Cyclic AMP-dependent Protein Kinase and Protein Kinase C Phosphorylate N-Methyl-d-aspartate Receptors at Different Sites*

A. Soren Leonard and Johannes W. Hell†‡

From the Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706-1532

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Ca2+ influx through N-methyl-d-aspartate (NMDA)-type glutamate receptors plays a pivotal role in synaptic plasticity during brain development as well as in mature brain. Cyclic AMP-dependent protein kinase (PKA) and members of the protein kinase C (PKC) family are also essential for various forms of synaptic plasticity and regulate the activity of different ion channels including NMDA and non-NMDA receptors. We now demonstrate that PKA and various PKC isoforms phosphorylate the NMDA receptor in vitro. The stoichiometry of [32P]phosphate incorporation per [3H]MK-801 binding site is greater than 1 for both PKA and PKC. Double immunoprecipitation experiments show that all three NMDA receptor subunits that are prevalent in the cortical structures, NR1, NR2A, and NR2B, are substrates for PKA as well as PKC. Two-dimensional phosphopeptide mapping reveals that the major phosphorylation sites for PKA and PKC differ for all three subunits. We predict that some if not most of these sites are phosphorylated in the central nervous system of rats in vivo. Evidence that some if not most of these sites are phosphorylated in the central nervous system of rats in vivo. The results presented in this article together with earlier electrophysiological experiments demonstrating that PKA and PKC activation increases the activity of NMDA receptors indicate that NMDA receptor potentiation can be mediated by direct phosphorylation by PKA and PKC. Collectively, these results strongly suggest that NMDA receptor functions such as control of neuronal development or expression of synaptic plasticity are modulated by PKA- and PKC-mediated phosphorylation of NMDA receptors.

Ionotropic glutamate receptors mediate fast synaptic transmission by glutamate, the prevailing excitatory neurotransmitter in the mammalian brain (1, 2). These glutamate receptors can be divided into two major families, N-methyl-d-aspartate (NMDA) receptors and non-NMDA receptors, based on their pharmacological and electrophysiological properties as well as sequence identity (1–3). NMDA receptors are key participants in the regulation of synaptic plasticity during brain development as well as in mature neurons (38) and in hippocampal microcultures (39). Finally, the effects of phosphatase inhibitors and direct application of phosphatase 1 and 2A suggest that phosphatases 1, 2A, and 2B are involved in the down-regulation of NMDA receptor activity under various conditions (40–42). Most of these studies are based on electrophysiological methods that do not reveal whether the NMDA receptor itself or another protein or enzyme modifying NMDA receptor activity has been phosphorylated.

The work presented in this study addresses the question of...
whether the NMDA receptor itself is a substrate for PKA and PKC and whether the corresponding phosphorylation sites are targeted in vivo. No previous evidence has demonstrated that NMDA receptors are directly phosphorylated by PKA, although it has been shown earlier by Tingley et al. (16) that NR1 is phosphorylated by PKC in vitro and in cell culture upon stimulation with phorbol ester. However, it has been unknown whether PKC also phosphorylates NR2 subunits. We now demonstrate that PKA as well as PKC phosphorylate NR1, NR2A, and NR2B. Two-dimensional phosphopeptide mapping reveals, however, different patterns for PKA and PKC phosphorylation, suggesting differential regulation of NMDA receptors by PKA and PKC. Back-phosphorylation of NMDA receptors immunoisolated from rapidly collected and homogenized rat brain in the absence and presence of phosphate inhibitors shows that NR1 and NR2 subunits are phosphorylated in vivo according to PKA and PKC in vitro.

EXPERIMENTAL PROCEDURES

Materials—MK-801 (1-propan-2-yloxy-4-(3-methyl-2-benzothiazolyl)phenylsulfonyl-1H-pyridine-2,4-dione) was obtained from RBI (Natick, MA), and [3H]MK-801 (884 GBq/mmol) and [3H]-3-aminopropyl-2-(5-methyl-10,11-dihydro-5H-dibenzo(c,e)pyrido[1,2-a]cyclohepten-5,10-imine maleate) was obtained from DuPont NEN. The ECL detection kit was purchased from Amer- sham Corp., protein A-Sepharose (PAS) and bovine serum albumin (BSA; IgG fraction) from Sigma, microcystin LR from Calbiochem, 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc) from Boehringer (Mannheim, Germany), sequencing grade trypsin from Promega (Madison, WI), and control antibodies from Zymed (South San Francisco, CA). PKA and PKC isoforms were isolated, and the absence of activity of other, contaminating protein kinases was confirmed by established procedures (43–46). PKA and Phc isoforms were kindly supplied by Dr. E. I. Rotman, Department of Pharmacology, University of Washington (Seattle, WA) and S.-M. Huang and Dr. P. J. Berics, Department of Biomolecular Chemistry, University of Wisconsin (Madison, WI), respectively. All other reagents were purchased from commercial suppliers and were of standard biochemical quality.

