NR2C-containing N-methyl-D-aspartate (NMDA) receptors are highly expressed in cerebellar granule cells where they mediate the majority of current in the adult. NMDA receptors composed of NR1/NR2C exhibit a low conductance and reduced sensitivity to Mg2+, compared with the more commonly studied NR2A- and NR2B-containing receptors. Despite these interesting features, very little is known about the regulation of NR2C function. Here we investigate the role of phosphorylation of NR2C in regulating NMDA receptor trafficking and ion channel properties. We identified a phosphorylation site, serine 1244 (Ser1244), near the extreme COOH terminus of NR2C, which is phosphorylated by both cAMP-dependent protein kinase and protein kinase C. This residue is located adjacent to the consensus PDZ ligand, a region that regulates protein-protein interactions and receptor trafficking in NR2A and NR2B. We show that Ser1244 on NR2C is phosphorylated in vitro, in heterologous cells, and in neurons. Moreover, we demonstrate for the first time that NR2C interacts with the PSD-95 family of PDZ domain-containing proteins but that phosphorylation of Ser1244 does not influence this PDZ interaction. Furthermore, Ser1244 phosphorylation does not regulate surface expression of NR1/NR2C receptors. However, we find that this site does regulate the kinetics of the ion channel: a phosphomimetic mutation at Ser1244 accelerates both the rise and decay of NMDA-evoked currents in excised patches from HEK-293 cells. Therefore, phosphorylation of Ser1244 does not regulate trafficking but unexpectedly affects ion channel function, suggesting that phosphorylation of Ser1244 on NR2C may be important in defining the functional properties of NMDA receptor-mediated currents in the cerebellum.

NMDA2 receptors are glutamate-gated ion channels that are critical for synapse formation, synaptic plasticity, and learning and memory (1, 2). They are members of the larger family of ionotropic glutamate receptors that also include AMPA and kainate receptors. Endogenous NMDA receptors are heteromeric channels containing two NR1 subunits combined with additional NR2 (NR2A–D) and NR3 (NR3A-B) subunits (2, 3). In contrast to the widespread distribution for NR1, NR2 subunits display unique spatiotemporal expression patterns throughout the central nervous system (4) and confer distinct pharmacological and functional properties on NMDA receptors (2). NMDA receptors in the cerebellum are composed of NR1 and NR2A–C subunits; the NR2C subunit is notably enriched in cerebellar granule cells and is up-regulated developmentally such that in the adult, NR1, NR2A, and NR2C are the predominant NMDA receptor subunits assembling to form functional receptors (2, 5). Moreover, NR2C-containing receptors have unique properties of low conductance and reduced sensitivity to Mg2+ compared with NR2A- or NR2B-containing receptors (2, 4, 6). Despite this, the specific regulation of NR2C-containing receptors has not been studied extensively.

Phosphorylation is an important mechanism regulating glutamate receptors and synaptic plasticity and occurs primarily in the cytosolic COOH-terminal domain of the receptor subunits. Phosphorylation of AMPA receptors by PKA and CaMKII directly affects channel function (7–9), receptor trafficking to synapses (10), and is critical for the expression of long term potentiation and long term depression (11, 12). Phosphorylation is an important regulator of NMDA receptor function and trafficking (13–15); however, the direct phosphorylation of NR2C has not been reported. Interestingly, NR2C contains a prototypic PKA phosphorylation site in the distal COOH-terminal region that is not conserved in NR2A and NR2B, suggesting that phosphorylation at this location might play a specific role in regulating NR2C-containing NMDA receptor complexes.

