Phosphorylation-dependent Regulation of N-Methyl-d-aspartate Receptors by Calmodulin*

Chihiro Hisatsune‡, Hisashi Umemori‡, Takafumi Inoue‡, Takayuki Michikawa§, Kazuhisa Kohda§, Katsuhiko Mikoshiba§, and Tadashi Yamamoto¶

From the Departments of ‡Oncology and §Molecular Neurobiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

The N-methyl-d-aspartate (NMDA) receptor plays important roles in synaptic plasticity and brain development. The NMDA receptor subunits have large intracellular domains in the COOH-terminal region that may interact with signal-transducing proteins. By using the yeast two-hybrid system, we found that calmodulin interacts with the COOH terminus of the NR1 subunit and inactivates the channels in a Ca\(^{2+}\)-dependent manner. Here we show that protein kinase C (PKC)-mediated phosphorylation on serine residues of NR1 decreases its affinity for calmodulin. This suggests that PKC-mediated phosphorylation of NR1 prevents calmodulin from binding to the NR1 subunit and thereby inhibits the inactivation of NMDA receptors by calmodulin. In addition, we show that stimulation of metabotropic glutamate receptor 1\(\alpha\), which potentiates NMDA channels through PKC, decreases the ability of NR1 to bind to calmodulin. Thus, our data provide clues to understanding the basis of cross-talk between two types of receptors, metabotropic glutamate receptors and the NR1 subunit, in NMDA channel potentiation.

Glutamate is a major excitatory neurotransmitter in the central nervous system. Glutamate receptors are divided into two types of receptors, ionotropic receptors and metabotropic receptors, based on their structural and functional properties. N-Methyl-d-aspartate (NMDA) receptors, which belong to the family of ionotropic glutamate receptors, are implicated in synaptic plasticity, synaptogenesis, and excitotoxicity (1, 2). NMDA receptors consist of two distinct types of subunits, the principal subunit NR1 and modulatory subunits NR2A–2D (3, 4). Both types of subunits have a large COOH-terminal intracellular domain (5–7), suggesting that the large cytoplasmic domain may directly interact with proteins that regulate NMDA receptor function. For example, recent studies have suggested that postsynaptic density protein PSD95 directly interacts with NMDA receptors, although the biological significance of the interaction has not been established. The intracellular domain of NR1 contains serine residues that can be phosphorylated by protein kinase C (PKC) (8). It has also been reported that the NMDA receptor-mediated current is regulated by several protein kinases and phosphatases (9–11).

Calmodulin (CaM) is a ubiquitous molecule that regulates the function of a large number of proteins including Ca\(^{2+}\)/CaM-dependent kinase II, adenylate cyclase, and phosphodiesterases. CaM also modulates the sensitivity of ion channels such as the olfactory cyclic nucleotide-gated channel and the retinal cyclic GMP-gated channel (12, 13). In addition, loading of CaM inhibitors at the postsynaptic neuron blocks the induction of long-term potentiation (LTP), a long-lasting enhancement of synaptic plasticity, and long-term depression, a long-lasting depression of synaptic plasticity, in the hippocampal CA1 region (14), suggesting that CaM is an important molecule for the induction of LTP and long-term depression.

We and Ehlers et al. (17) independently show that CaM binds to NR1 in a Ca\(^{2+}\)-dependent manner and that the binding inactivates NMDA channels. NMDA receptors show high permeability to Ca\(^{2+}\). However, the rise of the intracellular Ca\(^{2+}\) level causes a reversible inactivation of the NMDA channels (15, 16). This feedback system regulates the amount of Ca\(^{2+}\) influx through the NMDA receptors. These data together suggest that Ca\(^{2+}\)-dependent inactivation of NMDA receptors is mediated by CaM binding to NR1, namely, Ca\(^{2+}\) entering through the NMDA receptors binds to and activates CaM, and activated CaM, in turn, binds to NR1, resulting in inactivation of the NMDA channels.

