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N-Methyl-D-aspartate-induced α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptor Down-regulation Involves Interaction of the Carboxyl Terminus of GluR2/3 with Pick1

LIGAND-BINDING STUDIES USING Sindbis VECTORS CARRYING AMPA RECEPTOR DECOYS*

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The dynamics of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, as represented by their exocytosis, endocytosis and cytoskeletal linkage, has often been implicated in *N*-methyl-D-aspartate (NMDA)-dependent synaptic plasticity. To explore the molecular mechanisms underlying the AMPA receptor dynamics, cultured hippocampal neurons were stimulated with 100 μM NMDA, and the biochemical and pharmacological changes in the ligand binding activity of AMPA receptor complexes and its subunits, GluR1 and GluR2/3, were investigated. The NMDA treatment reduced the total amount of bound [^{3}H]AMPA on the surface of the neurons but not in their total membrane fraction. This process was mimicked by a protein kinase C activator, phorbol ester, but blocked by an inhibitor of the same kinase, calphostin C. The NMDA-induced down-regulation of the ligand binding activity was also reflected by the decreased AMPA-triggered channel activity as well as by the cells' reduced immunoreactivity for GluR1. In parallel, the NMDA treatment markedly altered the interaction between the AMPA receptor subunits and their associating molecule(s); the association of PDZ molecules, including Pick1, with GluR2/3 was enhanced in a protein-kinase-C-dependent manner. Viral expression vectors carrying GluR1 and GluR2 C-terminal decoys, both fused to enhanced green fluorescent protein, were transfected into hippocampal neurons to disrupt their interactions. The overexpression of the C-terminal decoy for GluR2 specifically and significantly blocked the NMDA-triggered reduction in [^{3}H]AMPA binding, whereas that for GluR1 had no effects. Co-immunoprecipitation using anti-Pick1 antibodies revealed that the overexpressed GluR2 C-terminal decoy indeed prevented Pick1 from interacting with the endogenous GluR2/3. Therefore, these observations suggest that the NMDA-induced down-regulation of the functional AMPA receptors involves the interaction between GluR2/3 subunits and Pick1.

Synaptic processes in hippocampal plasticity, such as long term potentiation (LTP)[1] and long term depression (LTD), are thought to involve the subcellular dynamics of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor channels. The most recent physiological studies have suggested that an exocytotic process of these receptor channels to the postsynaptic sites may form the basis of the NMDA-dependent hippocampal LTP (1). Conversely, the elimination of the receptors from the synaptic surface has been suggested to associate with hippocampal LTD (2). The former synaptic plasticity is known to require the activation of calmodulin-dependent protein kinase α (3, 4), whereas the latter involves protein kinase C (PKC) as well as various protein phosphatases (5, 6). Through the NMDA receptor channels, the influx of calcium ions can lead to the activation of these two protein kinases, each of which results in opposing synaptic changes. Although it remains to be established how both types of synaptic plasticity are switched on and off following the postsynaptic influx of calcium ions, postsynaptic density (PSD) proteins should contribute to such synaptic processes.

There are a large variety of PSD proteins and other molecules that can interact with AMPA receptor subunits. The AMPA receptor subunit GluR1 is known to interact with SAP97, Narp, and Lyn through various subdomains of this subunit (7–9). Other subunits (*e.g.* GluR2 and GluR3) can associate with GRIP, ABP, Pick1, and NSF at its carboxyl-terminal regions (4, 10–12). These adaptor molecules appear to regulate the subcellular distributions of AMPA receptor subunits, altering their cytoskeletal associations and/or metabolic stability (13, 14). Although individual subunits of the AMPA receptor channel complex have been shown to interact with different PSD proteins in parallel with AMPA receptor internalization (5, 6, 15–20), the real dynamics of the functional AMPA receptor complexes and the underlying molecular mechanism(s) have not been fully characterized. For instance, which AMPA receptor subunit governs the dynamics of the whole receptor complex? Which adaptor molecule accordingly regulates the pharmacological change of the AMPA receptors? In cultured hippocampal neurons, we attempted to address these questions by monitoring their ligand binding activity and mo-

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[1] The abbreviations used are: LTP, long term potentiation; LTD, long term depression; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EGFP, enhanced green fluorescent protein; NMDA, *N*-methyl-D-aspartate; PSD, postsynaptic density; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance.

lecular interactions. Using the Sindbis viral expression vector carrying AMPA receptor decoys, here we investigated the AMPA receptor subunits and domains responsible for the NMDA-induced pharmacological changes of AMPA receptors.

EXPERIMENTAL PROCEDURES

Hippocampal Culture—Hippocampal tissues were taken from embryos of Sprague-Dawley rats (embryonic day 18) and dissociated with 1 mg/ml papain solution. Hippocampal neurons were plated at a density of ~5.0×10^5 cells/ml with Dulbecco's modified Eagle's medium containing 10% calf serum, and grown for 6–7 days in serum-free conditions, as described previously (21). These cultures typically contained less than 5% nonneuronal cells, as monitored by antiglial fibrillary acidic protein immunostaining (22).

The stimulation of NMDA or PKC was carried out as follows. Hippocampal neurons were washed with warm phosphate-buffered saline and then exposed to 100 μM NMDA (Sigma) or 1 μM phorbol 12-myristate 13-acetate (PMA; Calbiochem) for 10 min in HEPES-buffered Hanks' solution containing 1 mM glycine but no magnesium ions. After 10 min, the stimulation was terminated by application of an NMDA receptor antagonist, D-2-amino-5-phosphonovaleric acid (50 μM; Sigma), and cultures were further incubated at 37 °C for 20 min. The treatment did not lead to severe neuronal damage; 98, 89, and 92% of hippocampal neurons were alive 40 min, 3 h, and 24 h after the NMDA stimulation, respectively, as revealed by the dye exclusion assay (23, 24). Prior to the NMDA treatment, cultures were preincubated with the protein kinase inhibitors K252b (100 nM; Calbiochem), calphostin C (1 μM; Wako), or KN93 (370 nM; Calbiochem).

