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The Phosphorylation State of GluR1 Subunits Determines the Susceptibility of AMPA Receptors to Calpain Cleavage*

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The α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is an ionotropic glutamate receptor that governs most of excitatory synaptic transmission in neurons. *In vitro* biochemical assay has shown that calpain, a Ca^{2+}-activated protease, can cleave AMPAR GluR1 subunits. Our physiological study found that calpain, which was activated by prolonged stimulation of the *N*-methyl-D-aspartate receptor (100 μM, 10 min), caused a substantial suppression of AMPAR currents in cortical neurons. Since the phosphorylation sites of GluR1 by several protein kinases are located in close proximity to the calpain cleavage sites, we investigated the effect of phosphorylation on the susceptibility of GluR1 to calpain cleavage. Interestingly, we found that the calpain regulation of AMPAR currents was diminished by inhibition of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) but was augmented by inhibition of protein phosphatase 1/2A (PP1/2A). In agreement with this, *in vitro* assay showed that the calpain-induced proteolytic cleavage of GluR1 C-terminal fusion protein was strongly potentiated by adding the purified active CaMKII, and GluR1 phosphorylated at Ser^{831} by CaMKII is much more sensitive to calpain cleavage. Taken together, our data suggest that calpain activation suppresses AMPA receptor currents via proteolytic cleavage of GluR1 subunits, and the susceptibility of AMPARs to calpain cleavage is determined by the phosphorylation state of GluR1 subunits, which is mediated by CaMKII-PP1/2A activity.

AMPA[2] receptors are glutamate-gated ion channels mediating fast excitatory synaptic responses in the mammalian brain. The AMPA receptor is a tetramer composed of subunits coded by GluR1, GluR2, GluR3, and GluR4 subunits (1, 2). Previous studies have reported that AMPA receptors are regulated by several mechanisms. One is to change the phosphorylation state of AMPARs by protein kinases/phosphatases (3, 4). Another one is to change the trafficking of AMPARs, thereby increasing or decreasing the number of functional AMPARs located at the synaptic membrane (1). *In vitro* biochemical assay also show that AMPAR GluR1 subunit is a potential substrate of the Ca^{2+}-activated protease calpain (5, 6), suggesting that AMPAR function could be regulated by another mechanism, *i.e.* calpain-mediated proteolysis.

As a Ca^{2+}-dependent protease, calpain has been implicated in many pathological conditions when calcium homeostasis is disrupted, such as stroke and epilepsy (7, 8). Calpain cleaves many targets, including ion channels such as AMPAR and NMDAR subunits (5, 9, 10), cytoskeletal proteins like MAP2 (11), and signaling molecules such as calcineurin (12). Biochemical studies have shown that the calpain-mediated proteolysis is often affected by the phosphorylation state of the substrates. For example, PKA phosphorylation of tau, a microtubule-associated protein, protects tau from calpain truncation (13). Similarly, Src-mediated tyrosine phosphorylation of NMDAR NR2 subunits prevents calpain-mediated cleavage of their C-terminal domains (14). Fyn-mediated tyrosine phosphorylation reduces calpain-induced truncation of GluR1 subunits, does not affect calpain proteolysis of NR2B subunits, but facilitates calpain cleavage of NR2A subunits (15).

The putative sites of calpain-mediated truncation of GluR1 subunits are at residues N^{833} and R^{837} (5, 6). The phosphorylation sites of GluR1 by several protein kinases are located in close proximity to the calpain cleavage sites, including PKA (Ser^{845}), CaMKII and PKC (Ser^{831}) (16, 17). Particularly, CaMKII is a major protein enriched in the postsynaptic density (18, 19), and plays a critical role in coordinating glutamatergic signaling (20). Activation of CaMKII can induce the increased AMPAR channel activity via GluR1 phosphorylation at Ser^{831} (16, 17) and the delivery of more AMPARs to the synaptic surface via a mechanism depending on GluR1 and PDZ domain interaction (21).

Our recent study has found that a prolonged (10-min) application of NMDA (100 μM) to cortical neurons induces the activation of calpain, which leads to the proteolysis of GluR1 subunits and the suppression AMPAR-mediated currents (9). Moreover, the calpain regulation of AMPARs is happening in excitotoxic conditions like ischemia *in vivo* (9). In this study, we examined the role of GluR1 phosphorylation in calpain regulation of AMPAR functions. We found that CaMKII phosphorylation of GluR1 determines the susceptibility of AMPARs to calpain-mediated cleavage.

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[2] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; PP1, protein phosphatase 1; GFP, green fluorescent protein; ANOVA, analysis of variance; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; siRNA, small interfering RNA; aa, amino acids.

EXPERIMENTAL PROCEDURES

Acute Dissociation Procedure and Primary Neuronal Culture—Cortical neurons from 3–4-week-old Sprague-Dawley rats were acutely dissociated as what we described previously (10, 22). In brief, brain slices (400 μm) were incubated in a $NaHCO_3$-buffered saline bubbled with 95% O_2, 5% CO_2. The frontal cortical areas were dissected and digested in an oxygenated chamber consisted of papain (0.4 mg/ml; Calbiochem) for 40 min at room temperature. Following washing, the tissue was mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The isolated cells were then dispersed into a 35-mm Lux Petri dish positioned on the stage of a Nikon inverted microscope. Rat cortical cultures from E18 embryos were prepared with procedures similar to what we used previously (22).

