors, activated calpain cleaves many other substrates, such as calcineurin (29), cyclin-dependent kinase 5 activators (43, 44), and caspase-12 (45). Calpain cleavage of these substrates plays a critical role in excitotoxic neurodegeneration (29, 42, 43). Moreover, the calpain-mediated release of lysosomal enzyme cathepsin also contributes to ischemic neuronal death (46). Because Ca^{2+}-induced calpain activation with the resultant proteolysis occurs immediately (within minutes) after the ischemic insult (47), whereas neuronal death occurs 3–5 days after ischemia (48), calpain may function as a mediator of the pathological process instead of as the ultimate destroyer. Following the hyperactivation of NMDA receptors, the initial response of calpain is to down-regulate NMDAR function by proteolysis of NR2 subunits, in order to limit calcium entry and minimize NMDAR-mediated excitotoxicity. If the protective mechanism is overridden by excessive calpain activation, the downstream cell death pathways will be triggered.

Acknowledgment— We thank Dr. David. R. Lynch (University of Pennsylvania School of Medicine) for helpful discussions.

REFERENCES

1. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) *Pharmacol. Rev.* **51,** 7–61
2. Choi, D. W. (1988) *Neuron* **1,** 623–634
3. Sattler, R., and Tymianski, M. (2001) *Mol. Neurobiol.* **24,** 107–129
4. Lieberman, D. N., and Mody, I. (1994) *Nature* **369,** 235–239
5. Salter, M. W., and Kalia, L. V. (2004) *Nat. Rev. Neurosci.* **5,** 317–328
6. Bi, R., Rong, Y., Bernard, A., Khrestchatisky, M., and Baudry, M. (2000) *J. Biol. Chem.* **275,** 26477–26483
7. Adamec, R., Kent, P., Anisman, H., Shallow, T., and Merali, Z. (1998) *Neurosci. Biobehav. Rev.* **23,** 301–318
8. Lynch, D. R., and Guttmann, R. P. (2002) *J. Pharmacol. Exp. Ther.* **300,** 717–723
9. Saido, T. C., Yokota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) *J. Biol. Chem.* **268,** 25239–25243
10. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) *FASEB J.* **8,** 814–822
11. Patrick, G. N., Xukerberg, L., Nikolic, M., Monte, S. D. L., Dikkes, P., and Tsai, L.H. (1999) *Nature* **402,** 615–622
12. Chen, M., He, H., Zhan, S., Krajewski, S., Reed, J. C., and Gottlieb, R. A. (2001) *J. Biol. Chem.* **276,** 30724–30728
13. Kim, M. J., Jo, D. G., Hong, G. S., Kim, B. J., Lai, M., Cho, D. H., Kim, K. W., Bandyopadhyay, A., Hong, Y. M., Kim, D. H., Cho, C., Liu, J. O., Snyder, S. H., and Jung, Y. K. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9870–9875
14. Bi, X., Rong, Y., Chen, J., Dang, S., Wang, Z., and Baudry, M. (1998) *Brain Res.* **790,** 245–253
15. Guttmann, R. P., Baker, D. L., Seifert, K. M., Cohen, A. S., Coulter, D. A., and Lynch, D. R. (2001) *J. Neurochem.* **78,** 1083–1093
16. Guttmann, R. P., Sokol, S., Baker, D. L., Simpkins, K. L., Dong, Y., and Lynch, D. R. (2002) *J. Pharmacol. Exp. Ther.* **302,** 1023–1030
17. Simpkins, K. L., Guttmann, R. P., Dong, Y., Chen, Z., Sokol, S., Neumar, R. W., and Lynch, D. R. (2003) *J. Neurosci.* **23,** 11322–11331
