Identification of a Phosphorylation Site for Calcium/Calmodulin-
dependent Protein Kinase II in the NR2B Subunit of the
N-Methyl-D-aspartate Receptor*

Ramakrishnapillai V. Omkumar, Melinda J. Kiely, Alan J. Rosenstein, Kyung-Tai Min,
and Mary B. Kennedy‡

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

The N-methyl-D-aspartate (NMDA) subtype of excitatory glutamate receptors plays critical roles in embryonic and adult synaptic plasticity in the central nervous system. The receptor is a heteromultimer of core subunits, NR1, and one or more regulatory subunits, NR2A-D. Protein phosphorylation can regulate NMDA receptor function (Lieberman, D. N., and Mody, I. (1994) Nature 369, 235–239; Wang, Y. T., and Salter, M. W. (1994) Nature 369, 233–235; Wang, L.-Y., Orser, B. A., Brautigan, D. L., and MacDonald, J. F. (1994) Nature 369, 230–232). Here we identify a major phosphorylation site on subunit NR2B that is phosphorylated by Ca2+/calmodulin-dependent protein kinase II (CaM kinase II), an abundant protein kinase located at postsynaptic sites in glutamatergic synapses. For the initial identification of the site, we constructed a recombinant fusion protein containing 334 amino acids of the C terminus of the NR2B subunit and phosphorylated it with CaM kinase II in vitro. By peptide mapping, automated sequencing, and mass spectrometry, we identified the major site of phosphorylation on the fusion protein as Ser-383, corresponding to Ser-1303 of full-length NR2B. The $K_m$ for phosphorylation of this site in the fusion protein was ~50 nM, much lower than that of other known substrates for CaM kinase II, suggesting that the receptor is a high affinity substrate. We show that serine 1303 in the full-length NR2B and/or the cognate site in NR2A is a major site of phosphorylation of the receptor both in the postsynaptic density fraction and in living hippocampal neurons.

Plasticity in the strength of transmission at synapses is essential for higher order brain functions such as learning and memory (1). In the hippocampus and cortex, activation of the NMDA subtype of glutamate receptors can trigger long lasting changes in synaptic strength. These changes include long term potentiation (2) and long term depression (3). NMDA receptors contain two classes of subunits in hetero-oligomeric associations, the core NR1 subunit and the regulatory NR2 (A–D) subunits (4, 5). The NR2 subunits are equipped with uniquely long carboxyl-terminal tails that are believed to extend into the cytoplasm (6). These tails may participate in transduction mechanisms or in forms of regulation of the NMDA receptor that are not yet fully understood. NR2A and NR2B are the major regulatory subunits of NMDA receptors in the forebrain (4, 5). Previous work from this laboratory has shown that NR2B is the principal NMDA receptor subunit found in the postsynaptic density fraction prepared from forebrain (7), suggesting that this subunit may participate in anchoring the NMDA receptor at postsynaptic sites. Indeed, recent work from our lab and the Seeberg lab (8) has demonstrated a direct association between NR2B and the postsynaptic density protein PSD-95.

NMDA receptors are ligand and voltage-gated Ca2+ channels (9, 10). Binding of glutamate released from the presynaptic terminal, coupled with strong depolarization of the postsynaptic membrane produces an influx of Ca2+ into the postsynaptic compartment (11). This Ca2+ influx initiates a wide array of biochemical events in the synapse that can lead to long term potentiation or long term depression (12, 13). One potential target for immediate activation by this Ca2+ influx is CaM kinase II. CaM kinase II has long been known to be concentrated in the postsynaptic density (14–17) and to be essential for expression of both long term potentiation and long term depression (18–20). One target for regulation by CaM kinase II at the postsynaptic site may be the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor subunit GluR1 (21), although uncertainty about the topology of receptor folding has cast some doubt on the significance of direct phosphorylation of GluR1 in vivo (22). Another potential target for phosphorylation is the NMDA receptor itself. Indeed, electrophysiological evidence indicates that NMDA receptor-mediated currents can be enhanced by protein phosphorylation (23–26). However, direct demonstration of the role of phosphorylation of the receptor itself in regulating its function requires identification of sites on the receptor subunits that are phosphorylated by particular protein kinases. Sites of phosphorylation of NR1 by protein kinase C have been located in the alternatively spliced C-terminal region, and their role in regulating receptor function in vitro has been demonstrated (27). Here we report the identification of a principal site of phosphorylation of the NR2B subunit of the NMDA-type glutamate receptor by CaM kinase II. We show that this site and/or its cognate site in NR2A is phosphorylated both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Acetonitrile, UV/HPLC grade, was purchased from Baxter; Optima/HPLC grade water from Fisher; HPLC/Spectra grade tri-fluoroacetic acid from Pierce; dithiothreitol (DTT) from Boehringer...
Mannheim; iodoacetamide from Sigma; [γ-32P]ATP from ICN Pharma-
col; sequencing grade trypsin from Serva; phos-
phate-buffered saline containing 1%
supernatant was saved, and the pellet was resuspended in 10 ml of
0.1 mM PMSF. The pellet was sonicated, and Triton X-100 and PMSF
X-100, and 0.1 mM PMSF overnight at 4°C on an end-over-end mixer.

mM, and Triton X-100 was added to a concentration of 1%. The cell
suspension was subjected to centrifugation at 15,000
g/ml of antipain, 0.4
m of 0.1 M NH4HCO3, and the supernatant (−0.6 ml) containing peptides was transferred to a fresh
tube. The gel pellets were washed with 0.4 ml of 0.1 M NH4HCO3,
and the washes were added to the previous supernatants. An additional 5 µg of sequencing grade trypsin was added to each supernatant along
with fresh DTT to a final concentration of 1 mM, and the tubes were incubated in a 37 °C shaker water bath for another 13 h. The supernatant
was then lyophilized to dryness. To recover additional peptides, 1 ml of 0.1 M NH4HCO3, 1 mM DTT containing 6 µg of sequencing grade
trypsin was added to the gel pellets, and they were incubated at 37 °C for another 13 h. Sequencing grade trypsin (3 µg) and 1 mM DTT were
added to the supernatants, and they were incubated at 37 °C for an additional 8 h. The final supernatants were pooled with the lyophilized
peptides from the previous supernatants and again lyophilized to
dryness.