[^{3}H]AMPA Binding Assay—Cultured neurons were rinsed with Tris buffer, pH 7.4, containing 100 μM sodium acetate, 2.5 mM $CaCl_2$, and 5 g/liter glucose. [^{3}H]AMPA (1561 GBq/mmol, 37 MBq/ml; Amersham, Japan) was adjusted to 50 nM with the Tris buffer and diluted with 0–1000 nM cold AMPA (RBI). Neurons were incubated with these AMPA solutions on ice for 60 min as described previously (25, 26). After washing with the cold Tris buffer, neurons were lysed with 0.5 N NaOH, and the radioactivity of the cell lysates was counted by the Liquid Scintillation System LSC-3050 (Aloka).

Whole-cell Patch Clamp—Electrophysiological analysis was performed at room temperature (24–26 °C) following the treatment with 100 μM NMDA. Membrane currents were monitored after being low pass-filtered at 3 kHz by the amplifier (Axoclamp 2B; Axon Instruments), and data were analyzed with pClamp6 software (Axon Instruments). The hippocampal culture medium was replaced with Krebs-Ringer solution, which consisted of 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM D-glucose, 20 mM sucrose, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.4. A hippocampal neuron was held with a glass electrode (6–8 MΩ) containing 130 mM $CsMeSO_2$, 10 mM CsCl, 10 mM HEPES, 0.5 mM ethylene glycol-bis-(oxonitrilo)tetraacetic acid, 5 mM QX-314·Cl^- (Alomone Laboratories), 3 mM Mg-ATP, pH 7.4, and clamped at −78 mV. AMPA (RBI) was dissolved in the same solution and applied locally to a neuron by positive air pressure (4 p.s.i., 20 ms) through a different glass pipette. If data exhibited changes in series resistance of greater than 20% during experiments, they were excluded from analysis.

Immunostaining—To visualize the AMPA receptors on the cell surface, hippocampal neurons were treated in culture with 20 μg/ml rabbit antibodies raised against the amino terminus of GluR1 (27). After fixation with 4% paraformaldehyde in phosphate-buffered saline, neurons were incubated with the Cy3-labeled secondary anti-rabbit immunoglobulin antibodies (1:500, Vector Laboratories) in the presence of 10% normal goat serum. Alternatively, the neurons that had been preincubated with the anti-GluR1 antibodies were similarly stimulated with NMDA, and the remaining anti-GluR1 antibodies on the cell surface were then removed with an acid-phosphate-buffered saline solution containing 0.25 M acetic acid (a process known as acid stripping) (28). Internalized GluR1 immunoreactivity was visualized by following fixation of the cell and subsequent immunostaining with secondary antibodies in the presence of 0.1% Triton X-100. Immunoreactivity was observed and pictured with the aid of a Zeiss fluorescence microscope (Axioskop).

Immunoprecipitation—Neurons in culture were harvested with a cell scraper and transferred into a microtube. Cell pellets were resuspended in 0.32 M sucrose solution containing a protease inhibitor mixture (0.5 mM leupeptin (Wako), 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5 mM NaF, and 1 mM $NaVO_4$ (Sigma)) and disrupted by sonication. After centrifugation at 15,000 rpm, the membrane fraction was obtained and solubilized with 1% sodium deoxycholate (12, 29). Total cell lysate (1 mg) was incubated at 4 °C for 12–16 h with 10 μg of anti-Pick1 (30), anti-GRIP1 (31), or anti-panPDZ antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). The resulting immunocomplexes were precipitated with Protein G-Sepharose beads (Amersham Pharmacia Biotech) and subjected to SDS-polyacrylamide gel electrophoresis.

Western Blot Analysis—Protein samples for Western blotting were prepared from cultured neurons or from immunoprecipitates. These samples were homogenized in 2% SDS by sonication and denatured in the presence of 100 μM dithiothreitol (Wako) at 95 °C for 5 min. In parallel, their protein concentrations were determined by a micro-BCA kit (Protein Assay Reagent; Pierce). Typically, 20 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schuell) by electrophoresis. The membrane was probed with anti-C-terminal GluR1 antibodies (1:1000; Chemicon), anti-C-terminal GluR2/3 antibodies (1:1000; Chemicon), or antibodies raised against enhanced green fluorescent protein (EGFP; 1:500; CLONTECH). After extensive washing, the immunoreactivity on the membrane was detected with an anti-rabbit/mouse immunoglobulin conjugated to horseradish peroxidase, followed by a chemiluminescence reaction. The immunoreactivity of the bands was quantified by densitometric analysis.

Sindbis Virus Expression Vector—The modified EGFP that carried either the carboxyl-terminal sequence of GluR1 (SSGMPLGATGL) or that of GluR2 (GYNVYGIESVKI) was constructed as follows. Using the EGFP expression vector pIRES/EGFP (CLONTECH) as a template, the polymerase chain reaction was performed with the forward primer for the PsG region, TACGGTGGTCCTAAATAGTC, and with the reverse primers corresponding to the stop codon of the fused proteins of EGFP and the above amino acid sequences: CTACTAAATTTTAACACTCTC-GATGCCATATACGTTGTAACCCTTGTACAGCTCGTC (for GluR1) and CTATTACAATCCTGTGGCTCCCAAGGGCATCCCTGAACTCTT-GTACAGCTCGTC (for GluR2). Polymerase chain reaction products were cut with the restriction enzymes *Xba*I and *Not*I, and these DNA cassettes were replaced by the 3′-translation region of the EGFP sequence in pSindRep5 (32). Viral genome RNA was transcribed *in vitro* from these constructs by SP6 RNA polymerase using an Invitroscript CAP kit (Invitrogen) and co-transfected with mRNAs coding viral coat proteins into BHK cells, according to the manufacturer's protocol (Invitrogen). The infectivity of the Sindbis virus particles was titered with BHK cells by serial dilution. Typically, we obtained a viral titer of more than 10^7 pfu/ml.