The NMDA receptor-mediated current is potentiated during LTP (18, 19). In addition, 2-amino-4-phosphonovaleric acid, an antagonist of NMDA receptors, blocks the induction of LTP. Thus, induction of LTP should be accompanied by fine-tuning of the level of Ca\(^{2+}\) influx through the NMDA channels. Since Ca\(^{2+}\) influx results in inactivation of NMDA channels, there should be a mechanism by which the inactivation of the NMDA channels by Ca\(^{2+}\) influx is prevented so that potentiation of NMDA receptors results in the induction of LTP. Several studies suggest that induction of LTP requires concomitant activation of metabotropic glutamate receptors (mGluRs) and NMDA receptors (20) and that trans-1-aminocyclopentane-1,3-dicarboxylate (t-ACPD), an agonist of mGluRs, potentiates NMDA currents in the hippocampus (21). Stimulation of mGluR1 and mGluR5 leads to the activation of PKC. There are reports that phorbol ester, an activator of PKC, enhances the amplitude of NMDA currents in Xenopus oocytes (22), hippocampus (21), and spinal dorsal horn neurons (23) and that intracellular injection of the selective PKC inhibitory peptide prevents the induction of LTP (24).

Here we show that PKC-mediated phosphorylation on serine residues of NR1 decreases its CaM binding affinity. Moreover, we provide evidence that activation of mGluR1\(\alpha\) decreases the affinity of NR1 for CaM. These results suggest that PKC-mediated phosphorylation of NR1 through mGluR1\(\alpha\) decreases...
NR1-CaM interaction and prevents inactivation of NMDA channels by Ca\(^{2+}\).

EXPERIMENTAL PROCEDURES

Screening by the Yeast Two-hybrid System—The NR1 cDNA encoding residues between the fourth transmembrane domain and the COOH terminus (Glu\(^{104}\)-Ser\(^{208}\)) was amplified by polymerase chain reaction with two primers: 5'-CTGCGAATTCCTGAGATGC-CC3', incorporating the EcoRI site (underlined), and 5'-GGGGCGGGTCGACT- CAGC-CTCC3', incorporating the SalI site (underlined). The amplified fragment was inserted into the EcoRI-SalI site of pBTM116. The resulting plasmid encodes a fusion protein of the LexA DNA-binding domain and the COOH-terminal domain of NR1. The yeast reporter strain L40 containing this plasmid was transformed with a mouse cDNA library in pVP16 by the lithium acetate method. The transformants were plated on medium lacking Trp, His, Ura, Leu, and Lys and containing 5 mM 3-amino-triazole. Nitrocellulose filters were placed on the plates and assayed for \(\beta\)-galactosidase expression as described (25).

DNA Sequence Analysis—DNA sequencing was performed by the Sanger chain termination method, employing a dideoxynucleotide kit (Takara).

Cell Culture—HEK 293T cells (simian virus 40 large T antigen-expressing human embryonic kidney cells) were grown in Dulbecco's modified Eagle's medium containing 2 mM glutamine. Cell culture reagents were from Life Technologies, Inc. mGluR1-expressing Chinese hamster ovary cells were grown in Dulbecco's modified Eagle's medium containing 2 mM glutamine (26).

**NR1 Expression Plasmid and Mutagenesis—** The NR1 expression plasmid (pNR1) contains NR1 cDNA from pN60 (27) at the EcoRI site of pME18S (27). The two mutagenic primers used were as follows. Primer 1 (5'-CTGCGCTCAAGCTTCCAGCAACAACAGTCCTCCAAA3', nucleotides 2659–2694) has a HindIII site (underlined) without changing the original amino acid sequence of NR1. Primer 2 (5'-ACT-AGTGCAGAAACAACTTCGCTC3') carries the same sequence as that of the multifunctioning site of pME18S, but contains an XbaI site of pBTM116. The modified fragment was inserted into the SalI site of pME18S, but contains an XbaI site of pBTM116. The two mutagenic primers used were as follows. Primer 1 (5'-CTGCGCTCAAGCTTCCAGCAACAACAGTCCTCCAAA3', nucleotides 2659–2694) has a HindIII site (underlined) without changing the original amino acid sequence of NR1. Primer 2 (5'-ACT-AGTGCAGAAACAACTTCGCTC3') carries the same sequence as that of the multifunctioning site of pME18S, but contains an XbaI site (underlined) of the original XbaI site. Site-directed mutagenesis was carried out according to the method of Deng and Nickoloff (28) using the above two primers and pNR1 as a template. Introduction of the mutant fragment into NR1 was confirmed by DNA sequencing.

