Characterization of Protein Kinase A and Protein Kinase C Phosphorylation of the N-Methyl-D-aspartate Receptor NR1 Subunit Using Phosphorylation Site-specific Antibodies*

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Ionotropic glutamate receptors mediate most rapid excitatory transmission in the central nervous system and play important roles in synaptic plasticity, neuronal development, and neurological disorders (1–5). Glutamate receptors have been divided into NMDA (N-methyl-D-aspartate) and non-NMDA (kainate or AMPA) receptors based on their pharmacological and physiological properties (1, 2). Non-NMDA glutamate receptors activate and desensitize rapidly and mediate excitatory synaptic transmission. NMDA receptors are more slowly activated and desensitized and have a high Ca2+ permeability and a voltage-dependent Mg2+ block, two properties thought to underlie use-dependent synaptic plasticity in the brain (1–3).

Molecular cloning studies have recently identified the genes encoding subunits for the NMDA and non-NMDA receptors (1, 2). NMDA receptors consist of two families of homologous subunits, the NR1 and NR2A-D subunits (6–9), and are thought to be pentameric or tetrameric complexes of the NR1 subunit with one or more of the NR2 subunits (10, 11). The differential expression of NR2 subunits in the various regions of the brain may account for the diversity of NMDA receptor subtypes (1). In addition, the NR1 subunit is highly alternatively spliced giving rise to at least seven forms of NR1 (NR1A-G) increasing the potential diversity of NMDA receptors in the brain (12–14).

Protein phosphorylation has been recognized as a major mechanism for the regulation of glutamate receptor function (15). NMDA receptors appear to be regulated by a number of protein kinases and phosphatases. Activation of protein kinase C (PKC) by phorbol esters have been demonstrated to activate (16, 17) or depress (18, 19) neuronal NMDA receptors. In addition, intracellular perfusion of purified PKC into spinal trigeminal neurons potentiates NMDA receptors (20), and PKC seems to mediate the modulation of NMDA receptors by μ-opioids in these cells (21). Activation of PKC by phorbol esters can also potentiate recombinant NMDA receptors expressed in Xenopus oocytes including homomeric NR1 receptors (13, 22, 23) and heteromeric receptors consisting of the NR1 subunit coexpressed with the NR2A or NR2B subunits (8, 24). In addition, recent studies in rat hippocampal neurons have suggested that cAMP-dependent protein kinase can also potentiate NMDA receptors (25).

Biochemical studies have demonstrated that NMDA receptors are directly phosphorylated by protein kinase C and protein tyrosine kinases (26–28). PKC has been shown to phosphorylate the NR1 subunit in neurons and in heterologous expression systems (26). This phosphorylation occurs on the C terminus of the NR1 subunit, and mutation of four serine residues in the C terminus dramatically reduce PKC phosphorylation of NR1 (26). These four serine residues are all contained within a single exon (C1) which is regulated by alternative splicing of NR1 mRNA (12, 13). Interestingly, the presence of the C1 exon induces the NR1 subunit to cluster into receptor-rich domains associated with the cell surface when expressed in heterologous cells, and PKC phosphorylation of the NR1 subunit disrupts these spontaneous aggregates of the receptor (29).

In the present study we have further characterized the phosphorylation of the NR1 protein in vitro and in situ using site-specific mutagenesis and phosphorylation site-specific antibodies. These phosphorylation site-specific antibodies recognize the NR1 protein only when specific serine residues are phosphorylated. We show that in addition to PKC, PKA also phos-
phorylates the C terminus of NR1. PKC specifically phospho-
ylates threonine 879 and serines 890 and 896, whereas PKA
phosphorylates serine 897. Moreover, only phosphorylation of
serine 890 by PKC induces the dispersal of the clusters of the
NR1 subunit in fibroblasts, suggesting that PKC and PKA may
differentially regulate NR1 subunits in the brain.