Hippocampal neurons were exposed to the Sindbis viral vectors at a titer of $>10^7$ pfu/ml for 1 h. After washing, the neurons were further incubated in regular growth medium at 37 °C for 6–12 h. The expression of EGFP from the vectors was monitored with the aid of the fluorescence microscope. At the viral titer used for transfection, more than 94% of neurons showed green fluorescence (see Fig. 6).

Statistical Analysis—One-factor ANOVA was employed to analyze the differences in [^{3}H]AMPA binding or immunoreactivity. Given distributions of the values of electrophysiological data and comparisons between NMDA-treated or control groups were made with the Mann-Whitney U test. The level of statistical significance was set at $p < 0.05$.

RESULTS

Down-regulation of the Ligand Binding Activities of AMPA Receptors Expressed on Neuronal Surfaces—The radiolabeled receptor assay method allows measurement of the amounts of radiolabeled ligands bound to cell membranes and enables characterization of the pharmacological properties of its receptors, such as the number of functional receptors (B_{max}) and the dissociation constant for their ligands (K_d) (25, 26). In the present study, this assay was used with ^{3}H-labeled AMPA to assess the effects of NMDA treatment on the ligand binding activity of functional AMPA receptors expressed on the surface of cultured hippocampal neurons. Hippocampal neurons were prepared from embryonic rats, grown in serum-free conditions for 7 days (21) and then treated with 100 μM NMDA for 10 min. Thirty minutes after the NMDA treatment, various concentrations of [^{3}H]AMPA were applied to the cultures and the binding of the radioactive ligand to neurons was measured. The values of bound [^{3}H]AMPA were subjected to Scatchard plotting, and the total amount (B_{max}) and the dissociation constant (K_d) of the functional AMPA receptors were estimated (Fig. 1).

The NMDA treatment significantly decreased the B_{max} of the AMPA receptors on the cell surface to 55% that of the control.

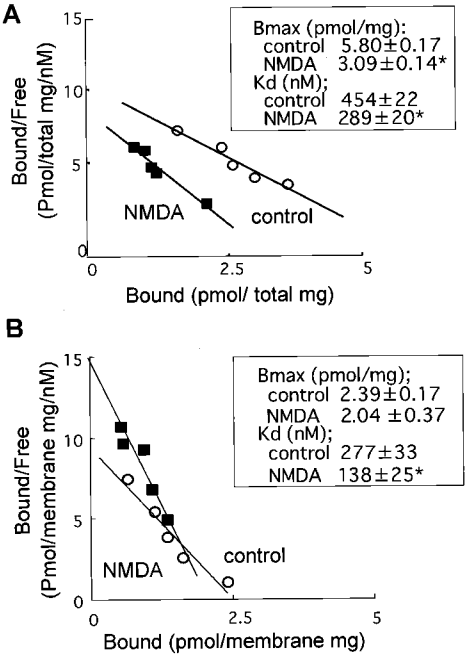

FIG. 1. **Scatchard analysis of [^{3}H]AMPA binding to cultured hippocampal neurons.** Hippocampal neurons were cultured in serum-free condition for 7 days and then treated with 100 μM NMDA for 10 min (*NMDA*) or with fresh Hank's solution (*control*). The cultured neurons (*A*) or the total membrane fractions (*B*) prepared from lysed neurons were exposed to mixtures of 50 nM [^{3}H]AMPA and 0–1000 nM cold AMPA. After washing, the resulting radioactivity of the membrane-bound [^{3}H]AMPA was measured and visualized graphically on a Scatchard plot. The set of binding experiments was repeated four times, and the maximum amount of bound [^{3}H]AMPA (B_{max}) and the dissociation constant (K_d) were calculated on each plot and averaged ($n = 4$). A typical Scatchard plot is shown for display. *, $p < 0.05$ with one-factor ANOVA.

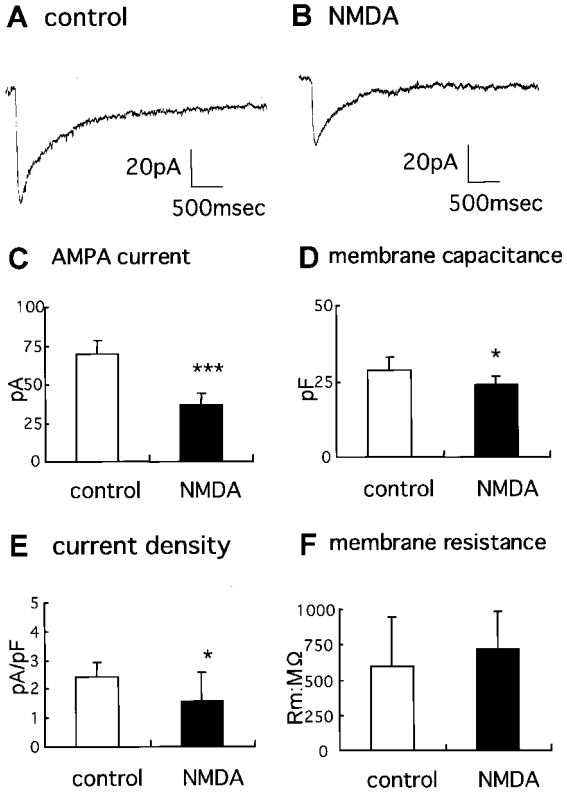

FIG. 2. **Reduction in AMPA-triggered currents revealed by the patch clamp method.** The effects of the NMDA treatment on the activity of functional AMPA receptor channels were assessed by the whole-cell patch clamp method. Hippocampal neurons that had been pretreated either with or without NMDA were voltage-clamped by Axoclamp 2B and exposed to 100 μM AMPA by pressure injection. *A* and *B*, typical responses to AMPA. *C*, averaged AMPA currents. *D* and *E*, cell membrane capacitance measurements, made by the integration of capacitive transients and the current density of the AMPA response. *F*, the membrane resistance of these cultured hippocampal cells was not influenced by the NMDA treatment, however. Significant difference from controls is shown as follows: *, $p < 0.05$; ***, $p < 0.001$ with Mann-Whitney *U* test.