**HPLC Fractionation of Phosphopeptides**—Lysylated peptides were
dissolved in 1 ml of 0.1% trifluoroacetic acid and fractionated in two
batches by HPLC on a C18 reverse phase column (4.1 × 250 mm). The
column was developed at 1 ml/min with a gradient of 0–42% acetonitrile
in 50 mM ammonium acetate, monitored at 214 nm, and 0.5-ml fractions were
collected. Radioactivity in each fraction was measured in a Beckman
LS 8000 scintillation counter by detection of Cerenkov radiation (34).

**Mass Spectrometry and Sequencing of Phosphopeptides**—Mass spec-
 trometry was conducted by the Protein/Pepptide Micro Analytical Labo-
 ratory at Caltech with a PerSeptive Biosystems/Vestec Lasertech II
reflector for matrix-assisted, laser desorption ionization, time-of-flight
mass spectrometry (MALDI-TOF). Concentrated peak fractions of pep-
tides were mixed with an α-cyano-4-hydroxycinnamic acid matrix solu-
don, dried, and placed in the mass spectrometer. Data were collected
both in linear and reflector modes.

Amino acid sequencing was performed in the Protein/Pepptide Micro-
Analytical Laboratory with a Perkin-Elmer/Applied Biosystems Inc.
model 476A automatic protein sequencer.

**Immunoprecipitation of NR2B from the Postynaptic Density Fraction**—The postynaptic density fraction was phosphorylated by endog-

eneous CaM kinase II in the assay solution described above without the
addition of exogenous kinase. For Fig. 1, the reaction was carried out
with [γ-32P]ATP (4500 cpm/μmol) for 2 min. For generation of tryp tic
peptides, the reaction was carried out with [γ-32P]ATP (80,000 cpm/
μmol) for 2 min. The reaction was stopped by the addition of a final
concentration of 0.2% SDS and boiled for 3 min. Immunoprecipitation
was carried out by the addition of RIPA buffer (10 ml Tris, pH 7.4, 1
ml EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate)
and rabbit antiserum against NR2B (final dilution 1:20 in a volume of
0.2 or 0.8 ml 150 mM Tris-EDTA). The reaction was monitored at 30°C.

**Tryptophan Determination**—Trp was determined by the spectrophotometric
analysis of tryptophan absorption at 280 nm (12). The sample was
mixed with the dye reagent and incubated for 15 min at 30°C. The absorbance
was read at 280 nm.

**Amino Acid Analysis**—Amino acid analyses were carried out on a model
476A automatic protein sequencer.
boiled for 5 min and subjected to SDS-PAGE on a 6% gel. The NR2B bands were excised and subjected to tryptic peptide mapping as per GST-NR2B except that the carboxymethylation was omitted.

Detection of the Phosphorylated Site on the NR2 Subunits with a Phospho-specific Antibody—A synthetic peptide with the sequence LRRKQSDYTDYFVC was obtained from the Peptide Sequencing Facility at Caltech. This mg was phospho-homogenized in 20 mM Tris-HCl, pH 8.0, 1 mM imidazole, 2 mM EDTA, 2 mM EGTA, 25 mg/liter soybean trypsin inhibitor, 1 mg/liter leupeptin, 2 mM PMSF, 0.1 mM EDTA, and 50 mg/ml PMSG in a final volume of 0.85 ml. The solution was brought to 0.4% trifluoroacetic acid, and the phosphopeptide was purified by HPLC and conjugated via succinimidyl 4-(N,N-dimaleoylaminopropyl)azepin-3-carboxylate to rabbit antiserum as described previously (7) by repeated injection of 0.33 mg of conjugated peptide in RIBI adjuvant (RIBI Immunocchemical Research Inc., Hamilton, MT) at several sites. Serum was collected and tested on immunobLOTS as described below against 0.2 

\[
\text{g of phosphorylated and 0.2 g of nonphosphorylated GST-NR2B.}
\]

The serum of one rabbit showed good specificity for phosphorylated GST-NR2B. To neutralize antibodies against nonphosphorylated GST-NR2B, the serum was preabsorbed with 6 mol eq of the nonphospho-tyr. The NR2B bands were excised and subjected to tryptic mapping as for GST-tNR2B except that the carboxymethylation was omitted. The plates were transferred to a 37°C CO2 incubator for 8 h. At the end of the incubation, the labeling medium was aspirated off. The slices were washed twice with Ringer buffer (125 mM NaCl, 2 mM KCl, 1.25 mM NaH2PO4, 2 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, 10 mM glucose, and 25 mM HEPES, pH 7.4), frozen on dry ice on glass slides, and transferred to a −70°C freezer overnight. The slices were thawed on ice and homogenized in 1 ml of homogenization buffer (20 mM Tris-HCl, pH 8.0, 1 mM imidazole, 2 mM EDTA, 20 mM sodium pyrophosphate, 25 mg/liter soybean trypsin inhibitor, 1 mg/liter leupeptin, 2 mM DTT, 0.1 mM PMSF, 2 mM EGTA) at 900 rpm with eight strokes. The homogenate was brought to 0.2% SDS, placed in a boiling water bath for 5 min, cooled in ice, and brought to 1X RIPA buffer, 0.1% SDS (final concentration). The sample was cleared by centrifugation at 

\[
16,000 \times g 
\]

for 5 min and then mixed with 100 

\[
\text{of antisera against NR2B in the cold room for 2 h. Protein A-Sepharose beads (2-mL suspension), washed once with RIPA buffer, and mixed and boiled for 5 min. The mixture was subjected to}
\]

SDS-PAGE on a 6% gel. The NR2B bands were visualized by autoradiography after drying the gel, excised, and subjected to tryptic digestion as described above for GST-NR2B.