EXPERIMENTAL PROCEDURES

Materials—NR1A cDNA was a generous gift of S. Nakanishi. PKC
and the catalytic subunit of PKA were purified from rat brain and
bovine heart, respectively, as described (30, 31). Other materials were
purchased from the following sources: radioisotopes, DuPont NEN;
phorbol esters and forskolin, Calbiochem; cellulose TLC (thin layer
chromatography) plates and XAR autoradiography film, Kodak; PVDF
membrane, Millipore; nitrocellulose membrane, VWR; tissue culture
plates and XAR autoradiography film, Kodak; PVDF

Phosphorylation of NMDA Receptors

Generation of Anti-phosphopeptide Antibodies—The peptides KTST-
LASSFKRRR and KKFRKRRSSKDTST, corresponding to amino acids
884–895 and amino acids 891–902 of NR1A, respectively, were synthe-
sized as described above. Lysine residues were included in the N-
terminal positions of the peptides to facilitate glutaraldehyde coupling
to the fusion protein, thyroglobulin. Phosphorylation of the purified
serine residues (Nova Biochemicals) were included in positions 889,
890, and 897, and 897. The resulting phosphopeptides were coupled
to thyroglobulin and used to immunize New Zealand White rabbits, and
sera were obtained periodically by Hazleton Research Products.
Poly
clonal anti-phosphopeptide antibodies were purified from sera by se-
queencing the antibody-bound fused protein with an amino-terminal
linked to unphosphorylated and phosphorylated peptides. Antibodies
were eluted from the peptide affinity columns using 100 mM glycine
(pH 2.7). Anti-NR1 C-terminal antibodies were described previously
(28).

NR1 Fusion Protein Phosphorylation—Bacterial fusion proteins con-
taining the C-terminal region of NR1A (amino acids 834–938) were
constructed by polymerase chain reaction amplification of NR1A cDNA
primers 5′-ACCATGATCCCGAGATCGCCTACAAGCGA-3′ and 5′-
ACCATGAATTCCTCAGCTCTCCCTATGACG-3′, subcloning the poly-
cerase chain reaction products into restriction endonuclease sites
(BamHI and EcoRI) in the gTrcHis vector (Invitrogen), and transform-
ing BL21 Escherichia coli. Transformed bacteria were grown in 400-
culture media and induced with isopropyl-1-thio-
lycosylated in 100 µl total volume. Phosphorylation reaction buffer
contained 50 µM Hepes (pH 7.4), 10 mM MgCl2, 1 mM CaCl2, 50 µg ATP,
50 µg/ml phosphatidylserine, and 5 µM diacetylglucorol. 5 µCi of
[γ-32P]ATP was included. In most cases, fusion proteins were resolved
by SDS-PAGE using 14% polyacrylamide gels and either e 
xcced from the gel for phosphopeptide mapping or transferred to PVDF membrane
by electrophoresis at 30 V. In some cases, fusion protein was applied
directly to nitrocellulose membranes ("slot blots"), using a Bio-Dot SF
apparatus (Bio-Rad) according to the manufacturer’s protocol.

Phosphopeptide Mapping—Polyacrylamide gel fragments containing
radiolabeled, phosphorylated NR1 C-terminal fusion protein were
incubated with 0.3 mg/ml trypsin (Sigma) in 1 ml of 0.4% NH4HCO3
for 20 h at 37 °C. After removal of NH4HCO3 by lyophilization, phos-
phorylated peptides were resuspended in 10 µl of H2O. The tryptic digests
were spotted onto TLC plates (Kodak) and separated by electrophoresis (500
V, horizontal) in the presence of acetic acid/pyridine/H2O, 19:1:89 (v/v),
and ascending chromatography in pyridine/butanol/acetic acid/H2O,
15:10:12 (32). Phosphopeptides were visualized by autoradiography.

Immunoblotting—PVDF or nitrocellulose membranes were fixed in
acetic acid/methanol/H2O, 10:25:65 (v/v), blocked (1 h) with 0.5% nonfat
dry milk (Carnation) and 0.1% Tween 20, incubated (1 h) with primary
antibodies (150–500 ng/ml in blocking buffer, washed (5 × 5 min,
blocking buffer), and incubated (1 h) with horseradish peroxidase-con-
jugated anti-rabbit Ig (1:5,000 in blocking buffer; Amersham Corp.).
After final washes in Tris-buffered saline (5 × 5 min) membranes
were immersed in chemiluminescence detection reagent (DuPont NEN) for
1 min and then exposed to XAR film. Exposure time ranged from 10 s to
10 min.