Whole Cell Recordings—Whole cell recordings of ion channel currents in acutely dissociated neurons employed standard voltage clamp techniques as what we described previously (10). The external solution contains (in mM): 127 NaCl, 20 CsCl, 1 $MgCl_2$, 10 HEPES, 5 $BaCl_2$, 12 glucose, 0.001 tetrodotoxin, pH 7.3–7.4, 300–305 mosM. Recording electrodes (2–4 megaohms) were filled with the following internal solution (in mM): 170 *N*-methyl-D-glucamine, 4 $MgCl_2$, 40 HEPES, 0.5 BAPTA, 12 phosphocreatine, 3 Na_2ATP, and 0.5 Na_2GTP, pH 7.2–3, 265–270 mosM. Recordings were obtained with an Axon Instruments 200B patch clamp amplifier that was controlled and monitored with an IBM PC running pCLAMP (v.8) with a DigiData 1320 series interface (Axon instruments). Following seal rupture, series resistance (4–10 mΩ) was compensated (70–90%). AMPAR-mediated currents were recorded with glutamate (1 mM) or AMPA (100 μM) application for 2 s every 30 s in cells (held at −60 mV) exposed to an Mg^{2+}-containing solution (to block NMDAR activation). During prolonged NMDA or glutamate treatment, NMDA (100 μM) or glutamate (100 or 500 μM) was continuously perfused for 10 min in the presence of 2 mM $CaCl_2$, 20 μM glycine, and Mg^{2+}-free external solution. Drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries (~150 μm inner diameter) was positioned a few hundred microns from the cell under study. Solution changes were effected by the SF-77B fast-step solution stimulus delivery device (Warner Instruments, Hamden, CT). Data were analyzed with AxoGraph (Axon instruments) and Kaleidagraph (Albeck Software).

Second messenger reagents KN-93, KN-92, autocamtide-2-related inhibitory peptide, okadaic acid, microsystin, cyclosporine A, myristoylated PKI_{14-22}, PKI_{5-24}, bisindolylmaleimide I, and calphostin C (Calbiochem) were made up as concentrated stocks in water or Me_2SO and stored at −20 °C. Stocks were thawed and diluted immediately prior to use. The CS peptide contains the functional sequence of TIPPKYR that is critical for inhibiting calpain activity (23). To make the CS peptide membrane permeable, we tethered it with 11 polyarginines, namely 11R-CS. Previous studies have shown that the 11R-CS peptide is a potent, membrane-permeable and specific inhibitor of calpain (10, 12). The amino acid sequence for the dynamin inhibitory peptide is: QVPSRPNRAP.

Small Interfering RNA—To suppress the endogenous calpain expression, we transfected cortical cultures with the small interfering RNA (siRNA) that specifically targets to the regulatory subunit of μ-calpain mRNA. The calpain siRNA oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) contained three sense strands: 5′-CCACGUAGUCAUUACUCUA-3′, 5′-GACUAUCGGUAGCCAUGAA-3′, and 5′-GUACCCAGCUUCCCAAUCA-3′. The α-CaMKII siRNA oligonucleotide (Ambion, Austin, TX) was 5′-GGAGUAUGCUGCCAAGAUUtt-3′ (sense). Transfection of siRNA was performed as what we described previously (22). The siRNA was co-transfected with enhanced GFP into cultured cortical neurons (8 days *in vitro*) using the Lipofectamine 2000 method. Electrophysiological experiments were performed in GFP-positive neurons after 2–3 days of transfection.

Immunocytochemistry—Cortical cultures (11 days *in vitro*) were fixed in 4% paraformaldehyde for 30 min at room temperature and were permeabilized with 0.2% Triton X-100 for 10 min. After incubating in 5% bovine serum albumin to block nonspecific staining, neurons were incubated with the anti-calpain polyclonal antibody (Santa Cruz Biotechnology) and the anti-MAP2 monoclonal antibody (Upstate Biotechnology) for 2 h at room temperature. After three washes, the cells were incubated with a fluorescein isothiocyanate-conjugated anti-rabbit and a TRITC-conjugated anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After three washes, the coverslips were mounted on slides with VECTASHIELD mounting media (Vector Laboratories, Burlingame, CA). Fluorescent images were detected using a 60× objective with a cooled CCD camera mounted on a Nikon microscope. All specimens were imaged under identical conditions and analyzed with identical parameters.

Western Blotting—After treatment, cultured cortical neurons (14 days *in vitro*) were lysed with the lysis buffer containing: 1% SDS, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 16,000 × *g*, and supernatant fractions were incubated with primary antibodies. Antibodies against the N terminus of GluR1 (1:200, Santa Cruz Biotechnology) and GluR2 (1:500, Chemicon), the C terminus of GluR1 (1:500, last 15 aa, Upstate Biotechnology) and GluR2/3 (1:500, aa 864–883, Upstate Biotechnology), α-spectrin (1:5000, Chemicon) and $GABA_AR\beta2/3$ subunits (1:500, Upstate Biotechnology) were used. After incubation with appropriate secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich), positive bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences). Quantitation was obtained from densitometric measurements of immunoreactive bands on films.

Calpain Cleavage Assay—The C terminus of GluR1 (residues 809–889) was cloned into pQE-80 (Qiagen. Inc.) as described previously (24). GluR1 C-terminal mutants (S831A, S831D, S831E, and S845A) were generated by site-directed mutagenesis using the QuikChange kit from Stratagene. The fusion protein was tethered with 6 consecutive His tags and a hemagglutinin tag at the N terminus and a FLAG tag at the C terminus. After expression and purification, the fusion protein was incu-