In this study, we have investigated the regulation of NR2C by phosphorylation. We demonstrate that Ser1244 is phosphorylated by both PKA and PKC in vitro and also on native NR2C in neurons. This serine is just upstream of the PDZ binding domain, which is conserved in NR2 subunits and is critical for NMDA receptor binding to PSD-95. We demonstrate that NR2C interacts directly with PSD-95; however, we find no effect of Ser1244 phosphorylation on PSD-95 binding to NR2C. Unlike the PKA regulation of AMPA receptors (10), we find no effect of Ser1244 phosphorylation on surface expression of NR1/NR2C NMDA receptors. However, we find that this site alters NMDA receptor channel function, because a phosphomimetic mutation (S1244E) accelerates channel kinetics. Thus, we find that this phosphorylation site in the distal COOH terminus regulates NR2C channel function.

**EXPERIMENTAL PROCEDURES**

DNA Constructs and Site-directed Mutagenesis—The rat NR2C cDNA was obtained from Dr. Peter Seeburg (Max Planck Institute, Heidelberg, Germany). The COOH terminus of NR2C (amino acids 1076–1250) was amplified by PCR using synthetic primers that include flanking EcoRI recognition sequences and subcloned into glutathione S-transferase (GST) fusion vector pGEX-6T-1 (Amersham Biosciences) and the LexA fusion vector pBHA (16). Full-length NR2C in the mam-
malian expression vector pRK5 was epitope tagged with the FLAG epitope (DYKDDDDK) between amino acids 36 and 37 using site-directed mutagenesis (Stratagene, La Jolla, CA). The FLAG-NR2C-iresEGFP plasmid was constructed by ligating the EcoRI-SalI DNA fragment of pRK5-FLAG-NR2C into pIREs2-EGFP vector (BD Biosciences). The rat PSD-95 and SAP102 cDNA were obtained from the yeast two-hybrid brain cDNA library. PSD-95 (amino acids 1–724), PSD-95-N (amino acids 1–404), PSD-95-C (amino acids 405–724), SAP102 (amino acids 1–849), SAP102-N (amino acids 1–490), and SAP102-C (amino acids 491–849) were amplified by PCR using synthetic primers that include flanking EcoRI recognition sequences and subcloned into the Gal4 activation domain fusion vector pGAD10. NR2C (S1244A and S1244E) mutations on GST fusion constructs, LexA fusion constructs, and pRK5-NR2C, pRK5-FLAG-NR2C, or FLAG-NR2C-iresEGFP were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. FLAG epitope insertion and mutations were verified by DNA sequencing.

**Cell Surface Biotinylation Assay**—HEK-293 cells expressing NR2C were rinsed twice with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS) and then incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS for 20 min at 4 °C. Cells were then quenched with PBS containing 50 mM glycine for 15 min at 4 °C. Cells were sedimented by centrifugation (100,000 g for 20 min) and solubilized in PBS containing 1% SDS for 15 min at 37 °C. Ten volumes of PBS containing 1% Triton X-100 were added to the lysate resulting in a final concentration of 1% SDS. Insoluble material was removed by centrifugation at 100,000 g for 20 min. The supernatant was incubated with antibody-bound protein A beads (Sigma) for 2 h at 4 °C and washed three times with PBS buffer. The fusion proteins were eluted with 1 ml of the 50% slurry of glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C and washed three times with PBS buffer. The fusion proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione) and dialyzed against BC100 buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethlysulfonyl fluoride). GST fusion proteins (GST alone, GST-NR2C WT, and GST-NR2C S1244A) were phosphorylated in 10 mM HEPES, pH 7.0, 20 mM MgCl2, 50 mM ATP, 1 pmol of [γ-32P]ATP (3000 Ci/mmol) with 50 ng of purified PKA catalytic subunit (Promega, Madison, WI) at 30 °C for 30 min. For PKC phosphorylation, reactions were performed in 20 mM HEPES, pH 7.0, 1.67 mM CaCl2, 1 mM dithiothreitol, 10 mM MgCl2, 50 mM ATP, 1 pmol of [γ-32P]ATP (3000 Ci/mmol) with 10 ng of purified PKC (Calbiochem) at 30 °C for 30 min. For CaMKII phosphorylation, reactions were performed following the protocol provided by the manufacturer (New England Biolabs, Beverly, MA) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol, 1 mM EDTA, 1.2 μM calmodulin, 2 mM CaCl2, 50 mM ATP, 1 pmol of [γ-32P]ATP (3000 Ci/mmol) with 25 ng of αCaMKII at 30 °C for 30 min. The phosphorylation reactions were stopped by adding SDS-PAGE sample buffer and boiling for 5 min. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