**In Vitro NR1-CaM Binding Assay—** HEK 293T cells in a 10-cm culture dish were transfected with 10 \(\mu\)g of NR1 expression plasmid by the standard calcium phosphate method. After 48 h, the cells were lysed with 500 \(\mu\)l of buffer A (10 mM Tris-HCl (pH 7.5), 1.0% Nonidet P-40, and 10 \(\mu\)g/ml aprotinin) containing 1.0 mM CaCl\(_2\) and 1% Triton for 1 h at 4 °C. The lysates were centrifuged at 15,000 rpm for 30 min, and 30 \(\mu\)l of CaM-agarose (Sigma) was added to the supernatants. After incubation for 1 h at 4 °C, the CaM-agarose was washed with the same buffer. For immunoblotting, proteins in the CaM-agarose complex were resolved by 6.0% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane filter was treated with blocking reagents containing 5.0% bovine serum albumin and probed with an antibody to the COOH terminus of NR1 (Chemicon International, Inc.).

**Immunoprecipitation—** HEK 293T cells were transfected with 20 \(\mu\)g of NR1 expression plasmid. After 48 h, the cells were treated with or without 100 mM TPA for 30 min. The cells were lysed with buffer B (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM Mg(OAc)\(_2\), 5 mM EDTA, 10 \(\mu\)g/ml aprotinin, 2 mM CaCl\(_2\) and 1% Triton) for 1 h at 4 °C. For analysis of the effect of CaM, the flow was continued for 30 min at 4 °C. The complexes were then resolved on nondenaturing 15% polyacrylamide gels containing 2 mM CaCl\(_2\) and visualized by Coomassie Blue staining. To phosphorylate the peptide, 3000 pmol of peptides was incubated with 0.3 milliunits of PKC (Pierce) in buffer D (120 mM NaCl, 5 mM Tris-HCl (pH 7.5), 0.5 mM Mg(OAc)\(_2\), 50 \(\mu\)g/ml phosphatidylerine, and 2 mM \(\gamma\)-\[^{32}\]P-ATP) for 3 h at room temperature. The peptide that was incubated with PKC in the above solution without \(\gamma\)-\[^{32}\]P-ATP was used as a nonphosphorylated peptide.

**RESULTS**

**Identification of CaM as an NR1-interacting Protein—** To identify proteins that interact with the COOH-terminal intracellular domain of NR1, we employed the yeast two-hybrid screening system (30, 31). To prepare a bait, we constructed a plasmid that expresses a fusion protein consisting of LexA and the COOH-terminal domain of NR1 (Glu\(^{104}\)-Ser\(^{208}\)) of NR1. The yeast reporter strain L40 containing the bait plasmid was transformed with a mouse cDNA library constructed in pVP16. By screening —10\(^6\) yeast transformants, we obtained 177 positive clones that activate expression of both the selection marker His and LacZ. Of these, 50 clones were subjected to nucleotide sequence analysis. The inserts of 16 clones encode the COOH-terminal amino acid sequence of CaM that corresponds to the amino acids between Lys\(^{77}\) and Lys\(^{146}\).

**Identification of the CaM-binding Site on NR1—** Most CaM-binding proteins contain conserved amino acid sequences that can form the amphipathic \(\alpha\)-helix (32, 33). Inspection of the NR1 sequence revealed the presence of the amphipathic structure. The MNR1-CaM binding assay revealed that the CaM-binding site on NR1 is the sequence between Lys\(^{77}\) and Thr\(^{200}\) (Fig. 1A). To examine whether this region is responsible for CaM binding, we constructed a mutant of NR1 (MNR1) with Gin\(^{98}\), Gin\(^{205}\), Gin\(^{206}\), and Gin\(^{207}\). As shown in Fig. 1A, the mutant (S65T) of green fluorescent protein (GFP) expression plasmids was transfected into HEK 293T cells (Simian virus 40 large T antigen-expressing human embryonic kidney cells) grown in Dulbecco’s medium containing 2 mM glutamine. Cell culture reagents were from Life Technologies, Inc. mGluR1 expressing Chinese hamster ovary cells were grown in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine (26).