Lyophilized tryptic peptides were dissolved in 0.1% trifluoroacetic acid in 50% acetonitrile, spotted onto 20 × 20 cm cellulose-coated thin layer plates, dried, and spotting was continued in a 95% O2, 5% CO2 atmosphere. The plates were dried in the Speed-Vac, redissolved in deionized water, and subjected to tryptic digestion as described above for GST-NR2B.

Phosphoamino Acid Analysis—Organoacetic hippocampal cultures were prepared and washed as described above and then incubated in minimal essential medium containing 100 

\[
\text{g of NaH2PO4 and 1 mCi of [gamma-32P]H3PO4 for 16 h at 37°C. The slices were then washed twice with Ringer buffer and homogenized in 600 
}\]

\[
\text{g of homogenization buffer with 10 mM MgCl2, 0.35 mM EGTA, 0.6 mM CaCl2, 0.26 mg/ml calmodulin, 2.2 mM DTT, 13.2 mM [gamma-32P]H3PO4 (final volume 2 ml). The sample was cleared by centrifugation at}
\]

16,000 

\[
\times g
\]

for 15 min. The supernatant was transferred to a new tube, and 20 

\[
\text{g of phosphate-buffered saline was added. The supernatant was transferred to SDS-PAGE on a 6% gel. The NR2B bands were visualized by autoradiography after drying the gel, excised, and subjected to tryptic digestion as described above for GST-NR2B.}
\]

The solution was mixed end-over-end for 2 h at 4°C. The homogenate was then cleared by centrifugation at 12,000 

\[
\times g 
\]

at 4°C for 15 min. The supernatant was transferred to a tube containing 50 

\[
\text{g of protein A-agarose beads previously washed with RIPA buffer and mixed end-over-end at 4°C for 1 h. The beads were pelleted by centrifugation at 12,000 
}\]

\[
\times g
\]

for 5 min and then washed three times with RIPA buffer and resuspended in 30 

\[
\text{g of SDS stop solution. Ten 
}\]

\[
\text{g of the sample was loaded onto a 6% SDS-polyacrylamide}
\]

\[
\text{gel. The gel was stained with Coomassie Blue and dried. Radioactive}
\]

\[
\text{NR2B was visualized by autoradiography. Gel bands were ex-}
\]

\[
\text{cised from the gel and placed in 1 ml of 30% methanol for 1 h, washed}
\]

\[
\text{twice in 50 mM NH4HCO3 and then incubated in the same solution}
\]

\[
\text{containing 20 
}\]

\[
\text{g of 1-tosylamido-2-phenylethyl chloromethyl ketone-}
\]

\[
\text{treated trypsin overnight at 37°C. The solution was removed from}
\]

\[
\text{the gel piece, dried under vacuum, and stored as a Speed-Vac centrifuge, re-}
\]

\[
\text{suspended in 100 
}\]

\[
\text{g of HCl, and incubated at 110°C for 1 h. The hydrolyzed}
\]

\[
\text{sample was dried in the Speed-Vac, redisolved in deionized water,}
\]

\[
\text{and dried again. It was then resuspended in 8 
}\]

\[
\text{g of deionized water containing}
\]

\[
\text{50 mM protein A-agarose beads previously washed with RIPA buffer and mixed end-over-end at 4°C for 1 h. The beads were pelleted by centrifugation at 12,000 
}\]

\[
\times g
\]

for 5 min and then washed three times with RIPA buffer and resuspended in 30 

\[
\text{g of SDS stop solution. Ten 
}\]

\[
\text{g of the sample was loaded onto a 6% SDS-polyacryl-
}\]

\[
\text{amide gel. The gel was stained with Coomassie Blue and dried. Radio-
}\]

\[
\text{active NR2B was visualized by autoradiography. Gel bands were ex-}
\]

\[
\text{cised from the gel and placed in 1 ml of 30% methanol for 1 h, washed}
\]

\[
\text{twice in 50 mM NH4HCO3 and then incubated in the same solution}
\]

\[
\text{containing 20 
}\]

\[
\text{g of 1-tosylamido-2-phenylethyl chloromethyl ketone-}
\]

\[
\text{treated trypsin overnight at 37°C. The solution was removed from}
\]

\[
\text{the gel piece, dried under vacuum, and stored as a Speed-Vac centrifuge, re-}
\]

\[
\text{suspended in 100 
}\]

\[
\text{g of HCl, and incubated at 110°C for 1 h. The}
\]

\[
\text{hydrolyzed}
\]

\[
\text{sample was dried in the Speed-Vac, redisolved in deionized water,}
\]

\[
\text{and dried again. It was then resuspended in 8 
}\]

\[
\text{g of deionized water containing}
\]

\[
\text{phosphoamino acid standards (2 
}\]

\[
\text{g each of phosphoserine, phosphothreonine, and}
\]

\[
\text{phosphotyrosine), and spotted onto a cellulose-coated thin-layer chromatography plate. The plate was subjected to}
\]

electrophoresis as described previously (37) at 500 V for 2 h. The plate was dried and sprayed with 0.25% ninhydrin in acetone to visualize the standard amino acids. Radioactive amino acids were visualized by PhosphorImage analysis (Molecular Dynamics).

