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

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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 Ca^{2+}/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[1] 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 Seeburg lab (8) has demonstrated a direct association between NR2B and the postsynaptic density protein PSD-95.

NMDA receptors are ligand and voltage-gated Ca^{2+} channels (9, 10). Binding of glutamate released from the presynaptic terminal, coupled with strong depolarization of the postsynaptic membrane produces an influx of Ca^{2+} into the postsynaptic compartment (11). This Ca^{2+} 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 Ca^{2+} 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 α-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 trifluoroacetic acid from Pierce; dithiothreitol (DTT) from Boehringer

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; GST-tNR2B, glutathione *S*-transferase fused to 334 amino acids from the C terminus of the NR2B subunit of the NMDA receptor; PSD; postsynaptic density; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization, time-of-flight; RIPA buffer, radioimmune precipitation buffer.

Mannheim; iodoacetamide from Sigma; [γ-^{32}P]ATP from ICN Pharmaceuticals Inc.; modified sequencing grade trypsin from Promega; sequencing grade trypsin from Boehringer Mannheim; C18 reverse phase HPLC columns (4.6 × 250 mm) from Vydac; cellulose-coated TLC sheets (20 × 20 mm) from EM Science; glutathione-agarose from Sigma; protein A-agarose from Pierce; phosphate-free minimal essential medium from Life Technologies, Inc.; and PhosphorImager screens and scanner from Molecular Dynamics. Calmodulin was purified by the method of Watterson *et al.* (28). CaM kinase II was purified from rat forebrain (29). Rabbit and mouse antiserum against NR2B was raised by immunization with GST-tNR2B as described previously (7). The rabbit antiserum against NR2B does not cross-react with the NR2A subunit (data not shown). Inhibitory monoclonal antibodies against CaM kinase II were raised and selected by the method of Ref. 30 after immunization of mice with purified CaM kinase II.

Preparation of Postsynaptic Density Fraction from Rat Brain—The crude PSD fraction was prepared as described previously (31) by a modification of the method of Carlin *et al.* (49).

Expression and Purification of the Fusion Protein GST-tNR2B from Escherichia coli—A vector for expression of a fusion protein containing the C-terminal 334 residues of NR2B fused to the C terminus of glutathione *S*-transferase (GST-tNR2B) was constructed in the pGEX3X plasmid (Pharmacia Biotech Inc.; Ref. 32) as described previously (7). A single colony of *E. coli* cells transformed with the plasmid was selected on an LB plate containing ampicillin and was grown in cultures containing 50 μg/ml ampicillin at 30 °C overnight. When 2-liter cultures reached mid-log phase (A_{600} of 0.5 units), the cells were induced by adding 0.1 mM isopropyl-thiogalactoside and grown to late log phase at 30 °C. Cells were harvested by centrifugation, and the cell pellets were frozen at −80 °C. The cell pellet from a 2-liter culture was resuspended in 20 ml of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3. (phosphate-buffered saline) containing 0.02 mg/ml aprotinin, 0.01 mg/ml leupeptin, 5 μg/ml antipain, 0.4 μg/ml pepstatin, 0.1 mM PMSF, 0.5 mM DTT, 1 mM EDTA, and 1 mM EGTA. Cells were lysed by sonication. PMSF was added again to a concentration of 0.1 mM, and Triton X-100 was added to a concentration of 1%. The cell lysate was subjected to centrifugation at 15,000 × *g* for 10 min. The supernatant was saved, and the pellet was resuspended in 10 ml of phosphate-buffered saline containing 1% *n*-lauroyl sarcosine (33) and 0.1 mM PMSF. The pellet was sonicated, and Triton X-100 and PMSF were added to concentrations of 1% and 0.1 mM, respectively. The suspension was subjected to centrifugation at 15,000 × *g*. This and previous supernatants were pooled and incubated at room temperature for 10 min with 2 ml of glutathione-agarose beads previously washed twice with phosphate-buffered saline. The supernatant solution was removed from the beads, and the beads were washed twice with phosphate-buffered saline. The beads were then mixed with elution buffer containing 50 mM Tris, pH 8.0, 20 mM reduced glutathione, 1% Triton X-100, and 0.1 mM PMSF overnight at 4 °C on an end-over-end mixer. The eluate was removed from the beads and stored at −80 °C. Additional fusion protein could be recovered from the first bead supernatant by a second round of affinity purification.

Phosphorylation of GST-tNR2B by CaM Kinase II—Phosphorylation of the fusion protein was conducted as described previously (34). The reaction (final volume 100 μl) contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.4 mM EGTA, 30 μM [γ-^{32}P]ATP (1500–3000 cpm/pmol), 10 μg/ml calmodulin, 10 mM DTT, GST-tNR2B, and 1.2 μg/ml rat brain CaM kinase II. Phosphorylation was initiated by the addition of 10 μl of kinase solution in Tris buffer, pH 8.0, containing 0.5–1.0 mg/ml BSA, to the reaction mixture, which was prewarmed to 30 °C for 1 min. The reaction was carried out for 1 min or as indicated and was stopped by the addition of either 50 μl of 3 × SDS sample buffer or 4 μl of 500 mM EDTA. When the reaction was stopped by the addition of SDS, the mixture was placed in a boiling water bath for 5 min before SDS-PAGE.