The dissociation constant of the AMPA receptors (K_d) decreased from 454 ± 22 to 289 ± 20 nM. This result indicates that the exposure of hippocampal neurons to NMDA decreased the total number of the AMPA receptors on their surface.

The above results raised the question of how the functional AMPA receptors were depleted from the cell surface. To address this question, we carried out the same radiolabeled receptor assay using the total membrane fraction of hippocampal neurons as a target material (26). The fraction contained total cell membranes, including intracellular membranes. The NMDA treatment failed to exert a significant influence on the B_{max} of the AMPA receptors in the total membrane fraction, although NMDA did increase the affinity of the AMPA receptors 2-fold. These observations imply that the reduction in surface [^{3}H]AMPA binding could be attributed to the internalization of AMPA receptors from the neuronal surface.

Treatment with NMDA Decreases Channel Activity of AMPA Receptors on Cultured Hippocampal Neurons—Electrophysiological studies have suggested that chemical exposure of hippocampal slice preparations to glutamate or NMDA induces long term depression of synaptic currents, namely chemically induced LTD (14). It has yet to be established whether the down-regulation of AMPA receptor binding is correlated with a functional decrease in their channel activities. In the present study, AMPA-induced currents were measured in hippocampal neurons by the whole-cell patch clamp method. Cultured hippocampal neurons, which were pretreated with or without NMDA, were voltage-clamped at −78 mV, and 100 μM AMPA was applied to their soma by pressure injection (Fig. 2). The AMPA currents were significantly decreased to 51.3 ± 17.1% ($n = 7$) of control values by the NMDA treatment. The magnitude of the reduction in AMPA currents was similar to that for the surface [^{3}H]AMPA binding, as shown earlier. These results further confirm that the down-regulation of their cell surface expression reflects the decrease in channel activities. In parallel, the membrane capacitance of hippocampal neurons and current densities were reduced to 82 ± 11.4 and 65 ± 9.8% of control values, respectively. The latter changes might be associated with the plasticity-related structural changes in hippocampal neurons such as membrane endocytosis or synapse elimination (5, 33, 34).

Effects of Protein Kinase Inhibitors on [^{3}H]AMPA Binding—Hippocampal synaptic plasticity is associated with the phosphorylation of AMPA receptor subunits, which depends upon various protein kinases (3, 4, 16, 35). To explore the intracellular signaling cascades leading to the AMPA receptor down-regulation, various kinase inhibitors were tested together with the NMDA treatment. Prior to the NMDA treatment, hippocampal neurons were pretreated with a PKC inhibitor, calphostin C (36), a broad protein-tyrosine kinase inhibitor, K252b (37), or a calmodulin-dependent protein kinase inhibitor, KN93 (38, 39). The ligand-binding activity of the AMPA receptors was estimated by measuring the total binding of [^{3}H]AMPA on the cell surface with the highest concentration (1 μM) of AMPA (Fig. 3). In the NMDA-treated culture, the surface binding of [^{3}H]AMPA decreased to 58 ± 8.2% of the control

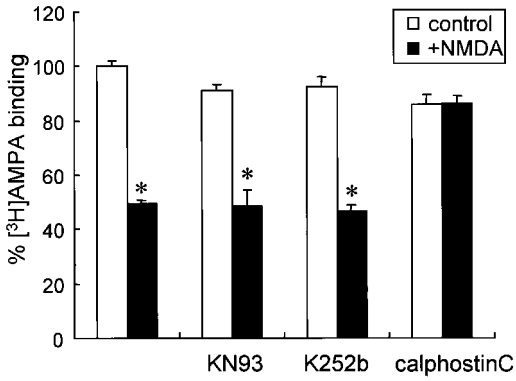

FIG. 3. **Effects of protein kinase inhibitors on the NMDA-induced down-regulation of AMPA receptors.** Hippocampal cultures were pretreated with several protein kinase inhibitors: KN93 (370 nM), K252b (100 nM), and calphostin C (1 μM). Neurons were then treated with or without NMDA and subsequently exposed to the mixture of 200 nM [^{3}H]AMPA and 800 nM AMPA (610 Bq/ml). A saturated amount of AMPA binding was then estimated. The radioactivity of [^{3}H]AMPA bound to the untreated hippocampal neurons was set as 100%, and relative amounts of [^{3}H] AMPA binding were calculated and averaged ($n = 4$). *, $p < 0.05$ with one-factor ANOVA.

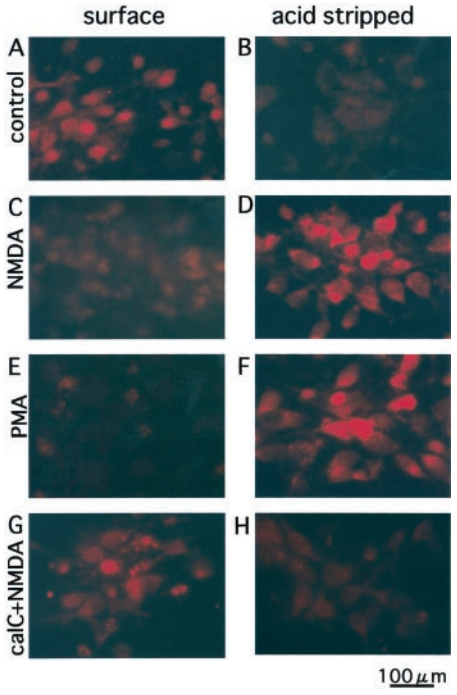

FIG. 4. **Internalization of AMPA-receptor-like immunoreactivity after NMDA treatment and PKC activation/inhibition.** Hippocampal neurons were treated with 100 μM NMDA (*C* and *D*), 1 μM phorbol ester (*PMA*; *E* and *F*), and NMDA plus calphostin C (*calC + NMDA*; *G* and *H*) or not treated (controls; *A* and *B*). The number of AMPA receptors remaining on cell surface was estimated by immunostaining of living neurons with antibodies raised against the extracellular domain of GluR1 (*A*, *C*, *E*, and *G*). AMPA receptors that had been internalized into cells were revealed by acid stripping of the anti-GluR1 antibodies remaining those on cell surfaces after NMDA treatment (*B*, *D*, *F*, and *H*) (27).

value. Only pretreatment with calphostin C significantly blocked the NMDA-triggered reduction in binding, whereas K252b and KN93 failed to inhibit the decrease. Thus, this pharmacological result implies that the down-regulation of AMPA binding requires the activation of PKC.