Other Methods—SDS-PAGE was performed as described in Ref. 38 on 6, 8, or 10% SDS running gels. Stacking gels contained 3.5% acrylamide-bis. Protein concentrations were measured by the method of Peterson (39) with bovine serum albumin as the standard. To estimate the proportion of intact GST-NR2B fusion protein (M, 63,000) in our purified preparations (Fig. 2), Coomassie-stained bands containing intact GST-NR2B were visually compared with those of known amounts of standard proteins. Because we estimated that intact GST-NR2B was about 30% of the total protein in the purified GST-NR2B solution, the
A Phosphorylation Site for CaM Kinase II in NR2B

**Fig. 1. Phosphorylation of NR2B by endogenous CaM kinase II in the PSD fraction.** Aliquots of the PSD fraction (50 μg) were incubated in the assay mixture for CaM kinase II as described under “Experimental Procedures.” Radiolabeled NR2B was immunoprecipitated from each tube, and the pellets were fractionated by SDS-PAGE. The figure shows an autoradiogram of the dried gel. Lane 1, incubation without Ca2+; lane 2, incubation with Ca2+; lane 3, incubation with Ca2+ and inhibiting monoclonal antibodies 4A11 (28 μg) and 6E9 (20 μg); lane 4, incubation with Ca2+ and 50 μg of nonimmune mouse IgG.

concentration of GST-tNR2B was taken as 30% of the total protein in the sample determined by the method of Peterson.

**RESULTS**

**Phosphorylation of NR2B by CaM Kinase II in the Postsynaptic Density Fraction**—To test whether NR2B is a substrate for endogenous CaM kinase II in the PSD fraction, proteins in the fraction were radiolabeled by phosphorylation with CaM kinase II, as described under “Experimental Procedures,” and then NR2B was immunoprecipitated from the radiolabeled PSD fraction. Incorporation of phosphate into NR2B was rapid and substantial, reaching a maximum of approximately 1 mol of phosphate/mol of NR2B in 2 min, assuming that NR2B is 0.5% of total PSD protein (7). Furthermore, the phosphorylation was stimulated by Ca2+ and was inhibited by monoclonal antibodies raised against CaM kinase II (Fig. 1; Ref. 40).

**Phosphorylation of GST-tNR2B by CaM Kinase II**—Because the amount of NR2B in the PSD fraction is less than 1% of total protein (7), we anticipated that isolation of sufficient quantities of phosphopeptide for automated sequencing of the endogenous site would be difficult. Therefore, we tested whether a recombinant fusion protein containing just the carboxyl terminus of NR2B could be phosphorylated by CaM kinase II. We constructed a pGEX plasmid in which cDNA encoding the C-terminal 334 amino acids of NR2B was fused to DNA encoding glutathione S-transferase. Fusion protein expressed in *E. coli* cells was purified by solubilization in 1% N-lauroyl sarcosine followed by affinity chromatography on a glutathione-agarose column (see “Experimental Procedures”; Fig. 2A). Purified GST-tNR2B was phosphorylated by CaM kinase II in *vitro* (Fig. 2B). As expected, the phosphorylation was dependent on the presence of Ca2+ and calmodulin. Recombinant glutathione S-transferase alone was not phosphorylated by CaM kinase II, indicating that phosphorylation of GST-tNR2B was likely occurring on the C-terminal NR2B portion of the fusion protein (data not shown). The phosphorylation was rapid and stoichiometric (Fig. 3A). The *K*ₘ for the fusion protein, calculated from double reciprocal plots, was unusually low, 47.2 ± 27.6 nM (Fig. 3B), suggesting that CaM kinase II has a very high affinity for the phosphorylation site(s) contained in the fusion protein.

**Purification of Tryptic Phosphopeptides from Phosphorylated GST-tNR2B**—To identify the phosphorylated site, 0.3 and 0.4 mg of fusion protein were exhaustively phosphorylated (in two separate experiments) by CaM kinase II. Tryptic peptides were generated from the phosphorylated protein and fractionated by HPLC on a C18 reverse phase column as described under “Experimental Procedures.” Seven major peaks of radioactivity appeared reproducibly (Fig. 4, peaks 2–8). Peaks 5 and 6 and peaks 7 and 8 always eluted as doublets migrating very closely on the column. The phosphopeptides in peaks 5–8 were pure enough for identification by mass spectrometry and/or automated sequencing. Peaks 2–4 contained multiple peptides revealed by mass spectrometry, none of which could be positively identified as a phosphopeptide (see below).

**Determination of Molecular Masses of the Tryptic Peptides of Phosphorylated GST-tNR2B by Mass Spectrometry**—Peaks 5–8 from two separate fractionations were concentrated and subjected to MALDI-TOF mass spectrometry in both the linear and reflector modes (41). In reflector mode, fragmentation of the phosphoryl group on phosphopeptides usually produces a new parent peptide peak with a mass 97 atomic mass units less than that of the phosphopeptide itself (41). Each of the four samples showed a second peak in reflector mode with a mass 97 atomic mass units less than that of the major peak observed in both linear and reflector mode (Fig. 5). The masses of the dephospho forms of peaks 5, 6, 7, and 8 were 1619, 1446, 2456,
The peptides in fractions 5 and 7 were sequenced with a molecular mass of an arginine residue. They present in samples 5 and 6 and in samples 7 and 8 differed in molecular mass by 2283, respectively. We also noted that the major peaks in samples 5 and 6 and in samples 7 and 8 differed in molecular mass by approximately 174 atomic mass units, the molecular mass of an arginine residue.