Trypsinization of Phosphorylated GST-tNR2B—GST-tNR2B was phosphorylated as described above with the following modifications. The reaction was conducted in a volume of 1 ml containing 16 or 54 μg/ml GST-tNR2B. The reaction mix was preincubated for 5 min, and the reaction was initiated by the addition of 1.2 μg of CaM kinase II. After 15 min, another 1.5 μg of CaM kinase II (in 0.5 μl) was added, and the reaction continued for 15 min at 30 °C. The reaction was stopped by the addition of EDTA, pH 8.0, to a final concentration of 19 mM. Several reactions were pooled into 6–8-ml batches and placed on ice. Protein was precipitated by the addition of trichloroacetic acid to a final concentration of 16%. After 1 h on ice, protein was pelleted by centrifugation for 15 min at 12,000 × *g*. The supernatants were discarded, and the pellets were washed twice with acetone to remove traces of trichloroacetic acid. The pellets were then dried in air and redissolved in 100–250 μl of carboxymethylation buffer containing 200 mM Tris-HCl, pH 8.0, 8 M urea, 2 mM EDTA, and 2 mM DTT. The tubes were flushed with nitrogen to remove air and incubated at 50 °C for 30 min to reduce cysteine residues. To carboxymethylate the cysteine residues, iodoacetamide, pH 8.0, was added to a final concentration of 4.5 mM, and the tubes were then flushed with nitrogen and incubated at 50 °C for 1 h, protected from light. 2-Mercaptoethanol was added to a final concentration of 1% (v/v), and the tubes were incubated at 50 °C for 10 min to quench excess iodoacetamide. The reaction mixtures were then diluted to a final concentration of 2 M urea, and protein was precipitated by the addition of a final concentration of 20% trichloroacetic acid. After 15 min on ice, protein was pelleted by centrifugation at 12,000 × *g* for 10 min. Pellets were washed twice with acetone to remove traces of trichloroacetic acid, air-dried for 10 min, redissolved in 1 × SDS-sample buffer, boiled for 5 min, and loaded onto an 8% SDS-polyacrylamide gel (1.5 mm thick). The GST-tNR2B bands were visualized by staining with Coomassie Blue. The bands were cut out of the gel and washed twice in 10 ml each of 50% methanol and 10% acetic acid for 3 h, 10 ml of 50% methanol overnight, 10 ml of 20% isopropyl alcohol for 1 h, and again in 10 ml of 50% methanol for 30 min. The bands were then lyophilized to dryness. Each band, containing about 50 μg of GST-tNR2B, was reswollen in 0.9 ml of 0.1 M NH_4HCO_3 and was homogenized using a mini homogenizer and pestle (Kontes Scientific Glassware/Instruments). Modified sequencing grade trypsin (25–30 μg in 1 mM DTT) was added to each gel suspension, the final volume was brought to 1 ml, and the tubes were incubated in a 37 °C shaker water bath for 24 h. After trypsinization, each gel suspension was pelleted by brief centrifugation, 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 NH_4HCO_3, 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 NH_4HCO_3, 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—Lyophilized 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. Absorbance at 214 nm was monitored, and 0.5-ml fractions were collected. Radioactivity in each fraction was measured in a Beckman LS 7800 scintillation counter by detection of Cerenkov radiation (34).

Mass Spectrometry and Sequencing of Phosphopeptides—Mass spectrometry was conducted by the Protein/Peptide Micro Analytical Laboratory 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 peptides were mixed with an α-cyano-4-hydroxycinnamic acid matrix solution, dried, and placed in the mass spectrometer. Data were collected in both linear and reflector modes.

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

Immunoprecipitation of NR2B from the Postsynaptic Density Fraction—The postsynaptic density fraction was phosphorylated by endogenous 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-^{32}P]ATP (4500 cpm/pmol) for 2 min. For generation of tryptic peptides, the reaction was carried out with [γ-^{32}P]ATP (60,000 cpm/pmol) 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 mM Tris, pH 7.4, 1 mM 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). The final SDS concentration was 0.1%. The antibody solutions were mixed in the cold room for 2–12 h, cleared by centrifugation at 12,000 × *g* for 15 min, mixed with protein A-agarose beads previously washed with 1 × RIPA buffer, and stirred again for 2 h in the cold room. The protein A beads were then pelleted and washed three times with 2 volumes of 1 × RIPA buffer. SDS sample buffer (50–100 μl/200 μl of protein A beads) was added to the beads. The mixture was

boiled for 5 min and subjected to SDS-PAGE on a 6% gel. The NR2B bands were visualized by either Coomassie staining or autoradiography. The NR2B bands were excised and were subjected to tryptic peptide mapping as for GST-tNR2B except that the carboxymethylation was omitted.

Detection of the Phosphorylated Site on the NR2 Subunits with a Phosphosite-specific Antibody—A synthetic peptide with the sequence LRRQHSYDTFVC was obtained from the Peptide Sequencing Facility at Caltech. Ten mg was phosphorylated for 2 h at 30 °C in 44 mM Tris-HCl, pH 8, 9 mM $MgCl_2$, 0.35 mM EGTA, 0.6 mM $CaCl_2$, 0.26 mg/ml calmodulin, 2.2 mM DTT, 13.2 mM [γ-^{35}S]thiophospho-ATP (1000 cpm/nmol), 0.53 mg/ml CaM kinase II 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*-maleimidomethyl)cyclohexane-1-carboxylate to keyhole limpet hemocyanin. Rabbit antisera were raised as described previously (7) by repeated injection of 0.33 mg of conjugated peptide in RIBI adjuvant (RIBI Immunochemical Research Inc., Hamilton, MT) at several sites. Serum was collected and tested on immunoblots as described below against 0.2 μg of phosphorylated and 0.2 μg of nonphosphorylated GST-tNR2B. The serum of one rabbit showed good specificity for phospho-GST-tNR2B. To neutralize antibodies against nonphosphorylated GST-tNR2B, the serum was preabsorbed with 6 mol eq of the nonphosphorylated antigen peptide at 4 °C overnight before dilution for use on immunoblots.