Down-regulation of AMPA Receptors Represents Internalization of AMPA Receptor Subunits—In our hippocampal culture, NMDA treatment led to a reduction in the surface AMPA binding and channel activities of the AMPA receptor complexes presumably through the activation of PKC. Previous immunohistochemical studies have demonstrated that similar chemical stimulation with glutamate results in the internalization of AMPA receptor subunits (15–18). These observations raised the question as to whether the down-regulation of AMPA receptor binding depends upon the internalization of these subunits. The total immunoreactivity to the AMPA receptor subunit, GluR1, on the surface of hippocampal neurons was visualized by labeling with antibodies raised against its extracellular domain, without cell fixation (*i.e.* permeabilization). Alternatively, hippocampal neurons were similarly preexposed to the anti-GluR1 antibodies and subsequently subjected to the NMDA treatment. Then the GluR1 antibodies remaining on the cell surface were washed away by a short exposure to acid solution (27, 28). Accordingly, the antibodies bound to the internalized AMPA receptor subunits escaped from the acid washing and were visualized by subsequent fixation and immunostaining for GluR1 (Fig. 4).

In the untreated control group, GluR1 immunoreactivity on the cell surface was much brighter than that inside the neurons. In the hippocampal neurons that were treated with NMDA or a PKC activator (PMA); however, the immunoreactivity on the cell surface was decreased in comparison with that on control cultures. Conversely, the intracellular immunoreactivity, which presumably reflected the internalized AMPA receptors, became more intense than the surface immunoreactivity (Fig. 4, *C–F*). In contrast, when the hippocampal neurons were exposed to NMDA in the presence of the PKC inhibitor, calphostin C, the surface immunoreactivity for the AMPA receptors was not translocated into cells (Fig. 4, *G* and *H*). Together with our pharmacological and electrophysiological findings on AMPA receptors, these immunohistochemical results indicate that, in cultured hippocampal neurons, the activation of NMDA-type glutamate receptors triggers AMPA receptor internalization leading to their down-regulation in a PKC-dependent manner.

Treatment with NMDA Enhances the Interaction between GluR2/3 and Pick1—Previous molecular studies have suggested that the postsynaptic compartment contains various PSD proteins that carry the PDZ domain(s) (29, 40–42). These proteins are known to interact with various types of glutamate receptors through the PDZ domain(s) and are implicated in the regulation of their distribution and stability in neurons (13, 43, 44). We aimed to confirm their contribution to the effects of the NMDA treatment in our culture system. Hippocampal neurons were grown for 7 days and transiently exposed to NMDA or PMA with or without pretreatment with the PKC inhibitor. Hippocampal neurons are known to contain two major compositions of AMPA receptor complexes: GluR1/GluR2 hetero-oligomers and GluR2/GluR3 hetero-oligomers (45). Accordingly, the interaction of these complexes with PDZ proteins was assessed by co-immunoprecipitation using anti-PDZ protein antibodies, followed by immunoblotting for GluR2/3 (Fig. 5). The amount of GluR2/3 immunoreactivity associated with PDZ proteins was significantly increased by the treatment with NMDA (155.4 ± 14.7%, $p < 0.05$) and with PMA (158 ± 11.6%, $p < 0.05$) compared with those left untreated (100 ± 21.7%). The increase in AMPA receptor immunoreactivity was inhibited by pretreatment with the PKC inhibitor, calphostin C. Among various PDZ proteins, GRIP and Pick1 are known to influence the distribution and/or subcellular dynamics of the AMPA receptors through their interactions. Using the same membrane fractions from the above hippocampal cultures, we carried out the co-immunoprecipitation experiments with anti-GRIP antibodies or with anti-Pick1 antibodies. The amounts of co-immunoprecipitated AMPA receptor complexes were estimated by

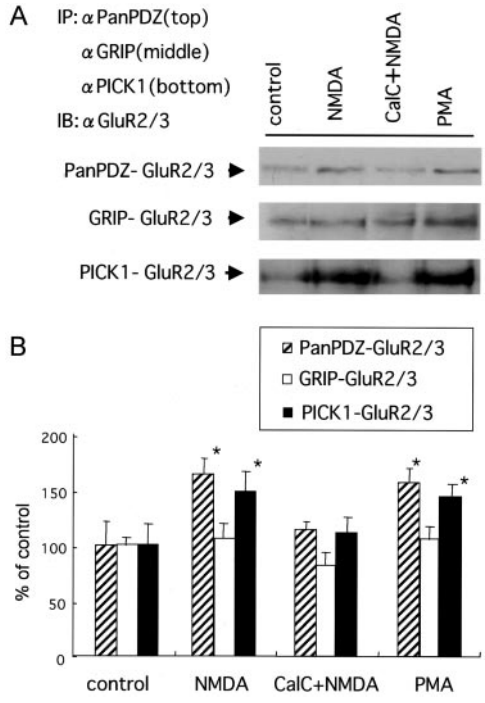

FIG. 5. **Altered interaction between the AMPA receptor and PDZ proteins including Pick1.** *A*, protein extracts were prepared from hippocampal neurons that were treated with NMDA, NMDA + calphostin C, and PMA. Hippocampal proteins were immunoprecipitated with anti-panPDZ antibodies, anti-GRIP antibodies, or anti-Pick1 antibodies. Immunoprecipitates were subjected to immunoblotting with anti-GluR2/3 antibodies. *B*, the effects of these stimulations on the interaction between panPDZ proteins and GluR2/3, between GRIP and GluR2/3, or between Pick1 and GluR2/3 are expressed as percentage ratios to the basal interaction in untreated neurons ($n = 4$). *, $p < 0.05$ with one-factor ANOVA.