Cell Culture and Transfection—Quail fibroblast (QT-6) cells or hu-
man embryonic kidney-293 (HEK) cells were maintained at 37 °C under
5% CO2 and passed every 4–6 days. QT-6 cell medium contained 199
Earle medium with 5% fetal bovine serum, 10% tryptose phosphate
broth, and 1% dimethyl sulfoxide (Sigma). HEK cell medium included
minimal essential media with 10% fetal bovine serum, 100 units/ml penicillin,
and 100 µg/ml streptomycin. For transfections, the pRK5
expression vector containing NR1 cDNA was precipitated onto cultured
cells by the calcium phosphate method (33) using 20 µg of DNA per
10-cm culture dish (1 × 105 cells). After incubation for 8–12 h under 5% 
CO2, remaining DNA was washed away with fresh media and cells were
returned to 5% CO2. For immunocytochemical experiments, cells were
immediately replated onto coverslips or coated glass coverslips for
dirty-five hours after transfection, cells were treated with 100 nm PMA,
20 µM forskolin, or vehicle solution, rinsed in PBS, and either fixed in
4% paraformaldehyde, or solubilized in SDS gel sample buffer and proteins resolved on 5% polyacrylamide gels.

Immunocytochemistry—QT-6 cells were fixed with 4% paraformal-
hyde, 4% sucrose in PBS for 10 min, and blocked with 6% bovine serum
Phosphorylation of NR1 Protein by PKA and PKC—In order to identify kinases that directly phosphorylate the NR1 subunit, bacterial fusion proteins containing the C-terminal region of NR1 protein were incubated with purified kinases in the presence of \(^{32}P\)ATP (Fig. 1). Previous results indicated that PKC phosphorylates the NR1 subunit in transfected cells and that mutation of four serine residues (serines 889, 890, 896, and 897) eliminated the majority of PKC phosphorylation (26). Consistent with these findings, PKC rapidly phosphorylated the C-terminal region of the NR1 subunit (Fig. 1C), with mutation of serine residues 889, 890, 896, and 897 to alanine dramatically reducing PKC phosphorylation (data not shown). Interestingly, based on the characterized consensus sequence of PKA substrates (34), the C-terminal region of the NR1 subunit also contains a potential site for PKA phosphorylation (serine 897). Indeed, PKA readily phosphorylated the NR1 C-terminal fusion protein (Fig. 1, C and D). Mutation of serine residues 896 and 897 to alanines (S896A, S897A) completely eliminated PKA phosphorylation of the fusion protein (Fig. 1D).

In order to characterize PKA phosphorylation of the NR1 subunit, tryptic phosphopeptide map analysis was performed on C-terminal NR1 fusion proteins. Fusion proteins were phosphorylated \textit{in vitro} by PKA and/or PKC, resolved by SDS-PAGE, and visualized by autoradiography. The phosphorylated NR1 fusion protein was excised from the SDS-PAGE gel, proteolyzed with trypsin, and the resulting tryptic phosphopeptides resolved by two-dimensional thin layer chromatography and visualized by autoradiography (see “Experimental Procedures”). PKA phosphorylation of the fusion protein produced phosphopeptides that differed remarkably from those produced by PKC phosphorylation (compare Fig. 2, A and B). However, tryptic phosphopeptides generated from fusion protein that had been phosphorylated by both PKA and PKC (Fig. 2C) closely resembled tryptic phosphopeptides of the NR1 subunit isolated from neurons in culture as indicated by the numbered phosphopeptides (compare with Fig. 2 from Ref. 26).

**RESULTS**

**Phosphorylation of NR1 Protein by PKA and PKC**—To associate the tryptic phosphopeptides with specific residues in the NR1 protein, tryptic phosphopeptide maps of mutated NR1 C-terminal fusion proteins were obtained. Conversion of serine residues 896 and 897 in the NR1 fusion protein to alanine residues resulted in tryptic phosphopeptide maps of PKC and PKA phosphorylated fusion protein that lacked phosphopeptides 1,4,4,5 and 6 (Fig. 2D). Similarly, phosphopeptide maps of a mutant fusion protein where serine residues 889 and 890 were mutated to alanine lacked phosphopeptides 3, 5, and 8, whereas mutation of threonine 879 to alanine eliminated phosphopeptide 2 (see Table I). Analogous mutants, expressed as full-length recombinant NR1 proteins in human embryonic kidney-293 (HEK) cells and phosphorylated \textit{in situ}, yielded results virtually identical to those seen \textit{in vitro} (data not shown). These results demonstrate that the majority of NR1 protein phosphorylation occurs on two pairs of serine residues (889−890, 896−897) in the C-terminal region of NR1 (see Fig. 1A). PKC phosphorylates both pairs of serines, whereas PKA phosphorylates only the second pair (896−897). PKC also phosphorylates threonine 879. In an attempt to determine which specific serine residues in the two pairs of