Nonphosphorylated and phosphorylated rat brain homogenates were prepared as follows. A rat brain was homogenized in 14 ml of homogenization buffer containing 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 DTT, 0.1 mM PMSF with eight strokes in a Teflon/glass homogenizer rotated at 900 rpm. The homogenate was cleared by centrifugation at 2000 × *g* for 10 min. Two ml of homogenate was incubated for 1 h at 30 °C to permit dephosphorylation of proteins by endogenous phosphatases. One ml was then removed and brought to 3% SDS, 5% glycerol, 2% β-mercaptoethanol, and 62 mM Tris-HCl, pH 6.7 (SDS-PAGE sample buffer). The other ml of dephosphorylated homogenate was added to an equal volume of prewarmed phosphorylation mixture containing (final volume) 50 mM Tris, pH 7.5, 0.7 mM CaCl, 0.3 mM EGTA, 10 mM MgCl, 5 mg/ml calmodulin, 10 mM ATP, 10 mM DTT, 0.06 mg/ml CaM kinase II, and 1 mM okadaic acid to inhibit protein phosphatases. The mixture was incubated for 5 min at 30 °C and then brought to 3% SDS, 5% glycerol, 2% β-mercaptoethanol, and 62 mM Tris-HCl, pH 6.7. Aliquots of both the nonphosphorylated and the phosphorylated homogenates were boiled for 2 min and stored at −80 °C for later application to SDS-PAGE gels.

Homogenates of hippocampal slices were prepared as described previously (35). Briefly, hippocampi were dissected from rat brains and placed in ice-cold, oxygenated Ringer buffer (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11 mM glucose). Slices (500 μm) were cut with a tissue chopper (Stolting, Wood Dale, Il), placed on filter paper on top of a Petri dish containing oxygenated, ice-cold Ringer buffer, and incubated at room temperature in a chamber flushed continuously with 95% O_2, 5% CO_2. After 1 h, groups of 12 slices were immersed in oxygenated Ringer buffer or in the same buffer containing either 5 μM okadaic acid or 0 $CaCl_2$/0.25 mM EGTA, for an additional hour at room temperature in a 95% O_2, 5% CO_2 atmosphere. The slices were then immediately transferred to a Teflon/glass homogenizer containing 600 μl of SDS-PAGE sample buffer plus 2 units of DNase and homogenized. Each homogenate was boiled for 5 min and frozen for later application to SDS-PAGE gels.

For detection of the phosphorylated site in homogenates, 25–40 μg of homogenate protein/lane was fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and blocked for 5 h in 5% dry milk (Carnation) diluted in TTBS (0.05% Tween-20, 20 mM Tris-Cl, pH 7.5, 0.5 M NaCl). After one wash in TTBS, blots were incubated overnight in anti-phosphosite-specific rabbit serum preabsorbed with nonphosphoantigen as described above and then diluted 1:2000 in TTBS plus 1% normal goat serum. Blots were washed three times in TTBS, then incubated for 1 h in alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (Jackson Immunochemicals) diluted in TTBS plus 1% normal goat serum. After three 10-min washes with TTBS, blots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reagent (Bio-Rad) according to the supplier's instructions.

Immunoprecipitation of NR2B from Hippocampal Slices—Organotypic hippocampal cultures were prepared and maintained for 4–6 weeks as described previously (36). Ten wells containing 4 or 5 cultures/well were washed with minimal essential medium containing 100 μM Na_2HPO_4, prewarmed to 37 °C. Then 1 ml of the same medium containing 0.8 mCi of carrier free ^{32}P-phosphate was added to each well. The plates were transferred to a 37 °C CO_2 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 NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 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 3 min, cooled in ice, and brought to 1 × RIPA buffer, 0.1% SDS (final volume 2 ml). The sample was cleared by centrifugation at 16,000 × *g* for 5 min and then mixed with 100 μl of antiserum against NR2B in the cold room for 2 h. Protein A-Sepharose beads (2-ml suspension), washed once with RIPA buffer were added, and the incubation continued for 2 h in the cold room with mixing. The protein A beads were then pelleted by centrifugation and washed three times with 1 ml of RIPA buffer. SDS sample buffer (0.25–0.5 ml) was added to the washed beads and boiled for 5 min, and 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-tNR2B.

Lyophilized tryptic peptides were dissolved in 0.1% trifluoroacetic acid in 50% acetonitrile, spotted onto 20 × 20-cm cellulose-coated thin layer plates, and fractionated in two dimensions (37). Electrophoresis in the first dimension was at 600 V for 4 h, in a pH 3.5 buffer containing 5% acetic acid and 0.5% pyridine. The plates were dried and subjected to thin layer chromatography in the second dimension in a solvent containing *n*-butanol, pyridine, acetic acid, and water in the ratio 37.5:25:7.5:30. Phosphopeptides were visualized by PhosphorImager analysis (Molecular Dynamics).