**Single Channel Recording of the NMDA Currents—** HEK 293T cells were cotransfected with a DNA mixture containing NR1, NR2A, and a mutant (865T) of green fluorescent protein (GFP) expression plasmids (0.1, 0.3, and 0.5 \(\mu\)g/cm\(^2\), respectively) (29). After 12 h, 50 \(\mu\)M \(\alpha\)-amino-5-phosphopontetic acid was added to the medium to prevent cell death caused by NMDA receptor activation. After 12–48 h of transfection, the expression of GFP was confirmed with an epifluorescence microscope, and cells expressing GFP were used for electrophysiological recordings. Fire-polished borosilicate glass pipettes (4–5 megohms) filled with an extracellular solution (110 mM Na\(_2\)SO\(_4\), 5 mM Ca\(_2\)SO\(_4\), 10 mM HEPES, 10 mM glucose, 1 \(\mu\)M probenecid, 10 mM mg, 5 mM glycine were used for single channel recording. Inside-out patches were excised from HEK 293T cells in the extracellular solution and moved into the flow of an intracellular solution (140 mM cesium gluconate, 10 mM HEPES, and 1 \(\mu\)M CaCl\(_2\) (pH 7.25)). For analysis of the effect of CaM, the flow was changed to the intracellular solution containing 250 mM Ca (Sigma). Currents through the NMDA channels were measured by using the
found in the presence of Ca\(^{2+}\). However, the Ca\(^{2+}\)-peptide complex was not detected in the absence of Ca\(^{2+}\) (Fig. 1C, EDTA). Therefore, we concluded that the NR1-CaM interaction is Ca\(^{2+}\)-dependent.

**Decrease in NR1-CaM Interaction by TPA Stimulation**—The four serine residues in the CaM-binding site on NR1 can be phosphorylated with PKC by TPA stimulation (Fig. 2A) (8). PKC-induced phosphorylation of these serine residues alters their hydrophilicity and therefore might disrupt the amphiphilic structure of the CaM-binding site. Consequently, CaM would be unable to bind to phosphorylated NR1. To investigate this possibility, HEK 293T cells expressing NR1 were treated with 100 nM TPA. Using anti-phosphoserine antibodies, we detected the increment in serine phosphorylation of NR1 upon PKC-mediated phosphorylation of NR1. The peptide corresponding to the sequence from Asp\(^{873}\) to Thr\(^{902}\) of NR1 that includes the CaM-binding site was incubated with PKC to achieve its phosphorylation. Phosphorylation of the peptide was confirmed by radiolabeling (data not shown). CaM (300 pmol) was incubated with various amounts of the phosphorylated peptide complex was seen at a peptide/CaM ratio of 1:10. However, the CaM-phosphorylated peptide complex could be seen at a peptide/CaM ratio of 1:1. The data suggest that the affinity of NR1 for CaM binding is decreased by PKC-mediated phosphorylation of NR1.

**Quantitative Analysis of CaM Binding to NR1 Phosphorylated by PKC**—To quantitate CaM binding to NR1 phosphorylated by PKC, we performed the binding assay between CaM and the NR1 peptide phosphorylated at the CaM-binding site. The peptide corresponding to the sequence from Asp\(^{873}\) to Thr\(^{902}\) of NR1 that includes the CaM-binding site was incubated with PKC to achieve its phosphorylation. Phosphorylation of the peptide was confirmed by radiolabeling (data not shown). CaM (300 pmol) was incubated with various amounts of peptide either nonphosphorylated or phosphorylated by PKC. As shown in Fig. 3B, the CaM-nonphosphorylated peptide complex was seen at a peptide/CaM ratio of 1:10. However, the CaM-phosphorylated peptide complex could be seen at a peptide/CaM ratio of 1:1. The data suggest that the phosphorylated peptide had a significantly lower affinity for CaM than the nonphosphorylated peptide. Thus, PKC-mediated phosphorylation of NR1 decreased the affinity of NR1 for CaM.