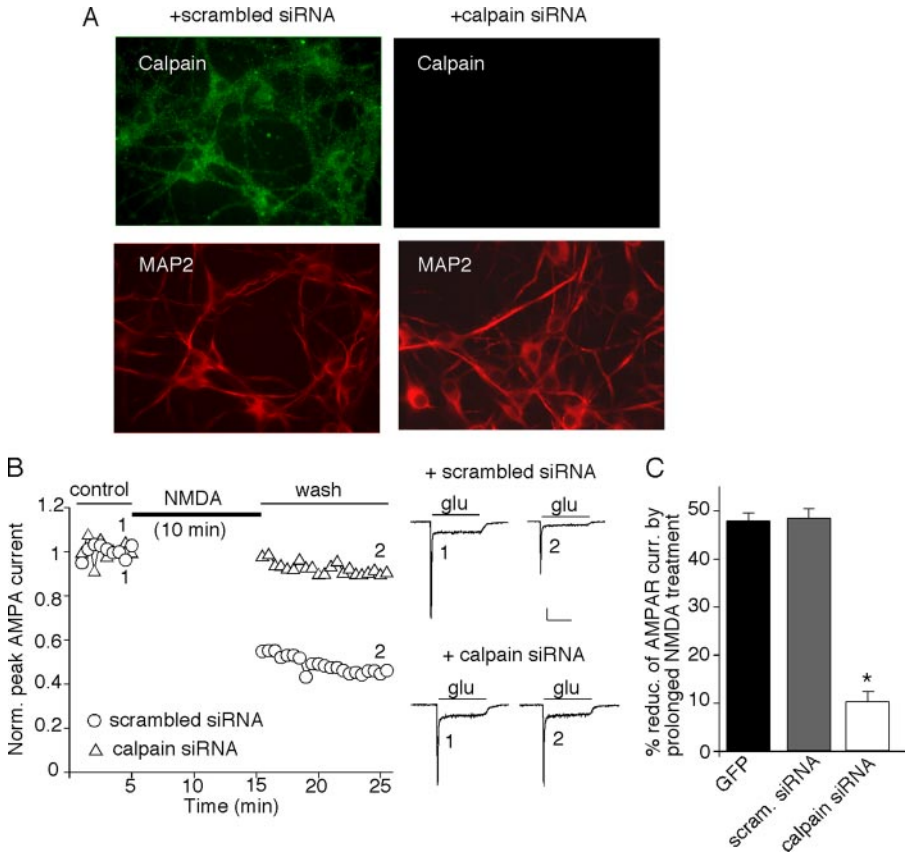

FIGURE 1. **Activation of calpain causes the down-regulation of AMPAR currents in cortical pyramidal neurons.** *A*, immunochemical images showing the co-staining of μ-calpain and MAP2 in cortical neurons transfected with calpain siRNA or a scrambled siRNA. *B*, plot of normalized peak AMPAR currents (I_{AMPA}) with a prolonged NMDA application (100 μM, 10 min) in GFP-positive neurons transfected with calpain siRNA or a scrambled siRNA. *Inset*, representative current traces taken at indicated time points. *Scale bars*, 100 pA, 1 s. *C*, cumulative data (mean ± S.E.) showing the percentage reduction of AMPAR currents by prolonged NMDA treatment in GFP-positive cells transfected with GFP alone, a scrambled siRNA or calpain siRNA. *, $p < 0.001$, ANOVA.

bated overnight at room temperature in a phosphorylation buffer containing (mM): 50 Tris, pH 7.4, 10 $MgCl_2$, 5 $MnCl_2$, 0.001 Na_2VO_4. GluR1 was phosphorylated by adding CaMKII (0.6 μg/ml, Calbiochem), CaM (30 μg/ml, Calbiochem), ATP (500 μM), and $CaCl_2$ (0.5 mM) for 5 min at 37 °C. Non-phosphorylated or phosphorylated GluR1 was incubated with calpain I (5 units/ml, Calbiochem) for 30 min at 37 °C in the presence of 2 mM $CaCl_2$ in each tube. Samples were denatured with SDS-sample buffer and resolved by SDS-PAGE.

RESULTS

Activation of Calpain Produces a Sustained Suppression of AMPAR Currents in Cortical Neurons—Our previous study has shown that calpain is activated in cortical neurons by prolonged NMDAR stimulation (9, 10). To investigate the role of calpain in AMPAR-mediated currents in neurons, we performed cellular knockdown of calpain in cortical cultures by transfecting a siRNA directed toward the regulatory subunit of μ-calpain. As shown in the immunocytochemical images (Fig. 1*A*), calpain, which is enriched at soma and dendrites of cortical neurons, was markedly suppressed in calpain siRNA-transfected neurons, while the staining of MAP2 (a dendritic marker) was unchanged.

Since the calpain siRNA has caused efficient and specific down-regulation of calpain expression, it is likely to cause functional inactivation of calpain. Then we examined the regulation of AMPAR currents by calpain, which is activated by prolonged NMDA treatment, in cortical neurons transfected with the calpain siRNA. Whole cell AMPAR-mediated currents was elicited by glutamate (1 mM) application in an Mg^{2+}-containing solution as what we described before (4). No difference in AMPAR current density (pA/pF) was detected in neurons transfected with the calpain siRNA or a scrambled siRNA (calpain siRNA: 28.4 ± 2.3; scrambled siRNA: 27.3 ± 2.2). As shown in Fig. 1*B*, when endogenous calpain was activated by prolonged NMDA treatment (100 μM, 10 min), AMPAR currents was substantially reduced in neurons transfected with a scrambled siRNA (48.2 ± 2.0%, $n = 7$, Fig. 1*C*) or GFP alone (44.7 ± 1.5%, $n = 9$, Fig. 1*C*), while this effect was significantly abolished in neurons transfected with the calpain siRNA (10.2 ± 2.1%, $n = 6$, Fig. 1*C*). These data suggest that calpain, which is activated by prolonged stimulation of NMDA receptors, down-regulates AMPAR function in cortical neurons.