Phosphoamino Acid Analysis—Organotypic hippocampal cultures were prepared and washed as described above and then incubated in minimal essential medium containing 100 μM NaH_2PO_4 and 1 mCi of [γ-^{32}P]H_3PO_4 for 16 h at 37 °C. The slices were then washed twice with Ringer buffer and homogenized in 600 μl of homogenization buffer with eight strokes at 900 rpm. Nonidet P-40 and Triton X-100 were added to the homogenate to final concentrations of 1 and 0.1%, respectively. The homogenate was mixed end-over-end for 2 h at 4 °C. The homogenate was then cleared by centrifugation at 12,000 × *g* at 4 °C for 15 min. The supernatant was transferred to a new tube, and 20 μl of a polyclonal mouse antiserum against NR2B was added (7). The solution was mixed end-over-end at 4 °C overnight and then subjected to centrifugation at 12,000 × *g* at 4 °C for 15 min. The supernatant was transferred to a tube containing 50 μl 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 × *g* for 5 min and then washed three times with RIPA buffer and resuspended in 30 μl of SDS stop solution. Ten μl of the sample was loaded onto a 6% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue and dried. Radioactive NR2B was visualized by autoradiography. Gel bands were excised from the gel and placed in 1 ml of 30% methanol for 1 h, washed twice in 50 mM NH_4HCO_3, and then incubated in the same solution containing 20 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin overnight at 37 °C. The solution was removed from the gel piece, dried under vacuum in a Speed-Vac centrifuge, resuspended in 100 μl of 6 N HCl, and incubated at 110 °C for 1 h. The hydrolyzed sample was dried in the Speed-Vac, redissolved in deionized water, and dried again. It was then resuspended in 8 μl of deionized water containing phosphoamino acid standards (2 μg each of phosphoserine, phosphothreonine, and 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 PhosphorImager 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. Protein concentration was measured by the method of Peterson (39) with bovine serum albumin as the standard. To estimate the proportion of intact GST-tNR2B fusion protein (M_r ~63,000) in our purified preparations (Fig. 2), Coomassie-stained bands containing intact GST-tNR2B were visually compared with those of known amounts of standard proteins. Because we estimated that intact GST-tNR2B was about 30% of the total protein in the purified GST-tNR2B solution, the

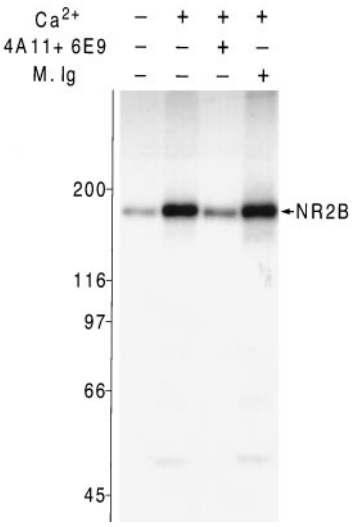

FIG. 1. **Phosphorylation of NR2B by endogenous CaM kinase II in the PSD fraction.** Aliquots of the PSD fraction (50 μg) were incubated in the assay mixture for CaM kinase II as described under "Experimental Procedures." Radiolabeled NR2B was immunoprecipitated from each tube, and the pellets were fractionated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

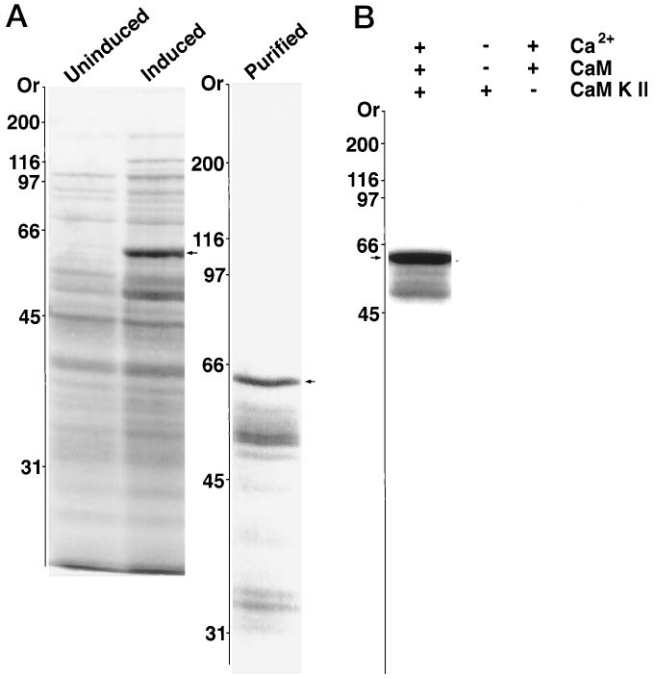

FIG. 2. **Characterization of the GST-tNR2B fusion protein.** *A*, the fusion protein GST-tNR2B was expressed from an engineered cDNA as described under "Experimental Procedures." Aliquots of bacterial lysates were fractionated on a 10% SDS gel, and proteins were visualized by staining with Coomassie Blue. *Lane 1*, uninduced bacteria containing the GST-tNR2B expression vector; *lane 2*, bacteria containing the expression vector after induction for 5 h with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside; *lane 3*, purified GST-tNR2B (3.7 μg; 6% gel). *Arrows* point to the 63-kDa fusion protein. *B*, GST-tNR2B (1 μg) was phosphorylated by CaM kinase II under three different conditions in the presence of radiolabeled ATP as described under "Experimental Procedures." Each reaction mixture was fractionated on a 10% SDS-gel. The *figure* shows an autoradiogram of the dried gel. *Lane 1*, complete incubation with Ca^{2+}, calmodulin, and CaM kinase II; *lane 2*, incubation without Ca^{2+} and calmodulin; *lane 3*, incubation without CaM kinase II.