**Inactivation of the NMDA Receptor by CaM Binding**—To investigate the functional regulation of NMDA channels by CaM, we measured the single channel activity from HEK 293T cells cotransfected with NR1, NR2A, and GFP expression plasmids. Fig. 4A shows a typical single channel current detected in...
inside-out patches excised from the HEK 293T cells expressing GFP. The slope conductance of the channel was 48 ± 6 pS/pF in the control patches, which corresponded closely to the single NMDA channel conductance (~50 picoSiemens) measured in adult rat dentate gyrus granule cells (11). The current was detected in patches from GFP-expressing cells, whereas it was not detected in untransfected cells (n = 4). Under the condition in which a saturating concentration (3 μM) of glycine was applied in the pipette solution, the current did not show a substantial rundown for at least 5 min.

We then tested the effect of CaM on the currents detected in the inside-out patches from GFP-expressing HEK 293T cells. As previously reported (17), application of 250 nM CaM in the intracellular solution containing 1 mM Ca²⁺ reduced the probability of channel opening (P_open) to 49.4 ± 25.3% (mean ± S.D.) of the control values (n = 9) (Fig. 4, A and B). CaM did not significantly affect the single channel conductance in all patches measured: 3.23 ± 0.47 pA (n = 9) at −60 mV in the absence of CaM and 3.49 ± 0.42 pA (n = 8) at −60 mV following the addition of CaM. Therefore, CaM decreases the activity of NMDA channels by reducing the frequency of channel openings. The NMDA receptors have high permeability to Ca²⁺, and Ca²⁺ influx through the channels, in turn, inactivates the NMDA receptors (15, 16). Since CaM binds to NR1 in a Ca²⁺-dependent manner (17), inactivation of the NMDA receptors by Ca²⁺ influx may be through the NR1-CaM interaction.

**Stimulation of mGluR1α Decreases the Affinity of NR1 for CaM—NMDA receptor-mediated currents are potentiated during LTP (18, 19). The potentiation of NMDA currents during LTP is opposed to inactivation of NMDA channels by Ca²⁺-dependent NR1-CaM interaction. Therefore, there should be a mechanism by which inactivation of the NMDA channels by Ca²⁺ influx is prevented during LTP. LTP is regulated by both mGluRs and NMDA receptors. In addition, stimulation of mGluRs by t-ACPD, an agonist of mGluRs, evokes a long-lasting enhancement of NMDA currents (34). Since stimulation of mGluR1α leads to activation of PKC, the activated PKC may be utilized to phosphorylate NR1, which prevents the interaction between NR1 and CaM. To investigate this, we examined the affinity of NR1 for CaM after stimulation of mGluR1α by t-ACPD. Chinese hamster ovary cells stably expressing mGluR1α were transfected with NR1 expression plasmids. The cells were then treated with or without 10 μM t-ACPD, and the NR1-CaM binding assay was performed using CaM-agarose. As expected, stimulation of mGluR1α decreased the affinity of NR1 for CaM (Fig. 5). These results suggest that the decrease in the affinity of NR1 for CaM underlies the potentiation of NMDA channels. Phosphorylation of NR1 by PKC may be one of the mechanisms for NMDA receptor potentiation.

**DISCUSSION**

We have shown that CaM inactivates the NMDA channels by binding to NR1 and that PKC-mediated phosphorylation of NR1 decreases the affinity of NR1 for CaM. Therefore, when the NMDA receptor is activated and permits Ca²⁺ influx, CaM binds to NR1 in a Ca²⁺-dependent manner and inactivates NMDA channels. However, when serine residues of NR1 are concomitantly phosphorylated by PKC, CaM cannot bind to NR1 and thus is unable to inactivate the NMDA channels, and this may result in the potentiation of LTP. Previous reports also show that CaM binding to target proteins is regulated by phosphorylation. Neuromodulin is a molecule that is present in neurons in the spinal cord, retina, and brain (35, 36). Neuromodulin releases CaM in response to its phosphorylation by PKC: neuromodulin binds to and localizes CaM at specific sites within the cell in the resting state and releases CaM locally in response to its phosphorylation by PKC (37, 38). Thus, in addition to Ca²⁺, phosphorylation also regulates the interaction of CaM and CaM-binding proteins.