Calpain Activation Causes the Proteolysis of AMPAR GluR1 Subunit in Cortical Neurons—To provide further evidence to complement our electrophysiological studies, we performed biochemical experiments to test whether overstimulation of NMDARs can indeed activate calpain and induce the cleavage of AMPAR subunits. Cultured cortical neurons transfected with the calpain siRNA or a scrambled siRNA were treated with glutamate (500 μM) or NMDA (100 μM) for 10 min, and then washed in culture medium for 10 min before collection. Antibodies against the N termini of GluR1 or GluR2 were used to detect both cleaved and uncleaved subunit, while those against the C termini of GluR1 or GluR2/3 were used to detect the full-length (uncleaved) subunit. As shown in Fig. 2*A*, in cells transfected with a scrambled siRNA, prolonged glutamate or NMDA treatment led to a dramatically reduced level of GluR1 subunit. The same stimulation cleaved α-spectrin (240 kDa), a well defined substrate of calpain, into two fragments (150 and 145 kDa). However, in cells transfected with the calpain siRNA, prolonged glutamate or NMDA treatment failed to reduce GluR1 levels and cleave α-spectrin. GluR2/3 subunit or $GABA_AR$ β2/3 subunit was not affected by prolonged glutamate or NMDA treatment in all the transfected cells. As sum-

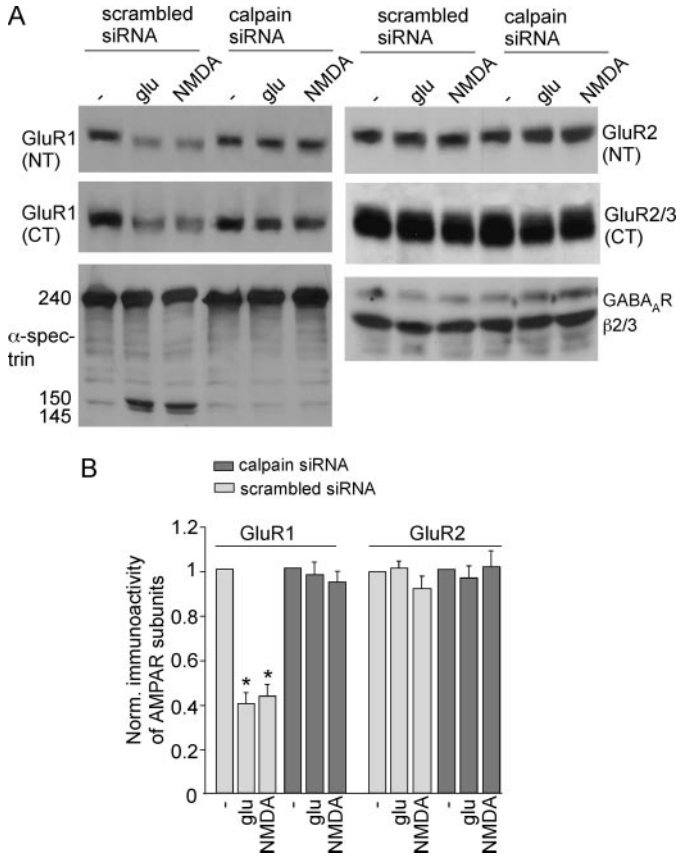

FIGURE 2. **Calpain activation causes the cleavage of GluR1 subunits in cortical pyramidal neurons.** *A*, Western blot analysis of various proteins in cultured cortical neurons transfected with calpain siRNA or a scrambled siRNA following glutamate (500 μM, 10 min) or NMDA treatment (100 μM, 10 min). Cells were collected after 10-min of washing. Antibodies against the N-terminal (*NT*) or C-terminal (*CT*) residues of GluR1, GluR2, or GluR2/3 were used. *B*, quantificative analysis (mean ± S.E.) showing the levels of GluR1 (detected with the N-terminal antibody) and GluR2 (detected with the N-terminal antibody) with glutamate or NMDA treatment in cortical cultures transfected with calpain siRNA or a scrambled siRNA. *, $p < 0.001$, ANOVA.