**Sequencing of the Tryptic Peptides of Phosphorylated GST-tNR2B**—The peptides in fractions 5 and 7 were sequenced with an automated gas phase sequencer (Table I). The sequences revealed that these two peptides contained the same phosphorylated site, serine 383 in GST-tNR2B, which corresponds to serine 1303 in full-length NR2B. The peptide present in fraction 7 is eight residues longer than the peptide in fraction 5 because of an uncleaved tryptic site at lysine 392 (Table I). The masses calculated from the sequences in Table I correspond to those determined by mass spectrometry (Fig. 5), confirming the identities of the phosphopeptides. Analysis of the phenylthiocarbamyl-derivatives produced from both peptides during the sequencing showed anomalously high yields of dehydroalanine compared with that of serine in the fourth sequencing cycle, which corresponds to serine 383. An anomalous high yield of dehydroalanine has been shown to be a reliable indicator of the presence of phosphoserine in a sequence (42). Serine 383/1303 is contained within a consensus sequence for phosphorylation by CaM kinase II, having an arginine at position –3 (43).

When we attempted to sequence the phosphopeptide contained in peak 6, we obtained two distinct sequences, neither of which corresponded to peak 6, without the requisite arginine at position 2283. Hence, we conclude that peak 8 contains a phosphopeptide with a blocked N terminus during the purification of peptides containing the principal autophosphorylation site of CaM kinase II, which contains a glutamine residue at position –2, just carboxyl to the requisite arginine at position –3 (34). Based upon the results of the mass spectrometry analysis, we conclude that the phosphopeptide in peak 6 is identical to that in peak 5 without the N-terminal arginine.

Peak 8 contained several phosphopeptides that were visible in the mass spectrometer and was judged too impure for sequencing. This peak appeared slightly behind peak 7 during reverse phase HPLC (Fig. 4) and contained a single phosphopeptide with a mass 173 atomic mass units less than the phosphopeptide present in peak 7 (Fig. 5). No other predicted tryptic phosphopeptide from the fusion protein has a molecular mass near 2382. Hence, we conclude that peak 8 contains a phosphopeptide with the same sequence as that in peak 7 with its N-terminal arginine removed (Table I). Thus, phosphopeptides 5–8 represent the same phosphorylation site, serine 383 of the fusion protein, corresponding to serine 1303 of the full-length NR2B subunit (6, 45).

Serine 383 Is the Principal Site Phosphorylated Rapidly on GST-tNR2B by CaM Kinase II—To study the rates of phosphorylation of GST-tNR2B at the sites observed after tryptic digestion (Fig. 4), phosphorylation was carried out for various times up to 10 min, and the amount of phosphate incorporated into each tryptic phosphopeptide was determined after HPLC. The amounts of phosphate incorporated into peaks 5–8 were added together, and the sum was plotted and compared with the incorporation of phosphate into other principal peaks (3 and 4) containing unidentified phosphopeptides (Fig. 6). The
most rapid phosphorylation occurs on serine 383, whereas incor-
poration of phosphate into peaks 3 and 4 is considerably
slower and reaches only about 10% of that into serine 383 after 10
min. (Recall that exhaustive phosphorylation of GST-tNR2B
prior to sequencing of the phosphopeptides (Fig. 4) was carried
out for 30 min.) Hence, serine 383 appears to be the preferred
site on GST-tNR2B for phosphorylation by CaM kinase II.

Serine 1303 in the NR2B Subunit Is Phosphorylated by Endoge-

ous CaM Kinase II in the Postsynaptic Density Fra-

c tion—To see whether Ser-1303 can be phosphorylated by CaM

kinase II in the full-length NR2B subunit, the rat brain

postsynaptic density fraction was incubated in the CaM kinase II

assay mixture described under “Experimental Procedures” to

allow endogenous kinase to catalyze phosphorylation of sub-

strate proteins. The NR2B subunit was then immunoprecipi-

tated from the mixture and subjected to SDS-PAGE (see

Fig. 1). The protein band was excised from the gel, and the

labeled NR2B subunit was subjected to tryptic peptide map-

ping. The major peaks of radioactivity correspond in mobility
to peaks 7 and 8 from phosphorylated GST-tNR2B (Fig. 7,
top and middle), and a smaller peak corresponding to peak 5 was

also present. The identities of the peaks were confirmed by

column chromatography of mixtures of tryptic peptides generated from

phosphorylated NR2B and those generated from phosphoryl-

ated GST-tNR2B (Fig. 7, bottom). Thus, serine 1303 is a major
target for phosphorylation by CaM kinase II in the full-length

NR2B subunit. The presence of additional minor peaks in the

NR2B digest (Fig. 7, top) indicates that a site (or sites) not

present in GST-tNR2B may also be phosphorylated by CaM

kinase II.

Serine 1303 in the NR2B Subunit Is Phosphorylated by CaM

Kinase II in the Hippocampus—A rabbit antisemum specific for

the amino acid sequence surrounding phosphorylated serine

1303 was raised by immunizing rabbits with a phosphopeptide
(Fig. 8A). When preabsorbed with the cognate nonphosphopep-
tide, this antisemum specifically recognizes a band at the posi-
tion of NR2B in immunoblots of rat hippocampus homogenates

subjected to phosphorylation conditions in vitro (Fig. 8B, first two lanes).