To date, at least eight splice variants of NR1 have been identified (39–41). These variants are generated by three exons: an exon near the N terminus and two exons near the C terminus. These variants differ in their sensitivity to factors such as Mg²⁺, glycine, polyamines, and protein kinases/phosphatases. Our study shows that CaM plays a role in regulating NMDA channel activity through the interaction of CaM with the sequence (Lys³⁶⁵–Thr³⁸⁰) in the C1 cassette (Asp³⁶⁴–Thr³⁸⁰) in the COOH-terminal region of NR1. Among the NR1 splice variants, there are variants that do not contain this cassette, suggesting that these variants have a different sensitivity to CaM. Indeed, Ehlers et al. (17) reported that splice variants that lack the C1 exon cassette, e.g. NR1-c and NR1-e, are much less sensitive to CaM than the variants that have the C1 cassette. They also reported that CaM binds to two sites of NR1: the lower affinity region (Lys³⁸⁳–Gln³⁹³) as well as the higher affinity region (Lys³⁷⁵–Lys³⁸⁶). Consistently, our data demonstrate that the NR1 mutant (MN1R) incorporating Gln instead of Lys or Arg at positions 892, 893, 894, and 895 significantly reduces the affinity for CaM, but still can bind to CaM very weakly (Fig. 1B). This residual ability of MN1R to bind CaM may be due to the other CaM-binding site (Lys³⁸⁶–Gln³⁸⁸) of NR1. Although the sensitivities to CaM are different among these variants at a low concentration of CaM, the effect of CaM on inactivation of the channels is not different at a high concentration of CaM (17). A high concentration of Ca²⁺/CaM that inactivates all the splice variants might be the limit of the intracellular Ca²⁺ concentration that induces excitotoxicity. Thus, whereas the low affinity CaM-binding site (Lys³⁸⁶–Gln³⁸⁸) might contribute to the prevention of excitotoxicity, phosphorylation and dephosphorylation of the high affinity CaM-binding site (Lys³⁷⁵–Lys³⁸⁶) in the C1 exon cassette might be important in regulating the sensitivity to CaM.
Then the lysates were subjected to the CaM binding assay with CaM-transfection, the cells were treated with (ordinate recorded without CaM. Values on the NP Mean values of NP of CaM on the open probability (technique. Membrane voltage was maintained at 60 mV (pipette interior, 0 mV). Recordings were filtered at 1 kHz for representation. B, effect of CaM on the open probability (NP) of the NMDA receptor in the inside-out patches excised from HEK 293T cells. Control channel activity was recorded without CaM. Values on the ordinate are the ratio (%) of the open probability treated with CaM to the open probability before treatment. Mean values of NP were 49.4 ± 25.3% with CaM treatment (CaM; mean ± S.D., n = 9) and 106.0 ± 40.3% without CaM treatment (Control; mean ± S.D., n = 8) (p < 0.01; Mann-Whitney test).

FIG. 4. Inactivation of NMDA channel activity by CaM. A. representative NMDA single channel currents from HEK 293T cells expressing NR1 and NR2A before (Before) and during (CaM) application of 250 nM CaM. NMDA single channel currents were recorded by the inside-out patch technique. Membrane voltage was maintained at ~60 mV (pipette interior, 0 mV). Recordings were filtered at 1 kHz for representation. B, effect of CaM on the open probability (NP) of the NMDA receptor in the inside-out patches excised from HEK 293T cells. Control channel activity was recorded without CaM. Values on the ordinate are the ratio (%) of the open probability treated with CaM to the open probability before treatment. Mean values of NP were 49.4 ± 25.3% with CaM treatment (CaM; mean ± S.D., n = 9) and 106.0 ± 40.3% without CaM treatment (Control; mean ± S.D., n = 8) (p < 0.01; Mann-Whitney test).