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 Ca^{2+} 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. 2*A*). Purified GST-tNR2B was phosphorylated by CaM kinase II *in vitro* (Fig. 2*B*). As expected, the phosphorylation was dependent on the presence of Ca^{2+} 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. 3*A*). The K_m for the fusion protein, calculated from double reciprocal plots, was unusually low, 47.2 ± 27.6 nM (Fig. 3*B*), 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,

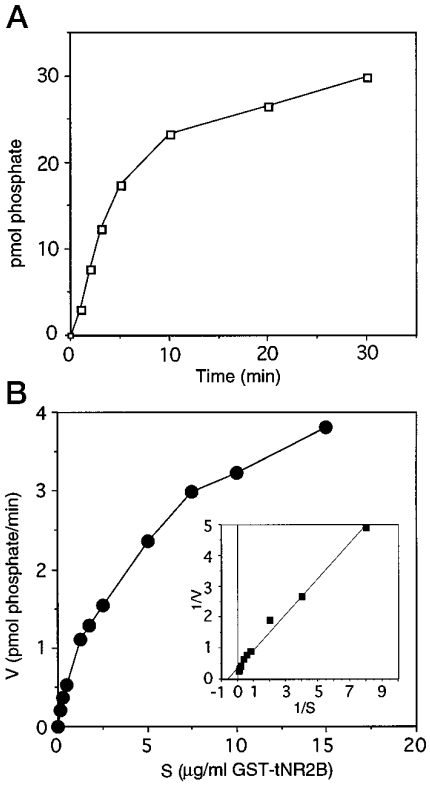

FIG. 3. **Kinetics of phosphorylation of GST-tNR2B by CaM kinase II.** *A*, GST-tNR2B (1 μg in 100 μl) was phosphorylated by CaM kinase II for the indicated times as described under "Experimental Procedures." Phosphate incorporated into the fusion protein was measured after fractionation of the mixture by SDS-PAGE. *B*, GST-tNR2B at the indicated concentrations was phosphorylated for 1 min as described under "Experimental Procedures." Phosphate incorporated into the fusion protein was measured after fractionation of the mixture by SDS-PAGE. The *inset* shows a Lineweaver-Burke double-reciprocal plot of the data from this experiment. The K_m values determined in three such experiments were 37, 27, and 79 nM. For these calculations, the concentration of full-length GST-tNR2B was determined as described under "Experimental Procedures."

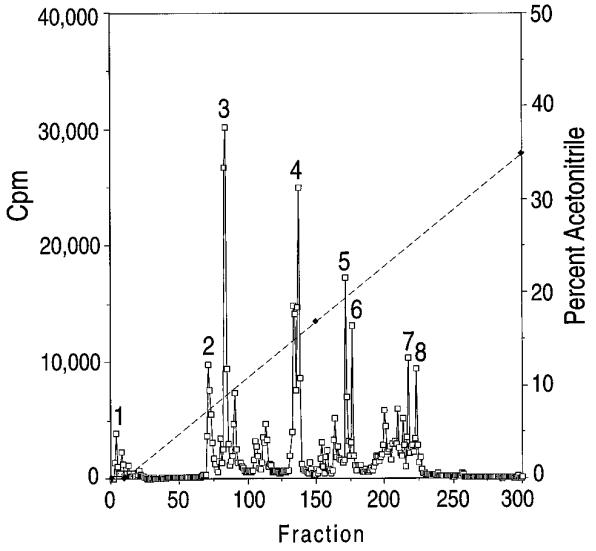

FIG. 4. **Tryptic peptides generated from exhaustively phosphorylated GST-tNR2B.** GST-tNR2B (0.4 μg) was phosphorylated for 30 min as described under "Experimental Procedures." Tryptic fragments were generated, and half of the reaction was fractionated on a C18 reverse phase HPLC column as described under "Experimental Procedures." Radioactivity in each 0.5-ml fraction was measured by detection of Cerenkov radiation. □, radioactivity; ♦, acetonitrile concentration.

and 2283, respectively. We also noted that the major peaks present 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 contain 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 phenylthiohydantoin-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 in mass to the phosphopeptide observed by mass spectrometry. We thus concluded that this phosphopeptide has a blocked N terminus. The molecular masses of the phosphopeptide (1544 atomic mass units) and the parent peptide (1446 atomic mass units) from fraction 6 (Fig. 5) are those expected for a limit tryptic peptide corresponding to peptide 5 with the N-terminal arginine (173 atomic mass units) removed. The predicted limit peptide would have an N-terminal glutamine residue, which rapidly cyclizes to form pyroglutamate and is refractory to Edman degradation (44). We have previously observed the formation of 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 peptides 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