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**FIG. 1.** PKA and PKC phosphorylation of the C-terminal region of NR1. A, schematic of the C-terminal region of NR1, including the fourth transmembrane domain (TMIV), the regions encoded by two alternatively spliced exons (CI and CII), and the protein sequence of the C1 exon. Serine and threonine residues subjected to site-specific mutagenesis are indicated by their residue numbers. B, schematic of a bacterial fusion protein containing amino acids 834–938 of NR1 protein. C, the wild-type fusion protein shown in B was incubated without kinase or with purified PKA, PKC, or both in the presence of \(^{32}P\)ATP for 30 min at 30 °C and resolved by SDS-PAGE and analyzed by autoradiography. D, PKA was incubated with wild-type fusion protein or a mutant fusion protein in which serine residues 896 and 897 had been converted to alanine residues (S896A, S897A) and then analyzed by SDS-PAGE and autoradiography.

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albumin, 0.5% Triton X-100, PBS for 1 h. Primary antibody (30 ng/ml) was applied overnight at 4 °C in 1% bovine serum albumin, 0.5% Triton X-100, PBS and then washed away with three buffer changes over 30 min. Fluorescein (fluorescein isothiocyanate)-conjugated donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch) secondary antibody was applied in the same buffer for 1 h at room temperature. After three more washes, cells were immersed in perfluor solution (Immuno) supplemented with 2.5% 1,4-diazabicyclo[2.2.2]-octane (Aldrich) and mounted on glass slides. Fluorescence was visualized and quantified using a Zeiss Axioshot microscope. In some cases, 1–2-μm thick optical sections were obtained in the x-y plane by scanning laser confocal microscopy using an argon laser and the Bio-Rad MRC-600 confocal microscope system. Excitation was at 488 nm, and emissions were taken between 510–515 nm. Confocal images were retained using CO-MOS version 6.03 software (Bio-Rad) and processed with Adobe Photoshop and ClarisDraw software.

**Hippocampal Slice Preparation**—The hippocampi of 6–8-week-old male Sprague-Dawley rats were dissected and chilled in ice-cold ACSF solution (126 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 10 mM d-glucose, 2 mM CaCl2, 2 mM MgCl2, saturated with 95% O2/5% CO2). Thick slices (400 μm) were transferred to a 35-mm diameter dish containing 1 ml of ACSF solution in a humidified 95% O2/5% CO2 gas saturated buffer A (10 mM Na3PO4 (pH 7.0), 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM Na3VO4, 10 mM Na2P2O7, 50 mM NaF, 10 units/ml trisylol) for 30 s. The homogenates were centrifuged at 12,000 × g for 5 min. After discarding the supernatant, the membrane pellet was resuspended in SDS sample buffer.
Serines are phosphorylated by the two kinases, we examined fusion proteins in which individual serines were converted to alanines. However, mutation of individual serines had little effect on phosphorylation of the mutant fusion proteins by PKA and PKC (data not shown) suggesting that both serines in each pair could be phosphorylated by the appropriate enzymes in these mutants. However, we were concerned that these results may not indicate which serine residues are phosphorylated by these kinases in the wild-type NR1 subunit. We therefore turned to phosphorylation site-specific antibodies to examine the physiological phosphorylation sites of NR1.

Phosphorylation Site-specific Antibodies for Serine Residues 890, 896, and 897—Phosphorylation site-specific antibodies has previously been used to examine the phosphorylation of individual proteins in vivo (35–41). We therefore attempted to generate phosphorylation site-specific antibodies that would specifically recognize the various phosphorylated forms of the NR1 subunit. Synthetic peptides corresponding to sequence of NR1 surrounding the pairs of serines were synthesized, and the peptides were chemically phosphorylated on serine residues 889, 890, 896, or 897 (see “Experimental Procedures”). These peptides were then used to immunize rabbits, and the resulting antibodies were affinity purified on phosphopeptide affinity columns.