Antibodies—The monoclonal antibody 54.2 (aR1) recognizes specifically NR1 (15) and was generously supplied by Dr. R. Jahn, Howard Hughes Medical Institute, Yale University (New Haven, CT). Polyclonal anti-peptide antibodies produced against the synthetic peptide corresponding to the COO-terminal 20 amino acid residues of NR2A recognize NR2A and NR2B (aR2Ap) (15, 19). Antibodies raised against the synthetic peptide corresponding to amino acid residues 1450–1429 of NR2A interact specifically with NR2A (a2Ap) (20). For the production of antibodies against polyhistidine fusion proteins containing the NR2A region spanning amino acids 934–1203 (a2A) or the NR2B region encompassing amino acids 935–1856 (a2B), oligonucleotides flanking the respective region and containing appropriate restriction sites were used to amplify corresponding sequences from cDNA clones of NR2A and NR2B by PCR. The DNA products were ligated into pQE30 (Qiagen, Chatsworth, CA), allowing the expression of respective polyhistidine fusion proteins that were subsequently purified on Ni2+ chelate resin.

NMDA Receptor Phosphorylation—NMDA receptors were solubilized and immunoprecipitated as described above. The resins with antibody-NMDA receptor complexes attached were treated with 20 μl of 50 mM Tris-CI, pH 8, 1.5% SDS, 5 mM dithiobisreitol, 200 μM Pefabloc, 1 μM pepstatin A, 20 μg of leupeptin, and 40 μg aprotinin for 20 min at 60 °C in a thermomixer, diluted with 300 μl of 1% Triton X-100, 0.2% bovine serum albumin, 200 μM Pefabloc, 1 μM pepstatin A, 2 μg of leupeptin, and 4 μg of aprotinin in TBS and centrifuged. Supernatants were incubated with various antibodies and PAS as described above. PAS immunocomplexes were washed, extracted with SDS sample buffer, and loaded onto SDS-polyacrylamide gels as detailed above.

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Nitroblotting—PAS, preswollen and washed three times with TBS (150 mM NaCl, 10 mM Tris-CI, pH 7.4) containing 1% Triton X-100, were added, and the samples were mixed for 2.5 h as before. The immunocomplexes were sedimented by centrifugation and washed three times with 1% Triton X-100 in TBS and then incubated for 20 min with 10 μM SDSPAGE, the gels were polymerized using 6% acrylamide), the proteins were transferred onto nitrocellulose, which was blocked by incubation for 2 h with 10% skim milk powder in TBS (TBS-milk). Blots were incubated with aR1 or control ascites (1:1000 in TBS-milk) or with a2A, a2B, a2A/B, or control antisemum (1:1000 in TBS-milk) for 1–2 h, washed five times with TBS-milk, incubated with horseradish peroxidase-la- beled sheep anti-mouse IgG (ascites) or protein A (sera) (both diluted 1:1000 in TBS-milk), washed for 5–6 h with 0.05% Tween 20 in TBS (8–10 changes), and developed with the ECL reagent.

For double immunoprecipitations, the resins with antibody-NMDA receptor complexes attached were treated with 20 μl of 50 mM Tris-CI, pH 8, 1.5% SDS, 5 mM dithiobisreitol, 200 μM Pefabloc, 1 μM pepstatin A, 20 μg of leupeptin, and 40 μg aprotinin for 20 min at 60 °C in a thermomixer, diluted with 300 μl of 1% Triton X-100, 0.2% bovine serum albumin, 200 μM Pefabloc, 1 μM pepstatin A, 2 μg of leupeptin, and 4 μg of aprotinin in TBS and centrifuged. Supernatants were incubated with various antibodies and PAS as described above. PAS immunocomplexes were washed, extracted with SDS sample buffer, and loaded onto SDS-polyacrylamide gels as detailed above.