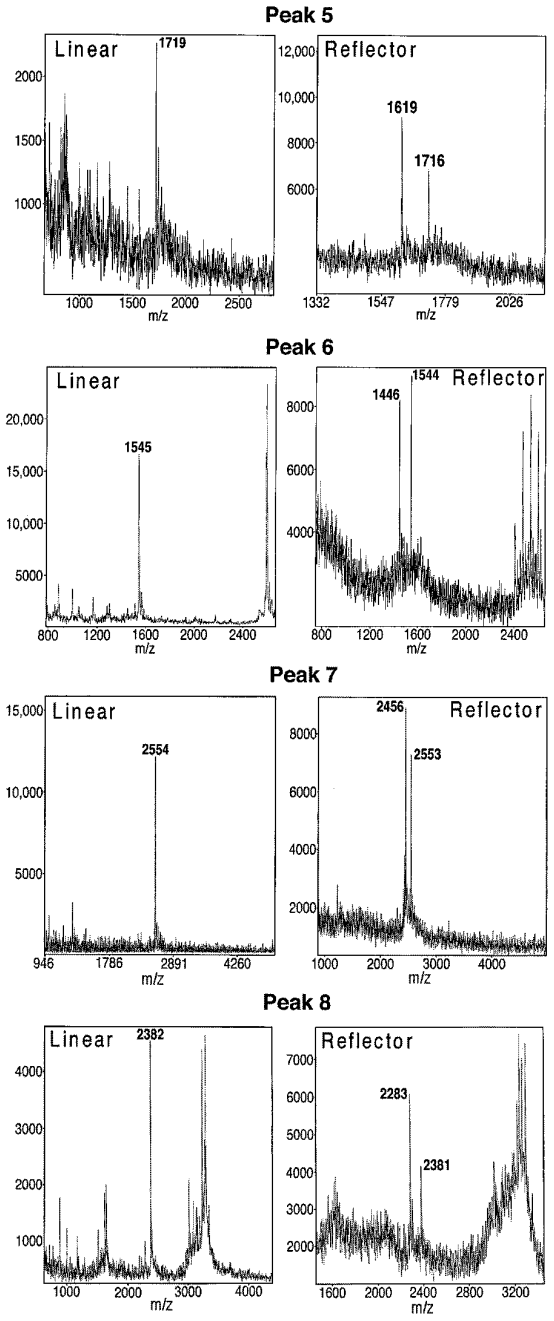

FIG. 5. **Mass spectra of the purified tryptic peptides.** Peptides in the indicated peaks (numbered as in Fig. 4) were subjected to MALDI-TOF mass spectrometry in linear and reflector modes as described under "Experimental Procedures." The masses in atomic mass units for each phosphopeptide and its 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.

most rapid phosphorylation occurs on serine 383, whereas incorporation of phosphate into peaks 3 and 4 is considerably slower and reaches only ~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 Endogenous CaM Kinase II in the Postsynaptic Density Fraction—To see whether Ser-1303 can be phosphorylated by CaM kinase II in the full-length NR2B subunit, the rat brain

TABLE I
Sequences of purified peptides from peaks 5–8

Sequences were determined by automated sequencing and/or MALDI-TOF mass spectrometry as described under "Experimental Procedures." The phosphoserine residue corresponding to Ser-383 of GST-tNR2B is marked with an asterisk. The sequences were determined from two different preparations of peaks 5–8.

Peak	Sequence
5	R Q H S* Y D T F V D L Q K
6	Q H S* Y D T F V D L Q K
7	R Q H S* Y D T F V D L Q K E E A A L A P R
8	Q H S* Y D T F V D L Q K E E A A L A P R

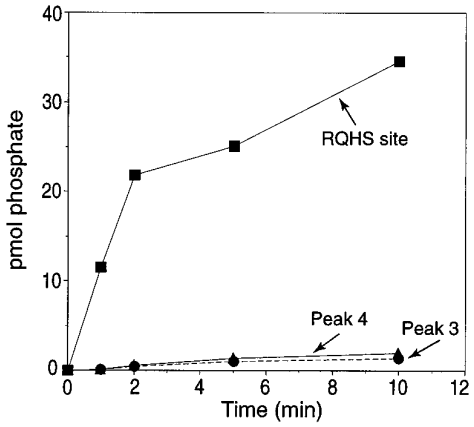

FIG. 6. **Rates of phosphorylation by CaM kinase II of different sites in GST-tNR2B.** GST-tNR2B (25 μg) was phosphorylated in the presence of CaM kinase II and labeled ATP for the indicated times, after which the phosphorylated GST-tNR2B was trypsinized as described under "Experimental Procedures" and 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.

postsynaptic density fraction was incubated in the CaM kinase II assay mixture described under "Experimental Procedures" to allow endogenous kinase to catalyze phosphorylation of substrate proteins. The NR2B subunit was then immunoprecipitated 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 mapping. The major peaks of radioactivity corresponded 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 chromatography of mixtures of tryptic peptides generated from phosphorylated NR2B and those generated from phosphorylated 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 antiserum specific for the amino acid sequence surrounding phosphorylated serine 1303 was raised by immunizing rabbits with a phosphopeptide (Fig. 8*A*). When preabsorbed with the cognate nonphosphopeptide, this antiserum specifically recognizes a band at the position of NR2B in immunoblots of brain homogenates subjected to phosphorylation conditions *in vitro* (Fig. 8*B*, *first two lanes*). We have previously shown that in acutely prepared slices of rat hippocampus, the basal level of active, autophosphorylated