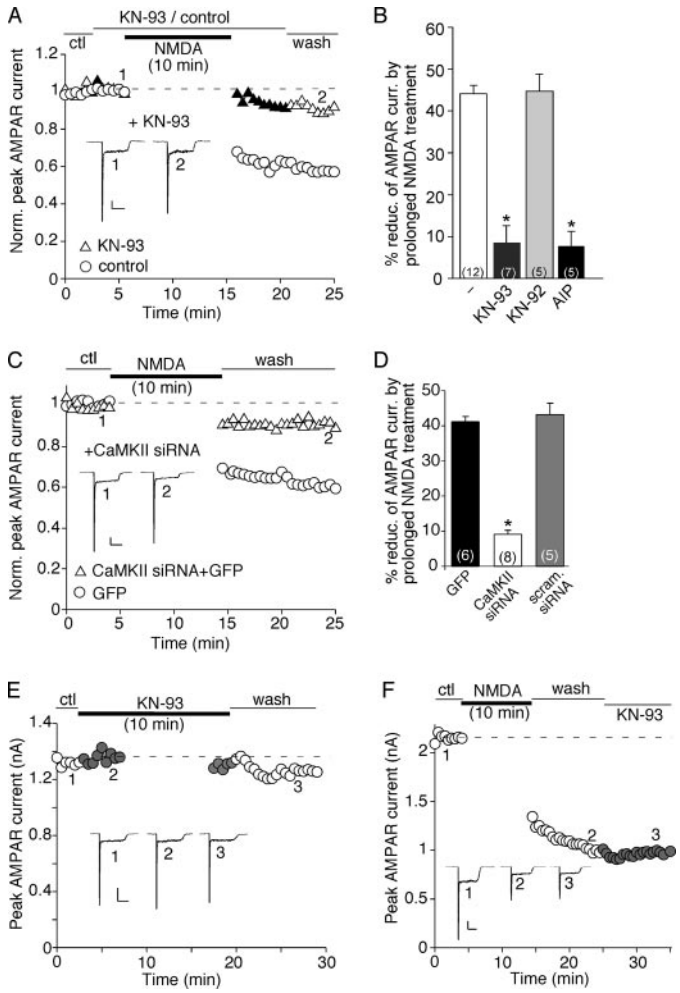

FIGURE 3. **Inhibition of CaMKII attentuates the effect of prolonged NMDA treatment on AMPAR currents.** *A*, plot of normalized I_{AMPA} with a prolonged NMDA application (100 μM, 10 min) in the absence (control) or presence of KN-93 (20 μM). *B*, cumulative data (mean ± S.E.) showing the percentage reduction of AMPAR currents by prolonged NMDA treatment with different agents that affect CaMKII activity. *AIP*, autocamtide-2-related inhibitory peptide. *C*, plot of normalized I_{AMPA} in GFP-positive neurons transfected with or without CaMKII siRNA. *D*, cumulative data (mean ± S.E.) summarizing the percentage reduction of AMPAR currents by prolonged NMDA treatment in GFP-positive cells transfected with CaMKII siRNA or a scrambled siRNA. *E*, plot of I_{AMPA} showing the effect of KN-93 (20 μM, 10 min) on AMPAR currents. *F*, plot of I_{AMPA} showing the effect of a prolonged NMDA application (100 μM, 10 min) and a subsequent application of KN-93 (20 μM). *Inset* (*A*, *C*, *E*, and *F*), representative current traces taken from the recordings at indicated times. *Scale bars*, 100 pA, 1 s. *, $p < 0.001$, ANOVA.

marized in Fig. 2*B*, GluR1 was significantly diminished by prolonged glutamate or NMDA treatment in scrambled siRNA-transfected neurons (Glu, 39 ± 5% of control; NMDA, 44 ± 6% of control, $n = 5$; $p < 0.001$, ANOVA) but not in calpain siRNA-transfected neurons (Glu, 98 ± 6% of control; NMDA, 95 ± 5% of control, $n = 5$). The GluR2 level was unaltered. These results suggest that calpain activated by prolonged stimulation of NMDA receptors induces the specific proteolysis of GluR1 subunits, which could account for the down-regulation of AMPAR currents.

CaMKII Phosphorylation of GluR1 Affects the Calpain Regulation of AMPAR Currents—The putative sites of calpain-mediated truncation of GluR1 subunit are at C-terminal residues Asn^{833} and Arg^{837} (5). These sites are in close proximity to PKA phosphorylation site (Ser^{845}) and CaMKII phosphorylation site (Ser^{831}) on GluR1 subunit (17, 25, 26). To test whether the phosphorylation state of GluR1 subunit alters the susceptibility to calpain proteolysis, we applied various agents that selectively inhibit these kinases. As shown in Fig. 3*A*, bath application of the CaMKII inhibitor KN-93 (20 μM) substantially blocked the reduction of AMPAR currents by prolonged NMDA treatment (8.6 ± 3.9%; $n = 7$, Fig. 3*B*), while the inactive analog KN-92 failed to do so (44.6 ± 4.1%; $n = 5$, Fig. 3*B*). Dialysis with autocamtide-2-related inhibitory peptide (5 μM), another mechanistically different CaMKII inhibitor, also significantly attenuated the effect of prolonged NMDA treatment on AMPAR currents (7.6 ± 3.5%, $n = 5$, Fig. 3*B*). Moreover, in the presence of KN-93, prolonged NMDA treatment had a substantially smaller effect on AMPAR-excitatory postsynaptic current amplitude (7.9 ± 2.6%, $n = 7$), compared with control cells (58.6 ± 3.9%, $n = 9$) in cortical slices (data not shown).

To further assess the involvement of CaMKII in calpain regulation of AMPARs, we suppressed the endogenous CaMKII level in cultured cortical neurons by introducing a siRNA directed against α-CaMKII (22) and tested the effect of prolonged NMDA treatment in these cultures. Our previous study