We have previously shown that in acutely prepared slices of rat

hippocampus, the basal level of active, autophosphorylated

most rapid phosphorylation occurs on serine 383, whereas incor-
poration of phosphate into peaks 3 and 4 is considerably
slower and reaches only about 10% of that into serine 383 after 10
min. (Recall that exhaustive phosphorylation of GST-tNR2B
prior to sequencing of the phosphopeptides (Fig. 4) was carried
out for 30 min.) Hence, serine 383 appears to be the preferred
site on GST-tNR2B for phosphorylation by CaM kinase II.

Serine 1303 in the NR2B Subunit Is Phosphorylated by Endoge-

ous CaM Kinase II in the Postsynaptic Density Fra-

c tion—To see whether Ser-1303 can be phosphorylated by CaM

kinase II in the full-length NR2B subunit, the rat brain

postsynaptic density fraction was incubated in the CaM kinase II

assay mixture described under “Experimental Procedures” to

allow endogenous kinase to catalyze phosphorylation of sub-

strate proteins. The NR2B subunit was then immunoprecipi-

tated from the mixture and fractionated by SDS-PAGE (see

Fig. 1). The protein band was excised from the gel, and the

labeled NR2B subunit was subjected to tryptic peptide map-

ning. The major peaks of radioactivity correspond in mobility
to peaks 7 and 8 from phosphorylated GST-tNR2B (Fig. 7,
top and middle), and a smaller peak corresponding to peak 5 was

also present. The identities of the peaks were confirmed by

column chromatography of mixtures of tryptic peptides generated from

phosphorylated NR2B and those generated from phosphoryl-

ated GST-tNR2B (Fig. 7, bottom). Thus, serine 1303 is a major
target for phosphorylation by CaM kinase II in the full-length

NR2B subunit. The presence of additional minor peaks in the

NR2B digest (Fig. 7, top) indicates that a site (or sites) not

present in GST-tNR2B may also be phosphorylated by CaM

kinase II.

Serine 1303 in the NR2B Subunit Is Phosphorylated by CaM

Kinase II in the Hippocampus—A rabbit antisemum specific for

the amino acid sequence surrounding phosphorylated serine

1303 was raised by immunizing rabbits with a phosphopeptide
(Fig. 8A). When preabsorbed with the cognate nonphosphopep-
tide, this antisemum specifically recognizes a band at the posi-
tion of NR2B in immunoblots of rat hippocampus homogenates

subjected to phosphorylation conditions in vitro (Fig. 8B, first two lanes).

We have previously shown that in acutely prepared slices of rat

hippocampus, the basal level of active, autophosphorylated

masses in atomic mass units for each phosphopeptide and its parent dephosphoryl form, measured after external calibration, are indicated above the peaks. The x axis (m/z) represents the ratio of mass to charge, and the y axis represents arbitrary units related to the size of the signal.

Fig. 5. Mass spectra of the purified tryptic peptides. Peptides in the indicated peaks (numbered as in Fig. 4) were subjected to MALDI-TOF mass spectrometry in linear and reflector modes as described under “Experimental Procedures.” The masses in atomic mass units for each phosphopeptide and its parent dephosphoryl form, measured after external calibration, are indicated above the peaks. The x axis (m/z) represents the ratio of mass to charge, and the y axis represents arbitrary units related to the size of the signal.

TABLE I

Sequences of purified peptides from peaks 5–8

| Peak | Sequence |
|------|----------|
| 5    | RQH$^*$YDTFVDLQK |
| 6    | QH$^*$YDTFVDLQK |
| 7    | RQH$^*$YDTFVDLQKEEAALAPR |
| 8    | QH$^*$YDTFVDLQKEEAALAPR |

Fig. 6. Rates of phosphorylation by CaM kinase II of different sites in GST-tNR2B. GST-tNR2B (25 μg) was phosphorylated in the presence of CaM kinase II and labeled ATP for the indicated times, after which the phosphorylated GST-tNR2B was trypsinized as described under “Experimental Procedures” and the tryptic peptides were fractionated by HPLC. The sum of the radioactivity in peaks 5–8 (equal to incorporation of phosphate into serine 383), and the radioactivity in individual peaks 3 and 4, were plotted at each time point. ■, sum of radioactivity in peaks 5–8 (ser-383); ●, radioactivity in peak 3; ▲, radioactivity in peak 4.
CaM kinase II can be manipulated by incubating the slices in a ringer buffer containing EGTA, which lowers the level of active kinase from an average of 9% (40) to about 4%, or in okadaic acid, which raises the level of active kinase to about 27% (34). To determine if serine 1303 is phosphorylated in intact hippocampal neurons under any of these conditions, we prepared homogenates of slices incubated in normal Ringer buffer and in Ringer buffer containing EGTA or okadaic acid, as described under "Experimental Procedures." We were able to clearly detect the presence of the phosphorylated site in a protein band at the position of NR2B in homogenates of slices incubated under each condition (Fig. 8B). The band detected by the antiserum was broader than that seen using monoclonal antibodies (see Fig. 2). The presence of the phosphorylated site in a protein band was excised from the gel and hydrolyzed as described under "Experimental Procedures." The acid hydrolsate was fractionated by electrophoresis on a cellulose thin layer plate, and radioactive amino acids were visualized in a PhosphorImager. A single radioactive spot was detected that co-migrated with the phosphoserine standard (Fig. 9), indicating that the major sites of phosphorylation of NR2B in vivo under these conditions are serine residues.