**Generation of Phosphorylation State-specific Antibody and Immunoblotting**—Rabbit phosphorylation state-specific antibodies recognizing phosphorylated Ser1244 of NR2C were generated by Biosource International (Camarillo, CA). Rabbits were immunized with a synthetic phosphopeptide Ac-C(Ahx)TWRRV[S]LESEV-OH corresponding to amino acids 1238–1250 of NR2C. Sera were collected and affinity-purified using the antigen phosphopeptide. Anti-NR2C antibody was purchased from Chemicon (Temecula, CA), and FLAG antibody was purchased from Sigma. HEK-293 cells or HeLa cells were transfected with full-length NR2C wild-type or NR2C S1244A using the calcium phosphate method (BD Biosciences), and the PKA activator forskolin (20 μM) was applied for 20 min at 37 °C. Cell membranes were prepared as described under “Cell Surface Biotinylation Assay.” Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Phosphorylation of Ser1244 on NR2C COOH-terminal fusion proteins was analyzed by immunoblotting with the NR2C Ser1244 phosphorylation state-specific antibody.

**Cerebellar Granule Cell Culture**—Primary cultures of cerebellar granule neurons were prepared as described previously (19). Briefly, the cerebella removed from postnatal day 7–9 rat pups were dissociated...
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**FIGURE 1.** NR2C is phosphorylated on Ser\(^{1244}\) by PKA in vitro. A, alignment of the last 20 amino acids of the NR2 subunits, NR2A, NR2B, and NR2C. The PDZ binding domain is depicted in italics, Ser\(^{1244}\) is indicated with an arrow, and Tyr\(^{1472}\) on NR2B is indicated with an asterisk. B, GST-NR2C and GST-NR2C S1244A were phosphorylated in vitro using [γ\(^{32}\)P]ATP with purified PKA catalytic subunit and analyzed by autoradiography. C, GST, GST-NR2C, and GST-NR2C S1244A were phosphorylated in vitro with purified PKA catalytic subunit and analyzed by immunoblotting with NR2C Ser\(^{1244}\) phosphorylation state-specific antibody. A faint nonspecific band was detected on longer exposures in lane 5. Equal loading of GST fusion proteins was confirmed by Coomassie Blue staining (data not shown). All data shown are representative of at least three independent experiments.

**RESULTS**

The NR2C COOH Termi

n Is Phosphorylated by PKA in Vitro—To investigate the regulation of NR2C by PKA phosphorylation, we first characterized the phosphorylation of the NR2C COOH terminus in vitro, specifically focusing on the distal COOH-terminal domain. The extreme COOH terminus of NR2C contains the PSD-95 binding motif, which is conserved in NR2A and NR2B; however, the adjacent upstream residues are quite divergent (Fig. 1A). Most notably, a tyrosine important in NR2B trafficking (Tyr\(^{1472}\), indicated with an asterisk; Fig. 1A) (22) that is conserved in NR2A, is not present in NR2C. Instead, NR2C contains a strong prototypical PKA consensus motif in this region (Ser\(^{1244}\), Fig. 1A), which is not conserved in NR2A or NR2B. Therefore, we generated GST fusion proteins containing the last 175 amino acids of the NR2C COOH-terminal domain (amino acids 1076–1250) of both wild-type NR2C (GST-NR2C) and NR2C containing an alanine mutation of Ser\(^{1244}\) (GST-NR2C S1244A). We incubated GST, GST-NR2C, and GST-NR2C S1244A with purified PKA and [γ\(^{32}\)P]ATP at 30 °C for 30 min to allow in vitro phosphorylation. The proteins were resolved by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography (Fig. 1B). We found that the NR2C COOH terminus was robustly phosphorylated by PKA, and mutation of serine 1244 to alanine (S1244A) reduced PKA phosphorylation. However, there was clearly residual PKA phosphorylation of the NR2C COOH terminus with the Ser\(^{1244}\) mutation, demonstrating that one or more additional residues were phosphorylated by PKA in vitro.