 Autoradiography—PAS, preswollen and washed three times with 1% Triton X-100 in TBS and once with basic phosphorylation buffer (50 mM HEPES-NaOH, pH 7.4, 10 mM MgCl2, 1 mM EGTA). Samples were phosphorylated with 0.5–1 μg of PKA or PKC in 50 μl of basic phosphorylation buffer containing 0.4 mM dithiobisreitol, 1 μM pepstatin A, 2 μg of leupeptin, 4 μg of aprotinin, and 200 μl of 5 mM [γ-32P]ATP. This buffer was supplemented with 1.5 mM CaCl2, 50 μg of diolene, and 2.5 μg of phosphatidylinositol for PKC. The samples were incubated for 30 min at 32 °C in a thermomixer, washed four times with 0.1% Triton X-100 in radioimmune assay buffer (10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 20 mM EDTA, 10 mM EGTA, 20 mM sodium pyrophosphate, 50 mM NaF, 20 mM 2-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride) and then in 10 mM Tris-HCl, pH 7.4. For quantitative phosphorylation as required for the measurement of the stoichiometry of [32P]phosphate incorporation by PKA or PKC, 10 μl unlabelled ATP was added to the phosphorylation reaction. At this ATP concentration, phosphorylation of NMDA receptor subunits by PKA or PKC was saturated. Extending the incubation time to 1 h also did not result in an increase in [32P]phosphate incorporation by PKA or PKC. The pellets were extracted with SDS sample buffer (see above) and used directly for SDS-PAGE. For double immunoprecipitations, pellets were treated as described in the previous paragraph except that radioimmune assay buffer was used rather than TBS for the dilution of the SDS extracts and the washes. Phosphorylated protein bands were visualized by autoradiography, and incorporated [32P] was quantified by Cerenkov counting or scintillation counting.

Phosphopeptide Mapping—The method for the generation of phosphopeptide maps (47) was modified as follows: NMDA receptors were solubilized from rat brain membranes with deoxycholate, immunoprecipitated with aR1, phosphorylated with PKA or PKC, and dissociated with SDS and, if required, with phosphatase inhibitors. The pellet was washed with SDS-PAGE and autoradiography, gel pieces containing [32P]-labeled...
NMIA Receptor Phosphorylation

NMIA Receptor subunits were excised from the gels, washed three times for 2–5 h with acetic acid/2-propanol/H2O (1:1:8) and twice for 2 h with 50% methanol, dried in a vacuum concentrator, rehydrated with 800 μl of 25 mM NH4HCO3 containing 0.5 μg trypsin (which is modified by reductive methylation to make it resistant to autoproteolysis) and a trace amount of phenol red, and digested overnight at 37 °C. After sedimentation for 1 min in a table top centrifuge, supernatants were removed, and gel slices were treated a second time with 0.5 μg of trypsin in 800 μl of 25 mM NH4HCO3 omitting phenol red in the digestion buffer, permitting a control for the extraction efficiency of the gel slices during the second cycle. In addition, 32P was quantified by Cerenkov counting of the gel pieces before and after the digests. Only samples with an extraction efficacy of at least 80% of 32P were further processed. Supernatants from both digests were combined, dried in a vacuum concentrator, resuspended in 200–300 μl of H2O, and dried again. Resuspension and evaporation was repeated until phenol red changed its color from red to yellow, reflecting a nearly complete removal of the basic NH4HCO3 salt. Tryptic phosphopeptides were solubilized in 5 μl of 1% pyridine, 10% acetic acid, pH 3.5, spotted onto thin layer chromatography cellulose plates, and subjected to electrophoresis until phenol red had migrated 5 cm toward the cathode (usually 2 h at 400 V). Plates were dried, developed in the second dimension by ascending chromatography in pyridine/1-butanol/acetatic acid/H2O (10:15:3:12), and dried, and the pattern of tryptic phosphopeptides was visualized by autoradiography or PhosphorImager analysis. To ensure that tryptic digest was complete, in 2–4 independent experiments tryptic digestes were performed for 3 full days rather than overnight, and each day 0.5 μg of trypsin was added to the reaction mixture. Phosphopeptide maps obtained from NR1, 2A, and 2B subunits phosphorylated with PKA or PKC were virtually identical after short and extended digests.

Quantification of NMIA Receptors by [3H]MK-801 Binding and Immunoblotting—[3H]MK-801 binding was performed with membrane fractions as described (48, 49). In brief, 500 μg of membrane protein in 0.1 ml of 5 mM Tris-Cl (pH 7.4) and 1 mM EGTA were incubated with 100 nM [3H]MK-801 to equilibrium (24 h, 4 °C). The Kd for [3H]MK-801 binding to NMIA receptors in membrane fractions is 2.6 nM (49). A concentration of 100 nM [3H]MK-801 will, therefore, saturate [3H]MK-801 binding to NMIA receptors. Nicotinic acetylcholine receptors are blocked by MK-801 with a low affinity. Therefore, at a concentration of 100 nM, MK-801 binding to nicotinic acetylcholine receptors is negligible, since the Kd value of this receptor for MK-801 is 70-fold higher than this concentration (50). Membranes were filtered through Whatman GF/C membranes and washed three times with 5 ml of ice-cold binding buffer. Nonspecific binding was defined by incubations in the presence of 10 μM unlabelled MK-801 and subtracted from total [3H]MK-801 binding to obtain specific binding. To determine the amount of NMIA receptor immunoprecipitated by αR1 for quantitative phosphorylation experiments, 33% of the precipitates were subjected to immunoblotting with αR1 as probing antibody. For comparison, various amounts of membrane fractions (e.g. 25, 50, 100, and 200 μg of protein) containing defined amounts of NMIA receptors as determined by [3H]MK-801 binding were loaded in parallel lanes. αR1 binding to the NMIA receptors on the membrane was detected with the ECL method and quantified by densitometry. ECL signals resulting from various exposure times were compared with each other to ensure that they were within the linear range of the film.