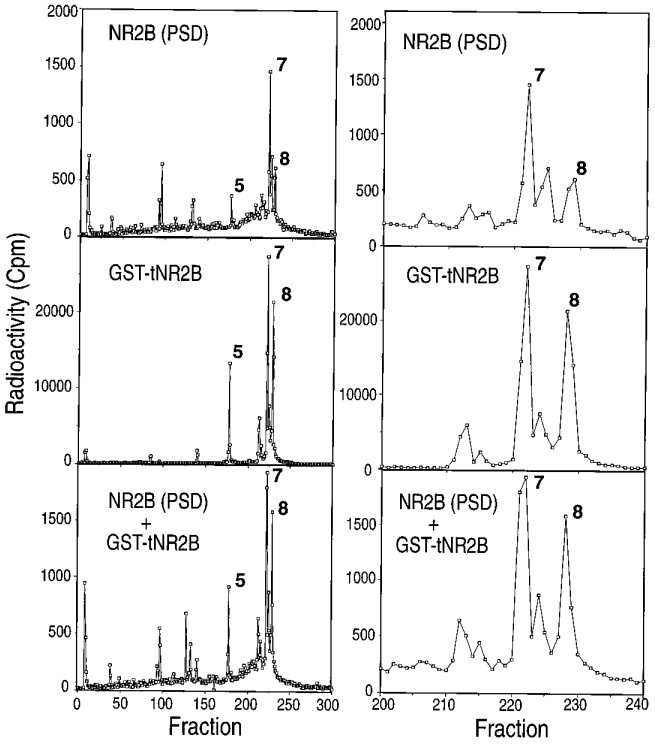

FIG. 7. **Serine 1303 in NR2B is phosphorylated by endogenous CaM kinase II in the PSD fraction.** An aliquot of the PSD fraction from forebrain (400 μg) was phosphorylated for 2 min in the presence of CaM kinase II and labeled ATP. Phosphorylated NR2B was immunoprecipitated as described under "Experimental Procedures." In a parallel reaction, GST-tNR2B (25 μg) was phosphorylated for 2 min. The immunoprecipitated NR2B and GST-tNR2B were purified by SDS-PAGE and digested with trypsin in parallel reactions. The tryptic peptides were subjected to HPLC on a C18 reverse phase column. Radiolabeled tryptic peptides are shown from phosphorylated NR2B from the equivalent of 300 μg of PSD fraction (*top*), phosphorylated GST-tNR2B (8 μg) (*middle*), and phosphorylated NR2B and GST-tNR2B mixed before application to the column (*bottom*). The *left column* shows the entire HPLC profile; the *right column* shows an expanded view of the fractions containing peptides 7 and 8.

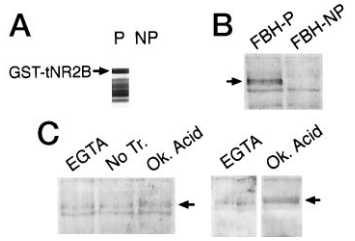

FIG. 8. **Detection of phosphorylation of serine 1303 in acute hippocampal slices with a phosphosite-specific antiserum.** *A*, a rabbit antiserum was raised against a 12-residue phosphopeptide mimicking the sequence surrounding phosphorylated serine 1303 in NR2B, as described under "Experimental Procedures." The serum was preabsorbed with a molar excess of the cognate nonphosphopeptide and was used to probe an immunoblot containing 0.2 μg of phospho- (*P*) or nonphospho- (*NP*) GST-tNR2B. The *arrow* shows the position of GST-tNR2B. The lower M_r bands are partial products formed during bacterial expression (see Fig. 2). *B*, aliquots of homogenates of rat forebrain were subjected to dephosphorylating conditions (*FBH-NP*), or to dephosphorylating conditions followed by phosphorylation by CaM KII (*FBH-P*) as described under "Experimental Procedures." Each homogenate (25 μg) was fractionated by SDS-PAGE and probed on immunoblots with a 1:2000 dilution of phosphosite-specific antiserum as described under "Experimental Procedures." *C*, three sets of 12 acute hippocampal slices were prepared and incubated in an oxygenated chamber for 1 h and then switched to oxygenated Ringer buffer containing 0 Ca^{2+}, 0.25 mM EGTA (*EGTA*), no additions (*No Tr.*), or 5 mM okadaic acid (*Ok. Acid*) (see "Experimental Procedures"). After 1 h of incubation in an oxygenated chamber, the sets of slices were homogenized immediately in SDS-PAGE sample buffer and boiled for 3 min. The first three lanes are immunoblots of 25 μg of each of the three homogenates with the phosphosite-specific antiserum diluted 1:2000. These lanes were contained in the same blot as those shown in *B*. The last two lanes are similar immunoblots of 40 μg of the EGTA and okadaic acid-treated homogenates. *Arrows*, the position of NR2B in *B* and *C*. It was determined by immunostaining an adjacent lane of homogenate with antiserum against NR2B.

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. 8*B*). The band detected by the antisera is darker in homogenates of slices incubated in okadaic acid, consistent with phosphorylation by CaM kinase II, which is more highly active in slices under that condition.

Six amino acids of the 12-residue peptide antigen used to raise the anti-phosphosite antibody are conserved in both the NR2A and NR2B subunits of the NMDA receptor (6). These two subunits have similar molecular weights and co-migrate on SDS-PAGE (data not shown). For this reason, it is possible that the phosphosite-specific antibody used for the experiments in Fig. 8 recognizes both NR2A and NR2B. We performed additional experiments in which radiolabeled NR2B was immunoprecipitated from homogenates of labeled organotypic hippocampal cultures with antibody specific for NR2B (Figs. 9 and 10). For identification of labeled phosphoamino acids, precipitated NR2B was subjected to SDS-PAGE, and the labeled protein band was excised from the gel and hydrolyzed as described under "Experimental Procedures." The acid hydrolysate 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. 10*A*). On separate plates, the digest was mixed with phosphopeptides 5, 7, and 8 purified by HPLC after trypsinization of radiolabeled GST-tNR2B (Fig. 10*C*; 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. 10*C*). 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