The specificity of the phosphorylation site-specific antibodies was characterized using immunoblots of the NR1 fusion protein. Because of its bacterial origin, the fusion protein should have a minimal level of basal phosphorylation. Appropriately, the anti-phosphoserine 890, anti-phosphoserine 896, and anti-phosphoserine 897 antibodies only poorly recognized NR1 fusion protein (Fig. 3, A–C). In contrast, phosphorylation of the NR1 fusion protein with PKA markedly increased its recognition by the anti-phosphoserine 897 antibody (Fig. 3, C), whereas phosphorylation of the NR1 fusion protein with PKC markedly increased its recognition by the anti-phosphoserine 890 and anti-phosphoserine 896 antibodies (Fig. 3, A and B). The anti-phosphoserine 898 antibody recognized both unphosphorylated and phosphorylated fusion protein equally well and was not investigated further (data not shown). These results indicate that the anti-phosphoserine 890, anti-phosphoserine 896, and anti-phosphoserine 897 antibodies recognize NR1 protein only when it is phosphorylated. In addition, these results suggest that PKA phosphorylates serine 897 and PKC phosphorylates serines 890 and 896.

Intriguingly, although the anti-phosphoserine 896 antibody readily recognized the NR1 fusion protein after PKC phosphorylation, it reacted poorly after combined PKC and PKA phos-

| Mutations     | Missing phosphopeptides |
|---------------|-------------------------|
| S896A, S897A  | 1, 4, 4', 6, 6'         |
| S889A, S890A  | 3, 5, 8                 |
| T879A         | 2                       |

TABLE I Phosphopeptide Identification

Fig. 2. Phosphopeptide map analysis of tryptic phosphopeptides from phosphorylated NR1 fusion protein. Phosphorylated NR1 fusion proteins were excised from gels similar to those in Fig. 1, digested with trypsin, and the resulting tryptic phosphopeptides spotted onto thin layer chromatography plates and resolved in two dimensions by electrophoresis (horizontal) and ascending chromatography (vertical) as described under “Experimental Procedures.” Tryptic phosphopeptides detected by autoradiography were numbered in accordance with similar phosphopeptides isolated from full-length NR1 protein phosphorylated in situ in neurons (see Tingley et al. (26)). Open circles indicate the origin. Phosphopeptide map analyses are shown for wild-type NR1 fusion protein (A–C) or mutant (S896A, S897A) fusion protein (D) phosphorylated with PKA (A), PKC (B), or both (C + D).
Fig. 3. Specificity of phosphorylation site-specific antibodies for phosphorylated NR1 fusion protein. Synthetic peptides corresponding to phosphorylation sites on NR1 protein were chemically phosphorylated and used to generate polyclonal antibodies. A–C and E, wild-type NR1 fusion protein was phosphorylated in the presence of unlabeled ATP, resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with phosphorylation site-specific antibodies generated against the phosphoserine residue(s) 890 (A), 896 (B), or 897 (C) or 896 and 897 (E). D, phosphorylation reactions containing wild-type fusion protein and no kinase, PKA, PKC, or both, were incubated for 30 min. In some reactions PKA or PKC were added sequentially, as indicated, and incubated for another 30 min, then proteins were applied directly to nitrocellulose membranes using a “slot-blot” device, and immunoblotted with phosphorylation site-specific antibodies.

Phosphorylation (Fig. 3B). One possible explanation of this result is that PKA phosphorylation of serine 897 inhibits PKC phosphorylation at serine 896. Alternatively, phosphorylation at both serines 896 and 897 may simply reduce recognition of phosphoserine 896 by the anti-phosphoserine 896 antibody. To test these two possibilities the order of addition of PKA and PKC to the phosphorylation reaction was varied. Interestingly, even when NR1 fusion protein was incubated first with PKC for 30 min before addition of PKA, the immunoreactivity of the anti-phosphoserine 896 antibody decreased (Fig. 3D). This result indicates that PKA phosphorylation of serine 897 inhibits recognition of phosphoserine 896 by the anti-phosphoserine 896 antibody. To directly examine whether serines 896 and 897 could be simultaneously phosphorylated, antisera against a synthetic NR1 phosphopeptide in which serine residues 896 and 897 were both chemically phosphorylated was generated. In immunoblot experiments, this antibody strongly recognized the NR1 fusion protein after preincubation with both PKC and PKA and only weakly recognized the NR1 fusion protein which was phosphorylated by either kinase alone (Fig. 3E). These findings showed that serines 896 and 897 could be simultaneously phosphorylated by two distinct protein kinases.