To specifically monitor the regulation of Ser\(^{1244}\) phosphorylation in vitro and potentially in vivo, we raised a phosphorylation state-specific antibody recognizing NR2C phosphorylated on Ser\(^{1244}\). We performed the in vitro phosphorylation assay of GST, GST-NR2C, and GST-NR2C S1244A, resolved the proteins by SDS-PAGE, and transferred the proteins to PVDF membrane. Immunoblotting with the NR2C Ser\(^{1244}\) phosphorylation state-specific antibody revealed that the antibody specifically recognized the NR2C COOH terminus containing phosphorylated Ser\(^{1244}\) (Fig. 1C). Importantly, the GST-NR2C S1244A mutant subjected to the same in vitro phosphorylation assay showed no specific immunoreactivity with this antibody, highlighting the specificity of this reagent for NR2C phosphorylated on Ser\(^{1244}\).
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NR2C Is Phosphorylated on Ser^{1244} in Heterologous Cells and in Neurons—To determine whether full-length NR2C is phosphorylated upon PKA activation in intact cells, we expressed both wild-type NR2C and NR2C S1244A in HEK-293 cells and treated the cells with or without forskolin, an activator of adenylate cyclase. By probing immunoblots of cell lysates with the NR2C Ser^{1244} phosphorylation state-specific antibody, we found that there was a very low level of phosphorylation on NR2C Ser^{1244} under basal conditions. However, phosphorylation of Ser^{1244} was dramatically increased upon treatment with forskolin (Fig. 2A). No signal was detected from lysates of cells expressing NR2C S1244A either with or without forskolin treatment when probed with the Ser^{1244} phosphorylation state-specific antibody, confirming the specificity of this antibody for NR2C phosphorylated on Ser^{1244}. Similar regulation of NR2C Ser^{1244} phosphorylation was observed when NR2C was expressed in HeLa cells (Fig. 2B), another non-neuronal mammalian cell line.

To investigate the possibility of NR2C phosphorylation in vivo, we next analyzed phosphorylation of endogenous NR2C on Ser^{1244} in cerebellum. We solubilized rat cerebella (P30), isolated NR2C, and probed immunoblots with our NR2C Ser^{1244} phosphorylation state-specific antibody. We found that endogenous NR2C was phosphorylated on Ser^{1244} (Fig. 2C). The NR2C band on the immunoblot was specific because no signal was detected when IgG was used as a control for NR2C immunoprecipitation (Fig. 2C). In addition, treatment of the membrane with λ-phosphatase prior to immunoblotting eliminated the NR2C band recognized with the Ser^{1244} phosphorylation state-specific antibody, confirming that the immunoreactivity was specific for phosphorylated NR2C. To determine whether endogenous NR2C is phosphorylated upon PKA activation in neurons, we examined Ser^{1244} phosphorylation in cultured cerebellar granule cells in the presence or absence of forskolin. In agreement with the results in heterologous cells, we detected strong phosphorylation of NR2C Ser^{1244} in response to forskolin treatment (Fig. 2D). Thus, Ser^{1244} is clearly regulated on endogenous NR2C in neurons when adenylate cyclase is activated.

NR2C Is Phosphorylated by PKC—The prototypical consensus sequence for PKA phosphorylation (RXRS) is consistent with consensus motifs for other serine/threonine kinases. For example, the CaMKII and PKC consensus sequence (RXRS) includes a positively charged amino acid preceding the phosphorylated residue. Therefore, we explored whether PKC and CaMKII could also phosphorylate NR2C on Ser^{1244}. We incubated GST, GST-NR2C, and GST-NR2C S1244A with purified PKA, PKC, or CaMKII at 30 °C for 30 min to allow in vitro phosphorylation. The proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblots were probed with Ser^{1244} phosphorylation state-specific antibody. Both PKA and PKC phosphorylated Ser^{1244} robustly, but we observed no phosphorylation by CaMKII (Fig. 3A).