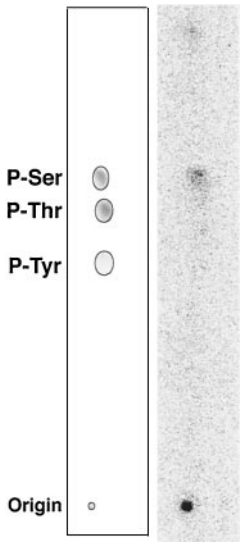

FIG. 9. **NR2B is phosphorylated primarily on serine residues in organotypic cultures.** Eight organotypic hippocampal cultures were prepared and labeled overnight with [^{32}P]PO_4, after which the cells were frozen and homogenized. NR2B was immunoprecipitated from the homogenate, purified on an SDS-PAGE gel, and hydrolyzed in acid as described under "Experimental Procedures." The acid hydrolysate was mixed with 2 μg each of standard phosphoserine, phosphothreonine, and phosphotyrosine and then spotted onto a cellulose thin layer plate and subjected to electrophoresis as described under "Experimental Procedures." *Left*, positions of the standards, detected with a ninhydrin spray reagent, are indicated by *circles*. *Right*, radiolabeled amino acids visualized by PhosphorImager analysis.

brain by the addition of Ca^{2+}. The Ca^{2+}-stimulated phosphorylation is inhibited by antibodies that specifically inhibit the activity of CaM kinase II (Fig. 1; Ref. 40). To facilitate identification 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 fusion 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 stoichiometry of 1.4 mol/mol of protein (Fig. 3*A*). All of the phosphorylation occurred on the NR2B portion of the fusion protein. The K_m for phosphorylation of GST-tNR2B was approximately 50 nM (Fig. 3*B*), 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 phosphorylation of synapsin I by CaM kinase II is 0.4 μM (46); for a peptide containing the amino-terminal 10 residues of glycogen synthase it is 7.5 μM, and for a peptide containing the amino-terminal 23 residues of smooth muscle myosin light chain it is 4 μM (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 corresponds 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) appeared more slowly and hence probably represent a lower affinity phosphorylation site or sites (Fig. 6).

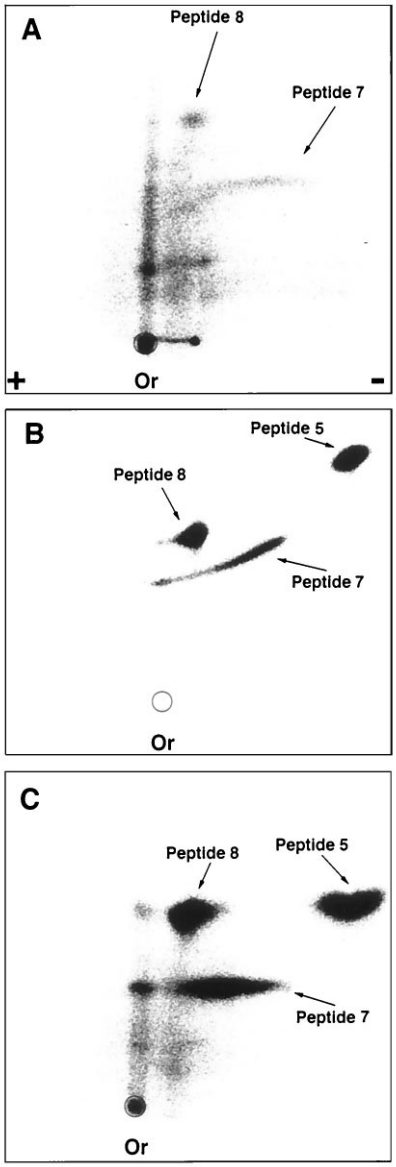

FIG. 10. **Serine 1303 in NR2B is phosphorylated in organotypic hippocampal cultures.** Organotypic hippocampal cultures (10 cultures each containing 4 or 5 slices) were labeled with [^{32}P]phosphate for 8 h, frozen, and homogenized. NR2B was immunoprecipitated from the homogenate, purified by SDS-PAGE, and digested with trypsin as described under "Experimental Procedures." Radiolabeled tryptic peptides generated from NR2B were fractionated on cellulose thin layer plates by electrophoresis in the first dimension and chromatography in the second dimension as described under "Experimental Procedures." The positive pole is to the *left*; the negative pole to the *right*. The positions of the peptides were visualized by PhosphorImager analysis. The mobilities of peptides generated from NR2B labeled *in vivo* were compared with those of standard purified peptides 5, 7, and 8 prepared by HPLC purification from phosphorylated GST-tNR2B as described in the legend to Fig. 7. *A*, 600 cpm of tryptic digest of NR2B from organotypic cultures; *B*, 200 cpm each of purified peptides 5, 7, and 8; *C*, 500 cpm of tryptic digest of NR2B plus 130 cpm each of peptides 5, 7, and 8 mixed before application to the cellulose plate.

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 postsynaptic 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, demonstrating 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 antiserum was raised against a phosphorylated synthetic peptide with the amino acid sequence surrounding serine 1303. We used this antiserum 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-tNR2B. NR2B labeled in the living neurons contained spots corresponding to peptides 7 and 8 from GST-tNR2B (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 hippocampal neurons suggests that its phosphorylation may regulate receptor channel properties or interactions with other proteins. Physiological studies have led to the hypothesis that some of the downstream effects of NMDA receptor activation are mediated by CaM kinase II. For example, the threshold for induction of long term potentiation *versus* long term depression may be governed by steady state CaM kinase II activity (47, 48). One mechanism by which the threshold could be modified would be through regulation of the NMDA receptor itself. Identification of a principal phosphorylation site will facilitate studies of the functional role of phosphorylation of the NMDA receptor by CaM kinase II.

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