Using the phosphorylation site-specific antibodies, we examined the phosphorylation of the full-length NR1 protein expressed in cultured cells. HEK cells were transiently transfected with NR1 cDNA and treated with combinations of two kinase activators, PMA (phorbol 12-myristate 13-acetate), a phorbol ester that stimulates PKC, and forskolin, which stimulates PKA indirectly by increasing the production of cAMP by adenylyl cyclase. In immunoblots of total cell lysates, each of the phosphorylation site-specific antibodies recognized a single 120-kDa protein (Fig. 4, A–D) that comigrated with the NR1 subunit (Fig. 4E). Mutation of the appropriate serine residues in the NR1 subunit completely eliminated the recognition of the NR1 subunit by the relevant phosphorylation site-specific antibody (data not shown). Treatment of HEK cells expressing wild-type NR1 protein with PMA increased the recognition of the NR1 subunit by the anti-phosphoserine 890 and the anti-phosphoserine 896 antibodies, whereas prior forskolin treatment had no effect on immunorecognition of the NR1 subunit by these antibodies (Fig. 4, A and B). In contrast, forskolin treatment, but not PMA treatment, increased the binding of the anti-phosphoserine 897 antibody to the NR1 subunit (Fig. 4C). Finally, the anti-phosphoserines 896/897 antibody gave a strong signal primarily after combined PMA and forskolin treatment of transfected cells (Fig. 4D). Immunoblots using a phosphorylation state-independent antibody against the C terminus of NR1 indicated that the amount of NR1 protein was not affected by either PMA or forskolin treatment (Fig. 4E). The detection of the phosphorylated NR1 subunit was completely eliminated when the antibody was incubated with the phosphorylated peptide used as immunogen but not by the non-phosphorylated peptide (data not shown).

To investigate the phosphorylation of the NR1 subunit in the brain, hippocampal slice homogenates were subjected to immunoblot analysis using the phosphorylation site-specific antibodies. In contrast to transfected cells, the phosphorylation site-specific antibodies cross-reacted with proteins in the total homogenates. However, these cross-reacting proteins could be minimized by isolating membrane preparations from the hippocampal slices (data not shown). In the isolated membrane preparations from acute slices of adult rat hippocampus, the phosphorylation site-specific antibodies recognized a 120-kDa protein that comigrated with the NR1 subunit, indicating that these sites are phosphorylated in vivo (Fig. 5). Phorbol ester treatment of the hippocampal slices increased the immunorecognition of NR1 by the anti-phosphoserine 890, the anti-phosphoserine 896, and the anti-phosphoserines 896/897 antibodies but had no effect on the signal detected by the anti-phosphoserine 897 antibody. However, forskolin treatment increased the immunorecognition of NR1 by both the anti-phosphoserine 897 antibody and the anti-phosphoserine 896/897 antibody. Taken together, these data demonstrated that the NR1 subunit is basally phosphorylated on serine residues 890 and 897 in untreated hippocampal slice preparations and has a lower basal phosphorylation of serine 896. Phorbol ester treatment increased the phosphorylation of serine 896 and caused serines 896 and 897 to become phosphorylated simultaneously. In addition, forskolin treatment increased both the phosphorylation of serine 897 and the simultaneous phosphorylation of 896 and 897. The detection of the phosphorylated NR1 subunit was completely eliminated when the antibody was incubated with the phosphorylated peptide used as immunogen but not by the non-phosphorylated peptide (data not shown).

Regulation of the Subcellular Distribution of NR1 Protein by PKC and PKA Phosphorylation—In addition to the phosphorylation sites, the C exon cassette in the C-terminal region of the NR1 subunit contains molecular cues that regulate its subcellular targeting in transfected cells (29). NR1 subunits
containing the C1 cassette spontaneously aggregate into receptor-rich domains near the surface of these cells, whereas NR1 splice variants that lack the cassette do not form these receptor-rich domains and are diffusely distributed throughout the cells. We have previously shown that phorbol ester treatment of QT6 cells expressing the NR1 subunits containing the C1 exon disrupts the NR1-rich domains resulting in a diffuse distribution of the NR1 subunits similar to that seen with NR1 splice variants missing the C1 exon. This phorbol ester-induced redistribution of the NR1 subunit is due to the phosphorylation of serine residues within the C1 cassette (29). Indeed, mutation of serines 889, 890, 896, and 897 to alanine residues abolishes both the vast majority of PKC phosphorylation and eliminates the phorbol ester-induced redistribution of the NR1 subunit (26, 29). These results suggest that the C1 cassette interacts with cytoskeletal proteins and that this interaction is disrupted by phosphorylation of the NR1 subunit.

In order to further investigate the effects of phosphorylation on the targeting of the NR1 subunit, we examined the subcellular distribution of wild-type and mutant NR1 proteins in transfected QT6 cells treated with kinase activators. The QT-6 cell line has been used as a model system for studying the clustering of neurotransmitter receptors such as the nicotinic acetylcholine receptor (42) and the NMDA receptor (29).