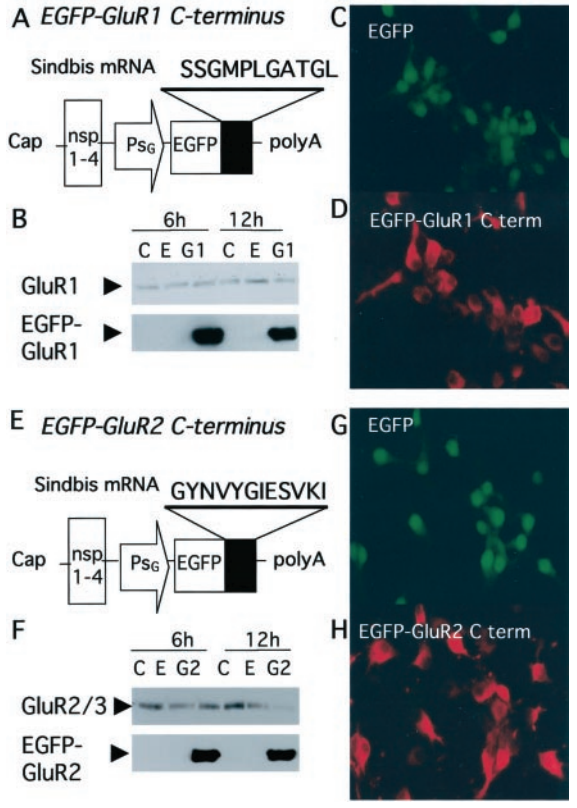

FIG. 6. **Neuronal expression of EGFP fused to the GluR1-terminal decoy or to the GluR2-terminal decoy using Sindbis virus vectors.** The fluorescent protein EGFP was modified by genetic engineering to have the extra amino acid sequences of SSGMPLGATGL and GYNVYGIESVKI. The sequences corresponded to the carboxyl termini of the rat AMPA receptor subunits GluR1 and GluR2 (59, 60). cDNA encoding the modified EGFP was introduced to the Sindbis expression vector, pSindRep5 (26) and transcribed into viral mRNA *in vitro* (*A* and *E*). After packaging in BHK cells, Sindbis virus vectors carrying EGFP-GluR1 C terminus and EGFP-GluR2 C terminus were transfected to cultured hippocampal neurons at a titer of $1.0–1.5 \times 10^7$ pfu/ml for 1 h. Total protein was extracted from the neurons 6 or 12 h after transfection and subjected to immunoblotting to monitor the expression of the GluR1-terminal decoy (*B*) or the GluR2-terminal decoy (*F*) as well as that of the endogenous receptors. Hippocampal cultures were examined for fluorescence of EGFP (*C* and *G*) and also immunostained with the anti-GluR1 C-terminal antibody (*D*) or with the anti-GluR2/3 C-terminal antibody (*H*). Note that the rat AMPA receptor subunit, GluR3, contains an almost identical C-terminal sequence, GYNVY**T**ESVKI (61).

the subsequent immunoblotting with anti-GluR2/3 antibodies. Consistent with the above observation, treatment with NMDA or with PMA significantly increased the amount of GluR2/3 that was co-immunoprecipitated with anti-Pick1 antibodies. In addition, the PKC inhibitor blocked this increase in Pick1-GluR2/3 interaction. In contrast, none of the treatments influenced the levels of GluR2/3 that were co-immunoprecipitated with anti-GRIP antibodies. These observations suggest that the NMDA stimulation and the subsequent PKC activation trigger the down-regulation of the AMPA receptor complexes through their interaction with Pick1.

Sindbis Virus Vectors Carrying Decoys of GluR2 but Not GluR1 Inhibit AMPA Receptor Down-regulation—The circumstantial evidence outlined earlier emphasizes the importance of the GluR2/3-Pick1 interaction in receptor down-regulation or internalization. Whether or not this interaction is essential for the down-regulation of functional AMPA receptors has yet to be determined. Most of the PDZ proteins are known to interact with various glutamate receptors through their binding to the carboxyl termini of the receptors (30, 31, 40). We addressed this matter by challenging these interactions with overexpression of the carboxyl-terminal peptides of the AMPA receptor subunits (*i.e.* AMPA receptor decoys). We attached the peptide sequences of rat GluR1 and GluR2 subunits to the carboxyl-terminal of EGFP and introduced them into a Sindbis virus vector (pSindRep5; Invitrogen). The viral expression vectors carrying EGFP + GluR1 C-terminal, EGFP + GluR2 C-terminal, or EGFP alone were introduced into cultured hippocampal neurons (Fig. 6, *A* and *E*). Their expressions were monitored by immunostaining as well as by immunoblotting of the culture lysates using antibodies raised against the carboxyl-terminal sequences of the AMPA receptor subunits (32). Strong immunoreactivity for EGFP as well as for the GluR1 C-terminal and GluR2 C-terminal was seen in more than 90% of cultured neurons, as early as 6 h following incubation (Fig. 6*C*, *D*, *G*, and *H*). In parallel, the transfection of the Sindbis viral vectors indeed led to the overexpression of the C-terminal decoy peptides for both AMPA receptors; the immunoreactivities of EGFP + GluR1 C-terminal and EGFP + GluR2 C-terminal were more intense (by >10-fold) than those of endogenous GluR1 and GluR2, respectively (Fig. 6, *B* and *F*). Under the conditions of overexpression, hippocampal neurons were subjected to NMDA treatment followed by the radiolabeled receptor assay with [^{3}H]AMPA (Fig. 7*A*). Transfection of these Sindbis vectors resulted in a modest reduction (by ~20%) in the endogenous receptor binding to [^{3}H]AMPA, irrespective of the overexpression of these C-terminal decoy peptides (see below). The overexpression of the EGFP + GluR2 C-terminal decoy specifically inhibited the NMDA-triggered reduction in [^{3}H]AMPA binding. In contrast, the overexpression of the EGFP + GluR1 C-terminal decoy or that of EGFP alone had no