RESULTS

Properties of NMIA Receptor Subunit-specific Antibodies—As described earlier (15), αR1 recognized a single band of about 110 kDa by immunoblotting of total brain homogenate (Fig. 1A, lane 1). This band appears elongated, probably reflecting the existence of closely comigrating NR1 isoforms as they are generated by differential splicing (19). The same band was detected when solubilized NMIA receptors were immunoprecipitated with αR1 (Fig. 1A, lane 2) and absent after immunoprecipitation with control antibody (Fig. 1A, lane 3), excluding a nonspecific precipitation of NMIA receptors under the experimental conditions. Double immunoprecipitation utilizing αR1 for both precipitation steps also yielded the immunoreactive 110-kDa band (Fig. 1A, lane 4), demonstrating that NR1 can be dissociated from the NMIA receptor complex with SDS and subsequently reprecipitated with αR1.

The specificity of antibodies directed against NR2 subunits was also demonstrated by immunoblotting. Similar to earlier observations (20), α2A/B labeled a band of about 190 kDa when either total brain homogenate (Fig. 1B, lane 1) or NMIA receptor complexes were solubilized with deoxycholate and immunoprecipitated, and subunits were separated by SDS-PAGE. Immunoblotting with αR1. Total brain homogenate was loaded in lane 1; NMIA receptors were immunoprecipitated with αR1 and directly loaded (lane 2) or dissociated with SDS and NR1, 2A/B labeled a band of about 190 kDa when

![Fig. 1. Immunoprecipitation and immunoblotting of NR1, NR2A, and NR2B.](http://www.jbc.org/content/121/10/12109)
in mature rat brains (11–13). Therefore, they were not included in our studies. To test, however, the possibility that α2A cross-reacts with NR2B and vice versa, NRMDA receptors were immunoprecipitated with αR1 (lanes 1 and 2), control antibodies (lane 3), α2A/B (lane 4), α2A (lane 5), or α2B (lane 6) before phosphorylation with PKA, SDS-PAGE, and autoradiography. PKA was omitted during the in vitro phosphorylation of the sample shown in lane 2. NMDA receptors were immunoprecipitated with αR1 (lanes 1, 3, 4, and 6–8) or with equivalent amounts of control antibodies (mouse ascites (lane 2) and control rabbit serum (lane 5)), phosphorylated with PKA, dissociated with SDS, and reprecipitated with αR1 (lanes 1 and 3), α2A/B (lane 4), α2A (lane 6), α2B (lane 7), α2Ap (lane 8), or control antibodies (mouse ascites (lane 2) or control rabbit serum (lane 5)). For the sample in lane 3, phosphorylation with PKA was performed after the second precipitation. Marker proteins are shown on the left.

NMDA Receptor Subunits NR1, NR2A, and NR2B Are Substrates for PKC—After one-step immunoprecipitation of NMDA receptors with αR1 and phosphorylation with either a PKC preparation containing PKCa, -β, and -γ or with purified PKCa or -γ isoforms, two phosphoprotein bands are apparent at 110 and 190 kDa (Fig. 3a, lanes 1, 3, and 5). No bands were detectable when precipitations were performed with control antibodies (Fig. 2b, lanes 2 and 5). These results clearly show that NR1, NR2A, and NR2B are phosphorylated in vitro by PKA and constitute, therefore, potential PKA substrates in intact neurons.