Examination of the time course of NR1 protein phosphorylation and redistribution was examined in QT6 cells expressing wild-type NR1 protein indicated that phosphorylation of serine 890 increased within 5 min of PMA treatment (Fig. 7A). In contrast, forskolin treatment had no effect on NR1-enriched domains (Fig. 7E), despite an increase in phosphorylation of serine 897 as revealed by the anti-phosphoserine 897 antibodies (data not shown). These findings indicate that the NR1 subunit rapidly and reversibly redistributes between sub-
cellular compartments in response to phosphorylation of serine 890.

**DISCUSSION**

Protein phosphorylation of ligand-gated ion channels is a primary mode of regulation of their function and may play a central role in the regulation of synaptic transmission (15, 43). NMDA receptors are ligand-gated ion channels that are critical for several forms of activity-dependent synaptic plasticity in the brain such as long term potentiation and long term depression (3). Thus, the regulation of NMDA receptor function by protein phosphorylation could have dramatic effects on these processes. PKC has been suggested to regulate NMDA receptor function since intracellular perfusion of PKC in spinal trigeminal neurons potentiates NMDA receptor function (20). In addition, PKA has recently been suggested to potentiate NMDA receptors in hippocampal neurons in culture (25). Phorbol ester activation of PKC can also potentiate NMDA responses in *Xenopus* oocytes expressing homomeric receptors consisting of the NR1 subunit (13, 23) and heteromeric receptors consisting of the NR1 and either the NR2A or NR2B subunit (8, 24). Interestingly, although homomeric NR1 receptors are potentiated by phorbol esters, receptors comprised of the NR1 and NR2C or 2D subunits are not affected by phorbol esters (8, 24). Moreover, NR1 splice variants which lack the C1 exon are dramatically potentiated by phorbol esters (13), in spite of the fact that most, if not all, of the PKC phosphorylation sites on NR1 appear to be contained within this exon (26). These results suggest that phorbol ester regulation of NMDA receptor function in *Xenopus* oocytes is complex and may be mediated in part by phosphorylation of endogenous proteins in the oocytes.

Previous results from our laboratory have demonstrated that PKC directly phosphorylates the NR1 subunit and that this phosphorylation can be eliminated by mutation of serines 889, 890, 896, and 897 in the C-terminal domain of NR1. Here we have further characterized the phosphorylation of the NR1 subunit using site-specific mutagenesis and phosphorylation site-specific antibodies. We have demonstrated that in addition to PKC, PKA directly phosphorylates the NR1 subunit of the NMDA receptor *in vitro*, in transfected cells, and in hippocampal slices. In addition, using site-specific mutagenesis and phosphorylation site-specific antibodies, we have shown that PKC phosphorylates serine 890, serine 896, and threonine 879, whereas PKA specifically phosphorylates serine 897 *in vivo*. Moreover, activation of PKA and PKC results in the simultaneous phosphorylation of neighboring serine residues 896 and 897.

NMDA receptors in the brain cluster in the postsynaptic membrane and are also found in intracellular pools of unknown function (28, 44–46). Although the molecular mechanisms behind synaptic targeting are poorly understood, increasing evi-
Evidence suggests that NMDA receptors may interact with the cytoskeleton (47). Recent studies have demonstrated that the NR1 subunit localizes to discrete receptor-rich domains in fibroblasts and that the formation of these receptor-rich domains is regulated by both alternative splicing and protein phosphorylation of the C1 exon (29). The C1 cassette has also recently been demonstrated to bind calmodulin (48). In this study we show that only phosphorylation of serine 890 by PKC, and not PKC phosphorylation of serine 896 or PKA phosphorylation of serine 889, affects NR1 distribution. One intriguing possibility is that PKC phosphorylation of serine 890 specifically regulates the interaction of the NR1 subunit with cytoskeletal proteins. It is interesting to note that the C1 cassette of the NR1 subunit is highly homologous to the effector domain of the cytoskeletal organizing protein MARCKS (myristoylated, alanine-rich, protein kinase C substrate), a protein initially identified as one of the major cellular substrates for PKC (49). The effector domain of MARCKS contains the major PKC phosphorylation sites as well as binding sites for actin and calmodulin. This region of the MARCKS protein is thought to mediate the reversible attachment of the actin cytoskeleton with the plasma membrane (49). In addition, the binding of the MARCKS effector domain to actin and calmodulin is inhibited by PKC phosphorylation. Thus, in a manner similar to the MARCKS protein, PKC phosphorylation of the C1 cassette of NR1 may play an important role in the regulation of the interaction of NR1 with the cytoskeleton. Future studies will address the role of the C1 cassette in regulating NR1 targeting in neurons and the role of PKC phosphorylation and calmodulin in regulating this process.
Essential to these studies was the generation of phosphorylation site-specific antibodies. These antibodies show remarkable specificity, with each antibody only recognizing NR1 when it was phosphorylated on a specific serine residues. These antibodies were useful for the study of NMDA receptor phosphorylation in vitro, in transfected cells, and in hippocampal slices. Moreover, these antibodies can differentiate between adjacent phosphoserine residues and are specific for the simultaneous phosphorylation of these two neighboring serine residues. These antibodies should be powerful reagents to study the regulation of NR1 phosphorylation in vivo during a variety of experimental paradigms such as model systems for the study of synaptic plasticity, activity-dependent neuronal development, and excitotoxicity.