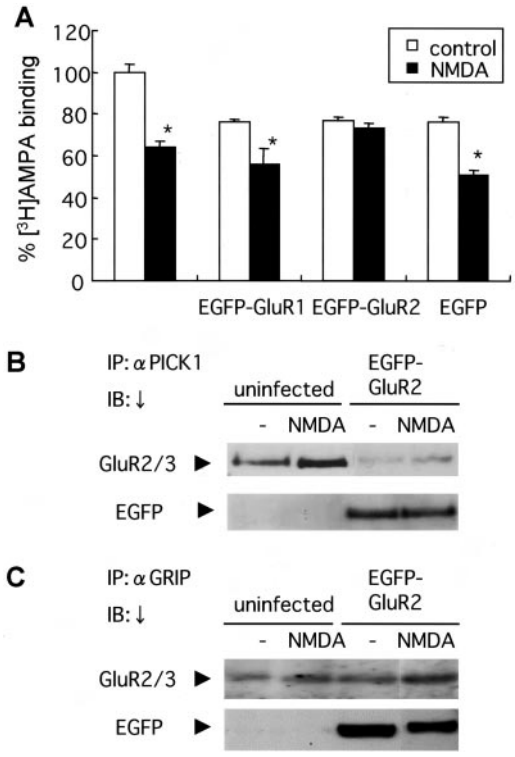

FIG. 7. **Effects of the GluR1 C-terminal decoy or the GluR2 C-terminal decoy on the down-regulation of surface AMPA binding.** *A*, hippocampal neurons expressed EGFP-GluR1 C-terminal decoy, EGFP-GluR2 C-terminal decoy, or authentic EGFP, infected with the Sindbis virus vectors carrying those EGFPs. Neurons were treated with or without NMDA and then exposed to [^{3}H]AMPA to estimate changes in AMPA receptor levels on cell surfaces. The radioactivity of [^{3}H]AMPA bound to the untreated and untransfected hippocampal neurons was set as 100%, and relative amounts of [^{3}H]AMPA binding were calculated and averaged in individual conditions ($n = 4$). *, $p < 0.05$ with one-factor ANOVA. *B*, in the place of [^{3}H]AMPA binding, the protein interaction of Pick1 with endogenous GluR2/3 or with the EGFP-GluR2 C-terminal decoy was monitored in parallel. Total protein was extracted from neurons that had been infected with or without the Sindbis virus vectors carrying the EGFP-GluR2 C-terminal decoy and had then been treated with or without NMDA. Immunoprecipitation was carried out with anti-Pick1 antibodies, and immunoprecipitates were subjected to immunoblotting with anti-GluR2/3 antibodies as well as with anti-EGFP antibodies. *C*, the protein interaction of GRIP with endogenous GluR2/3 or with the EGFP-GluR2 C-terminal decoy was also examined. Immunoprecipitation was carried out with anti-GRIP antibodies, and immunoprecipitates were subjected to immunoblotting with anti-GluR2/3 antibodies as well as with anti-EGFP antibodies as described above.

effects on the NMDA-induced reduction in [^{3}H]AMPA binding.

The interaction of Pick1 and GRIP with endogenous GluR2/3 or with the EGFP + GluR2 C-terminal decoy was monitored by immunoprecipitation using anti-Pick1 and anti-GRIP antibodies in parallel. In untransfected hippocampal cultures, the NMDA treatment indeed enhanced the interaction between GluR2/3 and Pick1. In contrast, the overexpression of the EGFP + GluR2 C-terminal decoy induced its association with Pick1 conversely, preventing Pick1 from binding to the endogenous GluR2/3 in the NMDA-untreated culture. The decrease in the interaction between Pick1 and endogenous GluR2/3 was also apparent in the NMDA-treated cultures. In contrast, the overexpression of the EGFP + GluR2 C-terminal decoys failed to interfere with the interaction between GRIP and endogenous GluR2/3 although the decoys indeed bound to endogenous GRIP in hippocampal cultures. Moreover, the NMDA treatment had no effects on the interaction between GRIP and endogenous GluR2/3 in both the absence and presence of the EGFP + GluR2 C-terminal decoys. All of these results suggest that the down-regulation of the whole AMPA receptor complexes almost depends on the molecular interaction of the carboxyl termini of the AMPA subunit(s), GluR2 and/or GluR3.

DISCUSSION

By monitoring [^{3}H]AMPA binding to the receptors, we were able to examine the subcellular dynamics of functional AMPA-type glutamate receptors in young hippocampal neurons. The present study revealed that subcellular traffic of AMPA receptors in the developing neurons is very dynamic and regulated by various molecules and their interactions. (i) Chemical stimulation with NMDA results in a reduction in the number of functional AMPA receptors on the cell surface of hippocampal neurons in a PKC-dependent manner. (ii) The reduction in the surface expression of AMPA receptors coincides with a reduction in their channel function. (iii) Treatment with NMDA also enhances the molecular interaction of the AMPA receptor subunits GluR2/3, but not GluR1, with their partner PDZ protein, Pick1. (iv) Expression of EGFP-GluR2/3 decoys, but not EGFP-GluR1 decoys, perturbs the NMDA-induced elimination of surface AMPA receptors. All of these results indicate that the down-regulation of AMPA receptor functions involves the PKC-dependent interaction between GluR2/3 subunit(s) and Pick1. This molecular process might underlie the NMDA-dependent synaptic development or plasticity.