We next investigated whether the activation of PKC could stimulate the phosphorylation of endogenous NR2C on Ser^{1244}. We treated cultured cerebellar granule cells (11 days in vitro) with (or without) 100 nM PMA for 20 min. We evaluated phosphorylation by immunoblotting with NR2C Ser^{1244} phosphorylation state-specific antibody and observed a robust increase in immunoreactivity with PMA treatment (Fig. 3B). Thus PKC activity increases phosphorylation of NR2C Ser^{1244} in vivo.

NR2C Interacts with PDZ Domain-containing Proteins Independent of Ser^{1244} Phosphorylation—The phosphorylation site that we identified on NR2C is particularly intriguing because it is located just a few amino acids upstream of the consensus PSD-95 binding motif. Members of the PSD-95 family of proteins contain PDZ domains, which are well-characterized protein-protein interaction motifs (23). The PDZ ligand, -XS/TVX, is conserved in NR2A, NR2B, and NR2C (Fig. 1A). The binding of PSD-95 and related proteins to NR2A and NR2B has been demonstrated by many groups; however, whether NR2C is capable of binding
the PSD-95 family of proteins has not been tested. Therefore, we investigated whether NR2C also binds directly to PSD-95 and the closely related protein SAP102 and, if so, whether or not phosphorylation of Ser\textsuperscript{1244} regulates the interaction. For this purpose, we used a yeast two-hybrid binding assay, a system that has been used to examine NMDA receptor binding to PDZ domain-containing proteins (24, 25). We co-transformed yeast with the NR2C COOH terminus and either PSD-95 or SAP102 and observed a strong interaction as monitored with expression of a reporter gene that allows growth on histidine-deficient media (−His) (Fig. 4A). Yeast were plated in serial dilutions to allow direct comparison of relative binding, and growth on medium containing histidine (+His) was used as a control for transformation efficiency of the yeast (Fig. 4A). NR2C bound exclusively to the amino-terminal half of PSD-95 (PSD-95-N) and SAP102 (SAP102-N), which contain the PDZ domains (Fig. 4A). In contrast, NR2C did not bind to either PSD-95 or SAP102 COOH-terminal domains (Fig. 4A). This is consistent with the previous characterization of the NR2A and NR2B interaction with the PSD-95 family of proteins (16, 26). In addition, we investigated a role for Ser\textsuperscript{1244} phosphorylation in protein binding by characterizing two Ser\textsuperscript{1244} mutations in the yeast two-hybrid assay, mutation of Ser\textsuperscript{1244} on NR2C to alanine to prevent phosphorylation or mutation to glutamate to mimic phosphorylation. In these studies we observed no change in binding (Fig. 4B), leading us to conclude that phosphorylation of Ser\textsuperscript{1244} does not dramatically affect binding affinity of NR2C for PDZ domain-containing proteins.