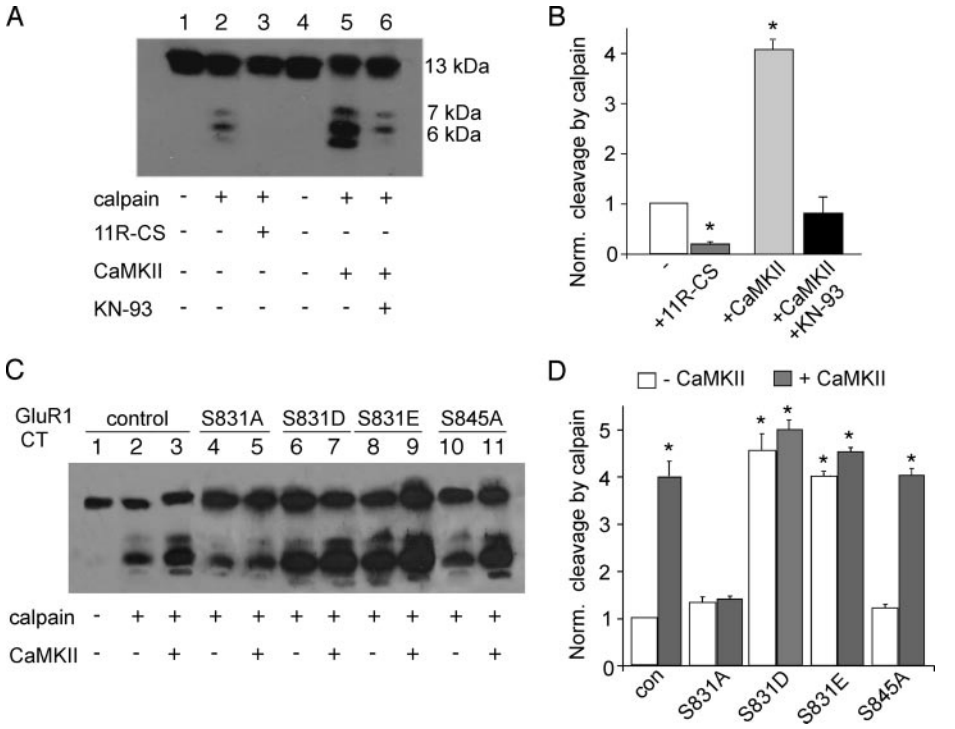

FIGURE 4. **Phosphorylation of GluR1 by CaMKII at Ser^{831} determines the susceptibility of GluR1 to calpain cleavage.** *A*, immunoblots of the GluR1 fusion protein treated with calpain (5 units/ml, 30 min) in the absence or presence of different agents that affect calpain cleavage (added 10 min before calpain treatment), including 11R-CS (10 μM), CaMKII (0.6 μg/ml), and KN-93 (20 μM). GluR1 was detected with an antibody against hemagglutinin, which labeled both the cleaved and uncleaved GluR1 fusion protein. *B*, quantitative analysis (means ± S.E.) showing the normalized level of calpain-cleaved GluR1 fusion protein in the presence of various agents. *, $p < 0.001$, ANOVA. *C*, immunoblots of different GluR1 fusion proteins (control, S831A, S831D, S831E, S845A) treated with calpain (5 units/ml, 30 min) in the absence or presence of CaMKII (0.6 μg/ml, added 10 min before calpain treatment). *D*, quantitative analysis (means ± S.E.) showing the normalized level of calpain-cleaved different GluR1 fusion proteins in the absence or presence of CaMKII. *, $p < 0.001$, ANOVA, compared with control (−CaMKII).

has reported that the CaMKII siRNA specifically and efficiently suppressed CaMKII expression in GFP-positive neurons (22). No difference in AMPAR current density (pA/pF) was found in neurons transfected with GFP alone or CaMKII siRNA (GFP, 19.0 ± 3.5; CaMKII siRNA, 19.6 ± 2.2). However, the effect of prolonged NMDA treatment on AMPAR currents was profoundly attenuated in GFP-positive neurons transfected with CaMKII siRNA (9.1 ± 1.3%, $n = 8$, Fig. 3, *C* and *D*), compared with control cells transfected with GFP alone (41.2 ± 1.5%, $n = 6$, Fig. 3, *C* and *D*) or cells transfected with a scrambled siRNA (43.3 ± 3.2%, $n = 4$, Fig. 3*D*). Taken together, these results suggest that CaMKII is involved in modifying the effect of calpain on AMPA receptors.

How does the suppression of CaMKII activity attenuate the calpain effect on AMPAR functions? Several potential mechanisms may underlie this observation. First, inhibition of CaMKII may reduce calpain activity, which results in diminished AMPAR cleavage. To test this, we compared the proteolysis of α-spectrin, a good indicator of calpain activity (27), in cells treated with glutamate in the absence or presence of CaMKII inhibitors. We found that application of glutamate (500 μM, 10 min) induced a similar breakdown of α-spectrin with or without KN-93 treatment (data not shown), indicating that inhibition of CaMKII does not affect calpain activity. Another possible mechanism is that suppressing CaMKII activity directly affects AMPAR channel properties. However, we found that bath application of KN-93 (20 μM) alone did not alter AMPAR currents throughout the entire recording (Fig. 3*E*). Moreover, after establishing the reduction of AMPAR currents by prolonged NMDA treatment, subsequent application of KN-93 failed to alter this effect (Fig. 3*F*).

Phosphorylation of GluR1 by CaMKII at Ser^{831} Affects the Calpain Cleavage of GluR1—Next, we performed *in vitro* calpain cleavage assay to confirm the effect of CaMKII phosphorylation on the susceptibility of GluR1 to calpain cleavage. We generated a GluR1 fusion protein by tethering hemagglutinin to GluR1 C terminus (aa 809–889). This fusion protein includes the calpain cleavage site aa 833–834 and aa 837–838 (5). As shown in Fig. 4*A*, application of purified calpain (5 units/ml, 30 min, in the presence of 2 mM Ca^{2+}) cleaved the 13-kDa GluR1 fusion protein into two fragments (6 and 7 kDa), which was significantly blocked by the specific calpain inhibitor 11R-CS (10 μM). Moreover, the calpain cleavage of GluR1 was markedly augmented by adding the purified active CaMKII (0.6 μg/ml), which was prevented by adding the CaMKII inhibitor KN-93 (20 μM). Summary data (Fig. 4*B*) indicate that CaMKII increased the calpain cleavage of GluR1 by 3.1 ± 0.3 fold ($n = 3$). There results provide direct evidence showing that CaMKII phosphorylation of GluR1 increases the sensitivity of GluR1 to calpain proteolysis.