In a separate experiment, the labeled NR2B was trypsinized as described under "Experimental Procedures." Because their specific activity was too low for detection in fractions from HPLC, the peptides were fractionated by two-dimensional thin layer chromatography (Fig. 10A). On separate plates, the digest was mixed with phosphopeptidases 5, 7, and 8 purified by HPLC after trypsinization of radiolabeled GST-tNR2B (Fig. 10C; see also Fig. 4). We found that NR2B was phosphorylated in vivo in the cultures and that its trypsinization gave rise to two phosphopeptides with mobilities similar to peptides 7 and 8 (Fig. 10, A–C). The two sets of peptides co-migrate when mixed before application to the plate, confirming their identities (Fig. 10C). This evidence indicates that NR2B itself can be phosphorylated on serine 1303 in vivo. In these experiments, as in those shown in Fig. 7, the presence of additional phosphopeptides in the digests of intact NR2B suggests that it may also be phosphorylated on sites not present in GST-tNR2B. The additional peptides labeled in vivo could also reflect phosphorylation anywhere in NR2B by kinases other than CaM kinase II.

**DISCUSSION**

We have found that phosphorylation of the NR2B subunit of the NMDA receptor is stimulated in the PSD fraction from rat...
brain by the addition of Ca\(^{2+}\). The Ca\(^{2+}\)-stimulated phospho-
ylation is inhibited by antibodies that specifically inhibit the
activity of CaM kinase II (Fig. 1; Ref. 40). To facilitate identi-
fi cation of the site(s) of phosphorylation on NR2B by CaM
kinase II we have used a recombinant fusion protein containing
the C-terminal 334 amino acid residues of the NR2B subunit
fused to the C terminus of glutathione S-transferase. The fu-
sion protein (GST-tNR2B) was purified in 9-mg quantities and
served as a substrate in vitro for purified CaM kinase II (Fig.
2). GST-tNR2B could be phosphorylated in 10 min to a stoi-
chiometry of 1.4 mol/mol of protein (Fig. 3A). All of the phospho-
ylation occurred on the NR2B portion of the fusion protein.
The \(K_m\) for phosphorylation of GST-tNR2B was approximately
50 nM (Fig. 3B), indicating that the affinity of the kinase for the
site on GST-tNR2B is considerably higher than for other
known protein substrates. For example, the \(K_m\) for phospho-
ylation of synapsin I by CaM kinase II is 0.4 \(\mu\)M (46); for a
peptide containing the amino-terminal 10 residues of glycogen
synthase it is 7.5 \(\mu\)M, and for a peptide containing the amino-
terminal 23 residues of smooth muscle myosin light chain it is
4 \(\mu\)M (43). The high affinity of the kinase for GST-tNR2B is
corroborated by the observation that the fusion protein inhibits
completely the phosphorylation of synapsin I when the two are
present at the same concentration in the assay (data not shown).

Four of the seven major tryptic phosphopeptides (numbered
5–8 in Fig. 4) generated after exhaustive phosphorylation of
GST-tNR2B were identified by mass spectrometry and gas
phase sequencing as originating from serine 383 of the NR2B
portion of GST-tNR2B (Fig. 5; Table I). This serine correspon-
des to serine 1303 in the full-length NR2B subunit. Analysis of the
time course of appearance of the tryptic phosphopeptide peaks
indicated that phosphorylation of this serine is rapid, whereas
the other major, unidentified phosphopeptides (3 and 4) ap-
ppeared more slowly and hence probably represent a lower af-
finity phosphorylation site or sites (Fig. 6).

To demonstrate that serine 1303 is phosphorylated in full-
length NR2B, we compared peptide maps of phosphorylated
GST-tNR2B with those from NR2B phosphorylated in the post-
synaptic density fraction by endogenous CaM kinase II and
purified by immunoprecipitation. NR2B labeled in this way
contained two major phosphopeptide peaks that co-migrated
during HPLC with peaks 7 and 8 from GST-tNR2B, demon-
strating that the major site of phosphorylation of NR2B by
CaM kinase II in the PSD fraction is serine 1303 (Fig. 7).
Finally, we used two strategies to examine whether serine 1303 is phosphorylated in living neurons. A phosphosite-specific antisem was raised against a phosphorylated synthetic peptide with the amino acid sequence surrounding serine 1303. We used this antisem to detect the presence of the phosphorylated site in a protein band from brain homogenates with the mobility of NR2B and NR2A (Fig. 8). Homogenates of hippocampal slices that had been incubated in a calcium-free buffer showed less antibody binding to this band compared with homogenates of slices incubated in the phosphatase inhibitor okadaic acid. These two conditions decrease and increase the basal level of activated CaM kinase II, respectively (35). Therefore, the results are consistent with the hypothesis that CaM kinase II phosphorylates serine 1303 and/or the cognate site in NR2A in vivo. Proof of the hypothesis and clarification of the physiological conditions that regulate phosphorylation will require additional experiments. We also prepared two-dimensional TLC peptide maps of immunoprecipitated NR2B labeled with $^{32}$P in situ in organotypic cultures. These maps were compared with those of HPLC-purified phosphopeptide standards from labeled GST-NR2B. NR2B labeled in the living neurons contained spots corresponding to peptides 7 and 8 from GST-NR2B (Fig. 10). Taken together, these experiments make a strong case that serine 1303 is a principal site of phosphorylation of NR2B in vivo.

Serine 1303 on NR2B is the first identified site of phosphorylation of the NMDA type glutamate receptor by CaM kinase II. The demonstration that this site is phosphorylated in living neurons is consistent with the hypothesis and clarifies the functional role of phosphorylation of the NMDA receptor by CaM kinase II. The demonstration that this site is phosphorylated in living neurons is consistent with the hypothesis and clarifies the functional role of phosphorylation of the NMDA receptor by CaM kinase II.