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REFERENCES
1. Hollmann, M., and Heinemann S. (1994) Annu. Rev. Neurosci. 17, 31–108
2. Seeberg, P. H. (1993) Trends Pharmacol. Sci. 14, 297–303
3. Nicoll, R. A., and Malenka, R. C. (1993) Nature 377, 115–118
4. Constantine-Paton, M., Cline, H. T., and Debski, E. (1990) Annu. Rev. Neurosci. 13, 129–154
5. Choi, D. W., and Rothman, S. M. (1990) Annu. Rev. Neurosci. 13, 171–182
6. Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991) Nature 354, 31–37
7. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1992) Science 256, 1217–1221
8. Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiyama, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., and Mishina, M. (1992) Nature 356, 36–41
9. Meguro, H., Mori, H., Araki, K., Kushiyama, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., and Mishina, M. (1992) Nature 357, 70–74
10. Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N., and Jan, L. Y. (1994)
11. Blahos, J., III, and Wenthold, R. J. (1996) *J. Biol. Chem.* 271, 15669–15674
12. Sugihara, H., Moriyoshi, K., Ishii, T., Masa, M., and Nakanishi, S. (1992) *Biochem. Biophys. Res. Commun.* 185, 826–832
13. Durand, G. M., Gregor, P., Zheng, X., Bennett, M. V. L., Uhl, G. R., and Zakin, R. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 9350–9363
14. Heilmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G., and Heinemann, S. (1993) *Neuron* 10, 943–954
15. Roche, K. W., Tingley, W. G., and Huganir, R. L. (1994) *Curr. Opin. Neurobiol.* 4, 383–388
16. Aniksztejn, L., Otani, S., and Ben-Ari, Y. (1992) *Eur. J. Neurosci.* 4, 500–505
17. Gerber, G., Kangrga, I., Ryu, P. D., Larew, J. S. A., and Randic, M. (1989) *J. Neurosci.* 9, 3606–3617
18. Markram, H., and Segal, M. (1992) *J. Physiol. (Lond.)* 457, 491–501
19. Courtney, M. J., and Nicholls, D. G. (1992) *J. Neurochem.* 59, 983–992
20. Chen, L., and Huang, L.-Y. M. (1992) *Nature* 356, 521–523
21. Yamazaki, M., Mori, H., Araki, K., Mori, K. J., and Mishina, M. (1992) *FEBS Lett.* 300, 39–45
22. Durand, G. M., Bennett, M. V. L., and Zakin, R. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 6731–6735
23. Mori, H., Yamakura, T., Masaki, H., and Mishina, M. (1993) *NeuroReport* 4, 519–522
24. Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) *Nature* 364, 70–73
25. Moon, I. S., Apperson, M. L., and Kennedy, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 3954–3958
26. Lau, L.-F., and Huganir, R. L. (1995) *J. Biol. Chem.* 270, 20036–20041
27. Ehlers, M. D., Tingley, W. G., Qu, Z., and Huganir, R. L. (1995) *Science* 269, 1734–1737
28. Woodgett, J. R., and Hunter, T. (1987) *J. Biol. Chem.* 262, 4836–4843
29. Reimann, E. R., and Behman, R. B. (1983) *Methods Enzymol.* 99, 51–55
30. Huganir, R. L., Miles, K., and Greengard, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 6968–6972
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 30–39, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Aderem, A. (1992) *Cell* 71, 713–716