To further confirm that it is the CaMKII phosphorylation site, Ser^{831}, that determines the sensitivity of GluR1 to calpain cleavage, we generated the GluR1 C-term fusion proteins with mutations at S^{831}. Ser^{831} was mutated to alanine to represent the non-phosphorylatable form (S831A), and Ser^{831} was mutated to aspartic acid or glutamic acid to represent the phosphomematic form (S831D, S831E). To examine the role of Ser^{845}, the PKA phosphorylation site, the GluR1 C-terminal fusion protein with Ser^{845} mutated to alanine (S845A), was used. As shown in Fig. 4, *C* and *D*, adding active CaMKII significantly increased the calpain cleavage of GluR1 C-terminal fusion protein (3.0 ± 0.3-fold increase, $n = 4$) but failed to enhance the calpain cleavage of GluR1 mutant S831A (0.07 ± 0.01-fold increase, $n = 3$). In the absence of CaMKII, the effect of calpain on the cleavage of GluR1 mutant S831D or S831E was significantly bigger (S831D, 3.5 ± 0.4-fold increase; S831E, 3.0 ± 0.1 fold increase, $n = 3$, compared with control). Adding CaMKII did not further enhance the calpain cleavage of GluR1

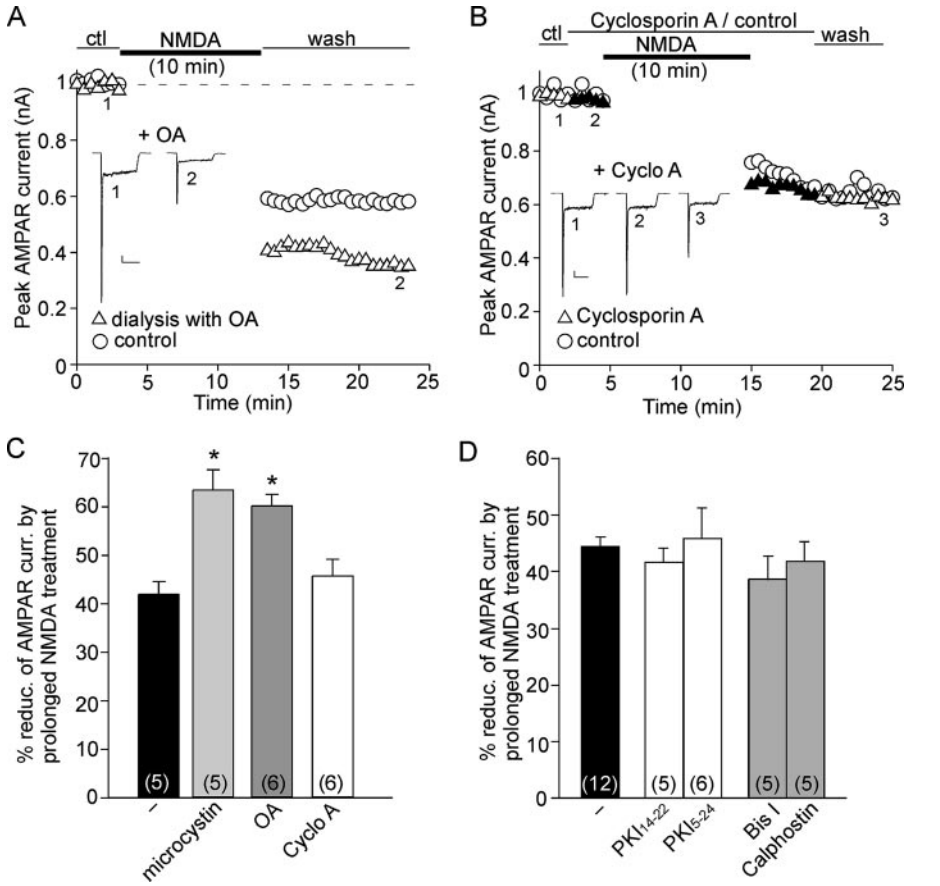

FIGURE 5. **Inhibition of PP1/2A augments the effect of prolonged NMDA treatment on AMPAR currents.** *A* and *B*, plot of I_{AMPA} with a prolonged NMDA application (100 μM, 10 min) in cells dialyzed without (control) or with the PP1/2A inhibitor okadaic acid (*OA*, 1 μM, *A*) or the PP2B inhibitor cyclosporin A (20 μM, *B*). *Inset* (*A* and *B*), representative current traces at indicated time points. *Scale bars*, 100 pA, 1 s. *C* and *D*, cumulative data (mean ± S.E.) summarizing the percentage reduction of AMPAR currents by prolonged NMDA treatment in the presence of various phosphatase inhibitors (*C*) or inhibitors for PKA and PKC (*D*). *, $p < 0.01$, ANOVA.

mutant S831D or S831E. Moreover, CaMKII significantly enhanced the calpain cleavage of GluR1 mutant S845A (3.1 ± 0.1 fold increase, $n = 3$). These results suggest that GluR1 phosphorylated at Ser^{831}, but not Ser^{845}, is much more sensitive to calpain proteolysis.

Changing Protein Phosphatase Activity Affects the Effect of Calpain on AMPAR Currents—Since inhibiting CaMKII phosphorylation of GluR1 diminished the calpain modulation of AMPARs, we then examined whether enhancing GluR1 phosphorylation would augment the calpain effect. To do this, we examined the effect of prolonged NMDA treatment on AMPAR currents in cells treated with protein phosphatase inhibitors. As shown in Fig. 5*A*, dialysis with the PP1/2A inhibitor okadaic acid (1 μM) potentiated the reducing effect of NMDA (100 μM, 10 min) on AMPAR currents. In contrast, bath application of the PP2B (calcineurin) inhibitor cyclosporine A (20 μM) failed to alter the NMDA effect (Fig. 5*B*). In the sample of neurons we tested (Fig. 5*C*), prolonged NMDA treatment reduced AMPAR current by 41.2 ± 3.1% ($n = 5$) under control conditions, and this effect was significantly ($p < 0.01$, ANOVA) enhanced in cells dialyzed with the nonspecific phosphatase inhibitor microsystin (5 μM, 60.0 ± 2.3%, $n = 5$), or okadaic acid (63.3 ± 4.1%, $n = 6$), but not cyclosporine A (45.5 ± 3.6%, $n = 6$). These results suggest that PP1/2A, but not PP2B, is involved in modifying the effect of calpain on AMPA receptors.