PKA Phosphorylation of Ser\textsuperscript{1244} on NR2C Does Not Influence NMDA Receptor Trafficking in Heterologous Cells—The distal COOH terminus of NMDA receptor subunits is an important determinant of receptor trafficking; for example, the tyrosine 1472 in this region of NR2B affects trafficking and endocytosis of NR2B without affecting the binding of PSD-95 (25). For this reason, we investigated whether PKA phosphorylation of NR2C affects receptor trafficking or surface expression in heterologous cells. PKA efficiently phosphorylates NR2C Ser\textsuperscript{1244} \textit{in vitro} and PKA activation dramatically up-regulates Ser\textsuperscript{1244} phosphorylation in heterologous cells; therefore, we explored the effect of forskolin treatment on NR2C trafficking and surface expression in HEK-293 cells. Functional NMDA receptors exist as heteromers of NR1 and NR2 subunits; therefore, we measured the surface expression of NR2C co-expressed with NR1 in HEK-293 cells using FACS analysis. A bicistronic expression vector containing the coding sequences of FLAG-tagged NR2C and EGFP (FLAG-NR2C-ires-EGFP) was used to monitor NR2C-transfected cells (GFP-positive cells). We found that the percentage of GFP-positive cells containing surface expression of NR2C co-expressed with NR1 was relatively low, only 22% (Fig. 5A). Incubation of the NR2C/NR1-expressing cells with forskolin (previously shown to lead to robust phosphorylation of NR2C on Ser\textsuperscript{1244} in Fig. 2) had no effect on NR2C surface expression (Fig. 5A). Furthermore, fluorescence intensities of surface-expressed NR2C in forskolin-treated and untreated cells were nearly identical, suggesting that PKA activation does not affect surface expression of NR2C.
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In addition to FACS analysis, we used a second method to evaluate surface expression of NR2C: a cell surface biotinylation assay of surface-expressed NR2C. We biotinylated HEK-293 cells expressing NR2C and NR1 for 20 min at 4 °C, isolated surface-expressed proteins with NeutrAvidin beads, and probed immunoblots with antibodies to detect surface-expressed pools of receptor. Tubulin, a cytosolic protein, was used as a negative control for surface biotinylation, confirming the specificity of our biotinylation procedure (Fig. 5B). Using this independent biochemical assay, we obtained results that were quantitatively consistent with FACS analysis, revealing that the proportion of NR2C expressed on the plasma membrane was low and unchanged with PKA activation (Fig. 5B). We also specifically evaluated NR2C phosphorylation on Ser1244 in the surface pool of receptors to investigate whether surface NR2C is preferentially phosphorylated. While forskolin treatment resulted in a dramatic increase in Ser1244 phosphorylation, the phosphorylation did not correlate with any change in plasma membrane expression and the relative amount of surface expressed NR2C was the same when measured using the phosphorylation state-specific antibody or an antibody recognizing all forms of NR2C (Fig. 5B). All of these data are consistent with Ser1244 phosphorylation playing a role distinct from regulating NR2C trafficking to the plasma membrane.

Ser1244 on NR2C Regulates Channel Function—Serine/threonine phosphorylation of AMPA receptors has been shown to affect channel function in a manner apparently distinct from its role in receptor trafficking (27). Therefore, we investigated whether phosphorylation of Ser1244 can regulate the channel function of NR2C-containing NMDA receptors. We used outside-out patches combined with ultra-fast solution exchange to enable the kinetics of NR1/NR2C receptors to be accurately measured. Whole-cell patch clamp recordings were obtained from HEK-293 cells expressing either NR1/NR2C wild-type or NR1/NR2C S1244E, a phosphomimetic mutant of Ser1244. Outside-out patches were excised, and NMDA (200 μM), in the presence of saturating glycine, was rapidly applied using a Piezo-electric ultra-fast solution exchange system. Previous studies have only investigated the kinetics of NR1/NR2C receptors using whole-cell recordings, most likely as a consequence of their low surface expression (28); therefore, the kinetics of NR1/NR2C receptors have not been reported for excised patches. Indeed we found that HEK-293 cells expressing NR1/NR2C do exhibit very small whole-cell NMDA receptor-mediated currents; however, we did observe detectable currents in a fraction (~10%) of excised patches. In these patches we found that averaged currents from NR1/NR2C S1244E-expressing cells consistently exhibited more rapid activation (Fig. 5F). Other properties such as reversal potential and absolute amplitude were not different between the wild-type and mutant NR2C-expressing cells (data not shown). These findings indicate that phosphorylation of this distal COOH-terminal phosphorylation site speeds the kinetics of NR2C-containing NMDA receptors. Thus we describe a novel modulation of NMDA receptor kinetics due to phosphorylation of NR2C.