FIG. 5. Stimulation of mGluR1A decreases the affinity of NR1 for CaM. mGluR1A-expressing Chinese hamster ovary cells were transfected with 10 μg of NR1 expression plasmid (pNR1). After 48 h of transfection, the cells were treated with (+) or without (−) 10 μM t-ACPD for 30 min and lysed in buffer A containing 10 μM okadaic acid. Then the lysates were subjected to the CaM binding assay with CaM-agarose in the presence of 3 mmol of synthetic peptide (Asp 767–Thr 792) as competitor. NMDAR1, NMDA receptor 1.

be important primarily in determining the sensitivity of channels to CaM.

The C1 exon cassette is also important in determining the subcellular distribution of NR1 (42). NR1 splice variants containing the C1 exon are aggregated and located in discrete, highly concentrated regions associated with the plasma membrane. NR1 splice variants lacking this exon cassette are distributed throughout the cell. Furthermore, PKC phosphorylation of serine residues within the exon disrupts the formation of the NR1 aggregation at the plasma membrane. The CaM-binding site (effector domain) of the cytoskeletal organizing protein MARCKS (myristoylated alanine-rich C kinase substrate). The effector domain of MARCKS binds both CaM and actin and contains the major site of PKC phosphorylation. PKC phosphorylation inhibits CaM and actin binding, and CaM binding prevents PKC phosphorylation and actin binding. The results show that CaM binds to the sequence in the region encoded by the C1 exon and that PKC-mediated phosphorylation of the serine residues regulates the NR1-CaM interaction. Therefore, as with the MARCKS protein, PKC phosphorylation and CaM binding may also contribute to the subcellular distribution of NR1 by interacting with cytoskeletal proteins.

We have shown that stimulation of mGluR1α, which can induce NMDA receptor potentiation, decreased the affinity of NR1 for CaM. Since stimulation of mGluR1 and mGluR5 activated PKC and since PKC-mediated phosphorylation of NR1 decreased the affinity of NR1 for CaM (Figs. 2 and 3), it is likely that phosphorylation of NR1 by activated PKC through mGluR1α results in inhibition of the NR1-CaM interaction. There are reports that stimulation of μ-opioid receptors or muscarinic receptors increases the NMDA currents through activation of PKC (21, 43). Therefore, activation of PKC through these G protein-coupled receptors may also contribute to phosphorylation of NR1 at sites within the CaM-binding region.

Although activation of NMDA channels is necessary every time LTP is generated, activation of mGluRs is not: activation of mGluRs is necessary the first time LTP is generated, and thereafter, mGluRs are no longer necessary to generate further LTP (44). Thus, mGluRs may activate some sort of “molecular switch.” Once activated, the molecular switch stays on during induction of LTP. Our results suggest that PKC-mediated phosphorylation of NR1 may be a molecular switch activated by mGluRs. Once serine residues of NR1 are phosphorylated by PKC, CaM cannot inactivate the NMDA channels by binding to NR1 until those serine residues are dephosphorylated. In the NR1 phosphorylated state, NMDA receptors remain potentiated without additional activation of mGluRs. If this hypothesis is correct, phosphorylation of NR1 should increase during LTP.

Recent reports indicated that phosphorylation of NR2B increases during LTP (45, 46). It would be worthwhile to examine whether the level of phosphorylation of NR1 increases during LTP.

Ca2+ influx through NMDA receptors affects synaptic plasticity and excitotoxicity. The extent of Ca2+ influx through NMDA channels is proposed to be responsible for the establishment of LTP and long-term depression: a large Ca2+ influx through NMDA channels results in LTP, and a small Ca2+ influx results in long-term depression. Furthermore, excessive Ca2+ influx through NMDA channels causes neuronal death. Ca2+-dependent inactivation of NMDA channels and release
from inactivation by NR1 phosphorylation would play important roles in these phenomena.

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