Molecular Signaling to AMPA Receptor Down-regulation—Our pharmacological approach using various chemical agents suggest that the down-regulation of AMPA receptors requires the activation of PKC following the stimulation of the NMDA receptors. Calcium influx through the NMDA receptors is known to activate calmodulin-dependent protein kinase α, which results in the phosphorylation of the AMPA receptor subunit GluR1. This pathway is often implicated in the LTP paradigm (3, 4, 46, 47). Similarly, it activates PKC to phosphorylate the other AMPA receptor subunit GluR2. But this signaling pathway is rather more often linked to the LTD paradigm (16–18, 48), although this argument is still controversial in electrophysiological studies (6, 49). In the present experiments, we found that the down-regulation of AMPA receptors requires both the activation of PKC and the recruitment of Pick1. Internalization of the AMPA receptors has been studied previously by immunochemical or electrophysiological approaches. The results of these studies suggest that glutamate analogues and insulin can trigger AMPA receptor internalization, but through distinct intracellular signal pathways; the insulin-dependent internalization requires the activation of PKC as well as that of protein phosphatases (5, 15, 20), while the NMDA-dependent internalization involves that of the latter enzymes (6, 15). On the other hand, culture studies have indicated that the activation of PKC with phorbol esters is enough to trigger the internalization of GluR2 immunoreactivity in both neocortical and cerebellar cultures (16, 18), which is consistent with our findings. Although it still remains to be determined which isoforms of PKC are involved in the receptor internalization in each type of neurons, the balance between the activities of PKC and protein phosphatase(s) appears to regulate phosphorylation states of GluR2, leading to the bidirectional regulation of the surface expression of functional AMPA receptors (5, 6, 27, 50). Future studies should elucidate detailed pictures of the potential signaling pathway to protein phosphatase(s) toward the AMPA receptor internalization in various developmental stages.

Molecular Machinery of Down-regulation of the AMPA Receptors—Postsynaptic sites or synaptic membranes are known to contain not only various transmitter receptors but also numerous kinds of PDZ proteins and cytoskeletal components to confine the postsynaptic machinery (2, 13, 43, 44). Such PDZ

proteins are implicated in the synaptic redistribution of AMPA-type glutamate receptor subunits (14, 19, 32). Among the many PDZ proteins, Pick1 is known to be phosphorylated by PKC and to associate with this enzyme (30, 51). Pick1 has a single PDZ domain that can bind to the AMPA receptor subunit GluR2/3 as well as to the metabotropic glutamate receptor mGluR7 and has been implicated in the subcellular distribution of these receptors (29, 52). In addition, Pick1 contains a coiled-coil domain that presumably enables it to self-assemble (29, 53). This coiled-coil domain appears to involve its dimerization, leading to the AMPA receptor internalization as monitored in the cerebellar LTD paradigm (18). The present results demonstrate that treatment with NMDA and PMA enhanced the interaction between GluR2/3 and various PDZ proteins, most apparently Pick1. This indicates that the effects of NMDA treatment involve PKC activation in the interaction between GluR2 and Pick1, which causes the internalization of the whole AMPA receptor complexes as evidenced by the decreased immunoreactivity of surface GluR1 subunits. However, we cannot completely rule out the possibility that not only Pick1 but also other PDZ molecule(s) may participate in the receptor down-regulation or internalization. It is mainly because the treatment with NMDA and the phorbol ester both increased the AMPA receptor levels in immunoprecipitates of the anti-pan-PDZ antibody that fails to recognize Pick1.[2] GRIP is also known to associate with the carboxyl-terminal domain of GluR2 and suggested to regulate the molecular behavior of the AMPA receptors on the postsynaptic sites (11, 31). When the Ser^{880} residue in GluR2 is phosphorylated by PKC, its interaction with GRIP but not with Pick1 is suggested to decline (16, 48). We detected a significant level of interaction between the AMPA receptor subunit and GRIP. However, we failed to observe any alteration in the interaction between GRIP and GluR2/3 after the NMDA treatment as well as after the overexpression of the GluR2 C-terminal decoy, suggesting that this interaction is resistant to NMDA stimulation as well as to the GluR2 decoy and thus appeared to be stable. The reason why a part of AMPA receptors, which might associate with GRIP, still escaped from internalization awaits further investigations.

Receptor Internalization and Synaptic Development/Plasticity—Strong electrical stimulation or membrane depolarization, both of which result in the activation of NMDA receptors, is required to induce up-regulation of synaptic AMPA receptors (2, 4, 54, 55). Rather weak stimulation of the NMDA receptors leads to LTD, instead of LTP, and is correlated with the down-regulation of AMPA receptors (2). Although chemical stimulation of the NMDA receptors should presumably be strong and effective in hippocampal neurons, it failed to induce the receptor up-regulation, as revealed by electrophysiological studies (6, 56). The reason why the chemical stimulation of NMDA receptors always results in the down-regulation and/or internalization of AMPA receptors remains to be established. The hippocampal neurons used in the present study were prepared from embryonic rats and grown only for 1 week in culture. They were forming synapses but had not obtained mature synapses as examined by immunohistochemistry (data not shown). Accordingly, the immunostaining of the AMPA receptor subunits revealed that the internalization was most apparent on the soma. Durand *et al.* (57) reported that the establishment of LTP requires synaptic maturation; in hippocampal slices from neonatal rats, LTD, but not LTP, was established. Immaturity of the hippocampal neurons might underlie the NMDA-induced down-regulation of the AMPA receptors examined in the present study. Alternatively, the simple chemical stimulation might fail to recruit other effector molecules important for LTP induction; such as neurotrophins, arachidonic acid, nitric oxide, etc. (58). Presumably, the missing involvement of such effectors might answer this question as well. Although the total number of surface AMPA receptors was down-regulated by NMDA stimulation, their ligand affinity in the cytoplasmic membrane fraction as well as on cell surface was rather elevated significantly. Their surface and intracellular increase in affinity would also contribute to synaptic plasticity, once the high affinity AMPA receptors are anchored onto the postsynaptic membranes. Future studies should provide an answer to the question of how the AMPA receptor complexes can avoid NMDA-triggered internalization, thus leading to postsynaptic maturation or potentiation.

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[2] Y. Hayashi, personal communication.

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