FIGURE 5. PKA phosphorylation of Ser1244 does not affect NR2C surface expression. A, HEK-293 cells expressing NR1-1a and FLAG-NR2C-IRES-EGFP were incubated with or without forskolin (FSK) for 20 min. Cells were collected and analyzed by FACS analysis (see “Experimental Procedures”). The percentage of cells in each subpopulation is shown in the respective delineated regions. Panel i, NR1-1a and FLAG-NR2C-IRES-EGFP without adding anti-FLAG antibody (negative control); panel ii, NR1-1a and FLAG-NR2C-IRES-EGFP in the absence of forskolin; panel iii, NR1-1a and FLAG-NR2C-IRES-EGFP in the presence of FSK; panel iv, table showing the percentage of NR2C and EGFP expressed cells (upper right region) versus EGFP expressed cells (upper right and lower right regions). B, HEK-293 cells expressing NR1-1a and NR2C were incubated with or without forskolin for 20 min before biotinylating surface proteins (see “Experimental Procedures”). Total lysates (lanes 1 and 3) were compared with surface pools of proteins (lanes 2 and 4) by immunoblotting with either the NR2C antibody or the NR2C Ser1244 phosphorylation state-specific antibody. The quantification of data was done using ImageJ software. All data shown are representative of at least three independent experiments.
We now report the first demonstration of NR2C phosphorylation. In the current study, we have characterized the phosphorylation of NR2C in several systems and investigated the functional role of phosphorylation on PSD-95 binding, NMDA receptor trafficking, and channel function. Using an in vitro phosphorylation assay, we showed that NR2C is directly phosphorylated on Ser^{1244} by PKA and PKC in vitro. We characterized PKA phosphorylation of NR2C expressed in heterologous cells. Although we found very little phosphorylation of Ser^{1244} on NR2C expressed in HEK-293 cells or HeLa cells under basal conditions, we observed a dramatic increase in Ser^{1244} phosphorylation after treating the cells with forskolin to activate PKA. Furthermore, we found that endogenous NR2C is phosphorylated in cerebellar extracts, and NR2C phosphorylation on Ser^{1244} is dramatically increased upon PKA or PKC activation in cerebellar granule cell cultures. This demonstrates that Ser^{1244} is physiologically phosphorylated by at least two distinct kinase pathways.

NR2A receptors directly interact with PDZ domain-containing proteins such as PSD-95. The binding affinity of PDZ domain-containing proteins is often regulated by phosphorylation within the PDZ ligand on receptors or channels. For example, PKC phosphorylation of the AMPA receptor GluR2 subunit on Ser^{880} within the PDZ ligand differentially modulates its interaction with PDZ domain-containing proteins (30). In addition, PKA phosphorylation of the inwardly rectifying K⁺ channel on a serine within the PDZ ligand inhibits binding to PSD-95 (31). Similarly, phosphorylation of NR2B by casein kinase II on Ser^{1480} within the PDZ ligand inhibits binding to PSD-95 and regulates surface expression of NMDA receptors (32). Of particular relevance is the finding that phosphorylation of a tyrosine residue a few amino acids upstream of the GluR2 PDZ ligand regulates its interaction with PDZ domain-containing proteins and affects AMPA receptor surface expression (33). Now we have identified a phosphorylation site on NR2C located just a few amino acids upstream of the consensus PSD-95 binding site. Although NR2C possesses the consensus motif for binding to PSD-95 conserved in NR2A and NR2B, this interaction has never been tested. Therefore, we used the yeast two-hybrid assay and found that indeed NR2C interacts with both PSD-95 and SAP102 and that the interaction is specific for the NH₂-terminal half of these proteins, which contains the PDZ binding domains. We also examined the effect of Ser^{1244} phosphorylation on protein binding by mutating Ser^{1244} to a phosphomimetic glutamate (S1244E) or to alanine (S1244A). We saw no change in PSD-95 binding with either mutation, suggesting that phosphorylation of this site does not regulate these protein-protein interactions.