Acknowledgments—We thank Dr. Gary Hathaway and Dirk Kräpf of the Caltech Protein/Peptide Micro Analytical Laboratory, and Leslie Shenker and Frank Asuncion for valuable assistance with this work.

REFERENCES

1. Squire, L. R., and Zola-Morgan, S. (1991) Science 253, 1380–1386
2. Morris, R. G. M., Anderson, E., Lynch, G. S., and Baudry, M. (1986) Nature 319, 774–776
3. Dudok, S. M., and Bear, M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4363–4367
4. Nakahishi, S. (1992) Science 258, 597–603
5. Seeburg, P. H. (1993) Trends Neurosci. 16, 359–365
6. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lameli, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1992) Science 256, 1217–1221
7. Moon, J. S., Apperson, M. L., and Kennedy, M. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3954–3958
8. Kornau, H.-C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) Science 269, 1737–1740
9. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., and Prochiantz, A. (1984) Nature 307, 462–465
10. Mayer, M. L., Westbrook, G. L., and Guthrie, P. B. (1984) Nature 309, 261–263
11. Mayer, M. L., MacDermott, A. B., Westbrook, G. L., Smith, S. J., and Barker, J. I. (1987) J. Neurosci. 7, 3220–3244
12. Lynch, G., Larson, J., Keho, S., Barrionuevo, G., and Schottler, F. (1983) Science 205, 719–721
13. Malek, R. C., Zucker, R. S., and Nicoll, R. A. (1988) Science 242, 81–84
14. Kennedy, M. B., Bennett, M. K., and Ermond, N. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 80, 7357–7361
15. Kennedy, M. B., Bennett, M. K., Bulleit, R. F., Ermond, N. E., Jennings, V. R., Miller, S. M., Molloy, S. S., Patton, B. L., and Schenker, L. J. (1990) Cold Spring Harbor Symp. Quant. Biol. 55, 101–110
16. Kelly, P. T., McGuinness, T. L., and Greengard, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 945–949
17. Goldring, J. R., McGuire, J. S., and De Lorenzo, R. J. (1984) J. Neurochem. 42, 1077–1084
18. Silva, A. J., Stevens, C. F., Tonegawa, S., and Wang, Y. (1992) Science 257, 201–206
19. Stevens, C. F., Tonegawa, S., and Wang, Y. (1994) Current Biol. 4, 687–693
20. Pettit, D. L., Perlman, S., and Malinow, R. (1994) Science 266, 1881–1885
21. McLaughlin, E., Yamamoto, H., Tan, S. E., Brickley, D. A., and Soderling, D. R. (1992) Science 306, 640–642
22. Hoffmann, M., Maron, C., and Heineman, S. (1994) NeuroReport 13, 1331–1343
23. Liederman, D. N., and Mody, I. (1994) Nature 369, 235–239
24. Wang, Y. T., and Salter, M. W. (1994) Nature 369, 233–235
25. Wang, L.-Y., Orser, B. A., Brautigam, D. L., and MacDonald, J. F. (1994) Nature 369, 230–232
26. Ramamoorthy, M., Teng, G., and Jahn, C. E. (1996) Neurotoxicology 16, 415–421
27. Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) Nature 364, 70–73
28. Watterstrom, M. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501–4513
29. Miller, S. G., and Kennedy, M. B. (1985) J. Biol. Chem. 260, 9039–9046
30. Kohler, G., and Milstein, C. (1976) Eur. J. Immunol. 6, 511–519
31. Cho, K.-O., Hunt, C. A., and Kennedy, M. B. (1992) Neuron 9, 929–942
32. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
33. Grieco, F., Hay, J. B., and Hull, R. (1992) BioTechniques 13, 856–857
34. Miller, S. G., Patton, B. L., and Kennedy, M. B. (1986) Neuron 1, 1593–1604
35. Kindler, S., and Kennedy, M. B. (1996) J. Neurosci. Methods, 68, 61–70
36. Patton, B. L., Molloy, S. S., and Kennedy, M. B. (1993) Mol. Cell. Biol. 4, 159–172
37. Boyle, W., Vandersra, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
38. Laemmli, U. K. (1970) Nature 227, 680–685
39. Peterson, G. L. (1983) Methods Enzymol. 91, 95–119
40. Molloy, S. S., and Kennedy, M. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4756–4760
41. Talbot, G., and Mann, M. (1994) in Techniques in Protein Chemistry (Crabb, J., ed) Vol. V, pp. 159–187, Academic Press, Inc, San Diego
42. Parten, B., McDowell, J., Nawrocki, J., and Hargrave, P. (1994) in Techniques in Protein Chemistry (Crabb, J., ed) Vol. V, pp. 159–187, Academic Press, Inc, San Diego
43. Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985) J. Biol. Chem. 260, 14471–14476
44. Podell, D. N., and Abraham, G. N. (1978) Biochem. Biophys. Res. Commun. 81, 176–185
45. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., and Nakanishi, S. (1993) J. Biol. Chem. 268, 2836–2843
46. Kennedy, M., McGuinness, T., and Greengard, P. (1983) J. Neurosci. 3, 818–831
47. Dudek, S. M., and Bear, M. F. (1993) J. Neurosci. 13, 2910–2918
48. Masu, L., Waz, J., Kandel, E. R., and O'Dell, T. J. (1995) Cell 81, 891–904
49. Carlén R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1988) J. Cell Biol. 106, 831–843