As controls, we also tested the potential involvement of other signaling molecules. As shown in Fig. 5*D*, the effect of prolonged NMDA treatment on AMPAR current was intact in neurons treated with PKA inhibitors (PKI_{14-22}, 1 μM, 49.0 ± 6.4%, $n = 5$; PKI_{5-24}, 20 μM, 45.8 ± 5.5%, $n = 6$). The effect was also unaltered by the PKC inhibitor bisindolylmaleimide I (4 μM, 36.0 ± 4.0%, $n = 5$) or calphostin C (1 μM, 41.6 ± 3.7%, $n = 5$). These results indicate that PKA and PKC, both phosphorylating GluR1 subunit at locations near the putative cleavage sites, are not involved in calpain regulation of AMPAR currents.

DISCUSSION

The present study provides functional evidence showing that activation of endogenous calpain by prolonged NMDA treatment reduces AMPAR-mediated currents. This *in vitro* phenomenon is pathophysiologically relevant because it is mimicked in animals exposed to transient ischemic insults, and the NMDAR-induced, calpain-mediated depression of AMPA responses is occluded in ischemic animals (9). Moreover, the effect of calpain on AMPAR currents is abolished by inhibiting CaMKII but is augmented by inhibiting PP1/2A. Consistently, *in vitro* assay shows that GluR1-CT is cleaved by application of calpain. Furthermore, activation of CaMKII enhances the ability of calpain to cleave GluR1 subunit. These results suggest that the phosphorylation state of GluR1 subunit, which is regulated by CaMKII-PP1/2A, controls its susceptibility to calpain cleavage.

Previous studies using repetitive synaptic stimulation or glutamate treatment (to drive long term potentiation/long term depression *in situ*) did not find the CaMKII-facilitated, calpain-mediated proteolysis of AMPARs. There are two major reasons for this. First, our stimulus (10-min glutamate) is more potent and persistent than long term potentiation/long term depression-driven stimuli (such as brief tetanic stimulus or 1–2 min glutamate application). We have compared the distinct effects of prolonged *versus* transient NMDAR stimulation on AMPARs (9). A brief NMDA treatment only triggers clathrin-mediated AMPAR internalization, while a prolonged NMDA treatment has a long term effect leading to calpain-mediated proteolysis (9). Only the strong stimulus (*e.g.* 10-min glutamate) will cause the activation of calpain. Second, most of electrophysiological studies used protease inhibitors (*e.g.* leupep-

tin) in their recording electrodes; therefore the effect of proteases (such as calpain) was blocked.

Calpain-mediated cleavage not only induces protein degradation but also modifies the substrate activity or generates new molecules. For example, in the brains of Alzheimer's disease patients, calpain cleaves cyclin-dependent kinase 5 (cdk5) activator p35 to p25, resulting in prolonged activation and mislocalization of cdk5, ultimately leading to neuronal death (28). Our electrophysiological and biochemical results suggest that calpain alters the function of another target, AMPA receptor, via proteolytic cleavage of GluR1 subunits. This leads to the suppression of functional AMPARs at the neuronal membrane and thus reduces AMPAR-mediated synaptic transmission. Thus, calpain could function as a feedback inhibitor of AMPAR activity when neuron is overstimulated.

Several studies have suggested that AMPAR functions can be influenced by protein kinases and phosphotases. For example, phosphorylation of GluR1 subunits (Ser^{845}) by PKA stabilizes AMPARs at the synapse, whereas dephosphorylation of the same site by phosphatase 2B promotes internalization of AMPARs (29). In the present study, we found that manipulating CaMKII activity alters the susceptibility of AMPARs to calpain regulation. This is not due to the direct phosphorylation at AMPAR channels, because application of CaMKII inhibitors did not affect basal AMPAR currents nor reverse the pre-established calpain suppression of AMPAR currents. We speculate that prolonged NMDAR stimulation may cause CaMKII phosphorylation of GluR1 subunits, therefore altering the calpain proteolysis of AMPARs. In agreement with it, we found that application of CaMKII inhibitors or suppression of CaMKII expression by siRNA prevented the calpain-induced reduction of AMPAR currents. *In vitro* biochemical assay also indicated that the calpain cleavage of GluR1 C-terminal fusion protein was substantially augmented by adding purified active CaMKII. These results suggest that the conformational change of GluR1 caused by CaMKII phosphorylation at Ser^{831} makes GluR1 more vulnerable to calpain cleavage.

Another important molecule that regulates the phosphorylation state of AMPA receptors is the serine/threonine phosphatase PP1. PP1 is enriched in dendritic spines of neurons (30) and plays an important role in modulating AMPAR functions (3). Appropriate NMDAR activation recruits more PP1 to synapses, allowing it to gain access to synaptic substrates, which is necessary for sustaining long term depression (31). PP1 could reduce GluR1 phosphorylation either directly or indirectly through inhibiting CaMKII Thr^{286} autophosphorylation and its autonomous activity (20). We found that suppressing PP1 activity (which would lead to elevated GluR1 phosphorylation) augmented the calpain effect on AMPAR currents, further suggesting that phosphorylated GluR1 subunits are more vulnerable to calpain cleavage.

Given the important role of calpain in the pathophysiology of multiple brain diseases, it is important to understand the cellular basis of calpain-mediated cleavage. Here we provide evidence suggesting that the phosphorylation state of one of the calpain substrates, AMPAR GluR1 subunit, controls its sensitivity to calpain cleavage. Thus, by increasing or decreasing calpain-mediated cleavage of AMPARs, CaMKII/PP1 activity could be especially critical for neurons to control excessive excitability as what occurs during stroke and ischemia.

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