Glutamate receptor phosphorylation can regulate receptor trafficking and surface expression by affecting PDZ binding (32) but can also regulate trafficking by mechanisms independent of PDZ proteins (10, 34, 35). The trafficking of glutamate receptors has been a major focus of research in the field for several years. Intense interest has resulted from the finding that AMPA receptor trafficking undergoes changes in synaptic plasticity. Phosphorylation of AMPA receptors by PKA regulates surface expression, channel function, and long term potentiation, leading us to speculate that PKA phosphorylation of NR2C-containing NMDA receptors might also affect surface expression. Therefore, using several independent assays, we investigated the role of PKA phosphorylation of Ser^{1244} on NR2C on protein trafficking. Using FACS analysis, we found that only a small percentage of NR1/NR2C receptors are expressed on the plasma membrane and that there was no change in surface expression of NR2C upon treatment with forskolin to activate PKA and phosphorylate Ser^{1244}. We also performed cell surface biotinylation experiments to evaluate surface expression of NR2C. As with the FACS analysis, we found that relatively little NR2C was present on
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the plasma membrane and that there was no change in surface expression with forskolin treatment, demonstrating that phosphorylation of Ser1244 on NR2C does not affect trafficking or surface expression of NR2C expressed in heterologous cells. We also analyzed oligomerization of NR1 and NR2C and found no change in the amount of NR1/NR2C receptor complexes with forskolin treatment (data not shown). However, phosphorylation of other residues on NR2C might regulate trafficking. Furthermore, it is still possible that NR2C phosphorylation on Ser1244 plays a specific role in regulating synaptic expression of NMDA receptors in neurons via mechanisms not present in heterologous cells. Therefore, future studies to address this issue will be important.

In addition to affecting protein binding and protein trafficking, the phosphorylation of glutamate receptors can directly affect channel function. For example, PKA and CaMKII phosphorylation of the GluR1 AMPA receptor subunit directly affects channel properties by changing the open probability (8) or single channel conductance (9), respectively. Therefore, we also evaluated the NMDA receptor channel kinetics of NR2C-containing receptors in excised patches from HEK-293 cells. We found that the phosphomimetic NR2C mutation, NR2C S1244E, accelerates the kinetics of the response, indicating that phosphorylation of this distal COOH-terminal phosphorylation site regulates ion channel gating. This is a surprising finding, since one would not expect such a distal region of the intracellular COOH terminus to regulate ion channel gating. Such a change in kinetics would likely affect the duration of NMDA receptor-mediated synaptic currents carried by NR2C-containing receptors, for example in cerebellar granule cells. This speeding of the kinetics is predicted to reduce Ca2+ influx through NMDA receptors and thus may modulate the induction of long term synaptic plasticity. Therefore, further investigation into the physiological consequences of the phosphorylation-dependent regulation of NR2C-containing NMDA receptors in neurons will be of great interest.

In conclusion, we report for the first time the phosphorylation of the NMDA receptor subunit NR2C, which is highly enriched in the cerebellum. NR2C is robustly phosphorylated on Ser1244 upon activation of PKA or PKC. Although Ser1244 is located in the distal COOH terminus adjacent to the PDZ ligand, we find no effect on PDZ binding or protein trafficking but instead find an exciting and unexpected change in channel kinetics of NR1/NR2C NMDA receptors. NR1/NR2C channels have distinctive properties, including low single channel conductance and reduced sensitivity to magnesium compared with the more commonly studied NR2A- and NR2B-containing receptors, supporting a unique role in modulating excitatory transmission and synaptic plasticity. Our findings that PKA and PKC directly phosphorylate NR2C, that this site is phosphorylated in cerebellum, and that it regulates channel kinetics adds to the unique functional regulation of this class of NMDA receptors and suggests that this may play an important role in defining the properties of NR2C-containing receptors that are specifically enriched in the adult cerebellum.

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