18. Tomizawa, K., Sunada, S., Lu, Y. F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F. Y., Matsushita, M., Li, S. T., Tsutsui, K., Hisanaga, S., Mikoshiba, K., Takei, K., and Matsui, H. (2003) *J. Cell Biol.* **163,** 813–824
19. Feng, J., Cai, X., Zhao, J. H., and Yan, Z. (2001) *J. Neurosci.* **21,** 6502–6511
20. Wang, X., Zhong, P., Gu, Z., and Yan, Z. (2003) *J. Neurosci.* **23,** 9852–9861
21. Tomizawa, K., Ohta, J., Matsushita, M., Moriwaki, A., Li, S. T., Takei, K., and Matsui, H. (2002) *J. Neurosci.* **22,** 2590–2597
22. Chen, G., Greengard, P., and Yan, Z. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **24,** 2596–2600
23. Legendre, P., Rosenmund, C., and Westbrook, G. L. (1993) *J. Neurosci.* **13,** 674–684
24. Vyklicky, L., Jr. (1993) *J. Physiol.* **470,** 575–600
25. Medina, I., Filippova, N., Bakhramov, A., and Bregestovski, P. (1996) *J. Physiol.* **495,** 411–427
26. Zhang, S., Ehlers, M. D., Bernhardt, J. P., Su, C. T., and Huganir, R. L. (1998) *Neuron* **21,** 443–453
27. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem.* **273,** 13367–13370
28. Wu, H. Y., Tomizawa, K., Matsushita, M., Lu, Y. F., Li, S. T., and Matsui, H. (2003) *Neurosci. Res.* **47,** 131–135
29. Wu, H. Y., Tomizawa, K., Oda, Y., Wei, F. Y., Lu, Y. F., Matsushita, M., Li, S. T., Moriwaki, A., and Matsui, H. (2004) *J. Biol. Chem.* **279,** 4929–4940
30. Hudmon, A., and Schulman, H. (2002) *Biochem. J.* **364,** 593–611
31. Kawasaki, H., Emori, Y., Imajoh-Ohmi, S., Minami, Y., and Suzuki, K. (1989) *J. Biochem.* (*Tokyo*) **106,** 274–281
32. Maki, M., Bagci, H., Hamaguchi, K., Ueda, M., Murachi, T., and Hatanaka, M. (1989) *J. Biol. Chem.* **264,** 18866–18869
33. Vissel, B., Krupp, J. J., Heinemann, S. F., and Westbrook, G. L. (2001) *Nat. Neurosci.* **4,** 587–596
34. Nong, Y., Huang, Y. Q., Ju, W., Kalia, L. V., Ahmadian, G., Wang, Y. T., and Salter, M. W. (2003) *Nature* **422,** 302–307
35. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., and Booker, G. W. (1993) *Cell* **75,** 25–36
36. Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7097–7102
37. Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G., and Moss, S. J. (2000) *J. Neurosci.* **20,** 7972–7977
38. Nakanishi, S. (1992) *Science* **258,** 597–603
39. Rosenmund, C., and Westbrook, G. L. (1993) *Neuron* **10,** 805–814
40. Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T., and Nishizuka, Y. (1989) *J. Biol. Chem.* **264,** 4088–4092
41. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2002) *Physiol. Rev.* **83,** 731–801
42. Wang, K. K. (2000) *Trends Neurosci.* **23,** 20–26
43. Lee, M. S., Kwon, Y. T., Li, M., Peng, J., Friedlander, R. M., and Tsai, L. H. (2000) *Nature* **405,** 360–364
44. Patzke, H., and Tsai, L. H. (2002) *J. Biol. Chem.* **277,** 8054–8060
45. Nakagawa, T., and Yuan, J. (2000) *J. Cell Biol.* **150,** 887–894
46. Yamashima, T. (2000) *Prog. Neurobiol.* **62,** 273–295
47. Lee, K. S., Frank, S., Vanderklish, P., Arai, A., and Lynch, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7233–7237
48. Garcia, J. H., Liu, K. F., Ye, Z. R., and Gutierrez, J. A. (1997) *Stroke* **28,** 2303–2309