NMDA Receptor Phosphorylation

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Phosphorylation of NR1, NR2A, and NR2B by PKA. A, NMDA receptors were solubilized with deoxycholate and immunoprecipitated with αR1 (lanes 1 and 2), control antibodies (lane 3), α2A/B (lane 4), α2A (lane 5), or α2B (lane 6) before phosphorylation with PKA, SDS-PAGE, and autoradiography. PKA was omitted during the in vitro phosphorylation of the sample shown in lane 2. B, NMDA receptors were immunoprecipitated with αR1 (lanes 1, 3, 4, and 6–8) or with equivalent amounts of control antibodies (mouse ascites (lane 2) and control rabbit serum (lane 5)), phosphorylated with PKA, dissociated with SDS, and reprecipitated with αR1 (lanes 1 and 3), α2A/B (lane 4), α2A (lane 6), α2B (lane 7), or α2Ap (lane 8), or control antibodies (mouse ascites (lane 2) or control rabbit serum (lane 5)). For the sample in lane 3, phosphorylation with PKA was performed after the second precipitation. Marker proteins are shown on the left.

low; Fig. 2b, lane 3). No bands were visible by autoradiography after precipitation with control antibody (Fig. 2a, lane 3), showing that the precipitations of the NMDA receptor had been specific.

NMDA receptor-associated proteins such as PSD-95 (51) or minor contaminants could coprecipitate with the receptor complex and comigrate during SDS-PAGE with one of the receptor subunits. If those proteins are preferred kinase substrates, they may appear as one of the major phosphorylated bands. Putative contaminating proteins can be removed efficiently, and the different subunits can be separated by a double immunoprecipitation method (46, 52). NMDA receptors were immunoprecipitated with αR1, phosphorylated with PKA, and dissociated with SDS. NR1 and NR2 subunits were specifically and separately reprecipitated with the corresponding antibodies. Reprecipitation with αR1 resulted in a 32P-containing band of about 110 kDa (Fig. 2b, lanes 1 and 3), corroborating the finding that this band corresponds to the NR1 subunit. Reprecipitation with α2A/B yielded a 32P-labeled band of 190 kDa, which may contain NR2A or -B (Fig. 2b, lane 4). More specifically, similar 32P-labeled bands were apparent after reprecipitation with α2A or with α2Ap (Fig. 2b, lanes 6 and 8), indicating that these bands correspond to NR2A. Also α2B reprecipitated a 32P-labeled band of 190 kDa, identifying this band as NR2B (Fig. 2b, lane 7). None of these bands were detectable when precipitations were performed with control antibodies (Fig. 2b, lanes 2 and 5). These results clearly show that NR1, NR2A, and NR2B are phosphorylated in vitro by PKA and constitute, therefore, potential PKA substrates in intact neurons.
before precipitation of NR2A with a2A (Fig. 3C, lanes 4–6).

PKA and PKC Phosphorylate NMDA Receptors at Different Sites—Two-dimensional tryptic phosphopeptide mapping was used to determine if PKA and PKC phosphorylate the NMDA receptor subunits at the same or different sites. NMDA receptor subunits were solubilized with deoxycholate, immunoprecipitated with αR1, washed, and split into two equal samples. One sample was phosphorylated by PKA and the other by PKC. Immunoprecipitates were washed, and the receptor subunits were dissociated with SDS. NR1, NR2A, and NR2B were specifically reprecipitated with αR1, α2A, or α2B, respectively, subjected to SDS-PAGE, and digested by trypsin. Fig. 4 shows the resulting two-dimensional phosphopeptide maps. For all three subunits, the pattern of the major phosphopeptides looks quite different after phosphorylation with PKA and PKC. For example, the three main phosphopeptides of PKA-phosphorylated NR1 are left of the origin toward the cathode and barely migrated during the ascending chromatography (Fig. 4A, spots 1, 3, and 4). The prevailing phosphopeptide of PKC-phosphorylated NR1, however, moved fairly far during this chromatography step (Fig. 4B, spot 5). Analogous considerations are true for NR2A and NR2B. However, spot 3 of PKA-phosphorylated NR2A could be identical with spot 1, 2, or 3 of PKC-phosphorylated NR2A. Similarly, spot 1 of PKC-phosphorylated NR2B could be identical with spot 2 or 3 of PKA-phosphorylated NR2B.

To further confirm that PKA and PKC phosphorylate NMDA receptor subunits at different sites, phosphorylation reactions were performed with PKA alone, PKC alone, or PKA and PKC together under assay conditions under which the incorporation of [32P]phosphate by PKA or PKC was saturated, followed by SDS-PAGE, autoradiography, and PhosphorImager analysis. For NR1 as well as NR2 subunits, the incorporation of [32P]phosphate was as high during incubation with a combination of PKA and PKC as the total incorporation of [32P]phosphate by PKA and PKC during separate incubation reactions taken together. Accordingly, phosphorylation of NMDA receptor subunits is additive, corroborating the findings described in the previous paragraph that indicate that PKA and PKC target different sites.

Stoichiometry of PKA and PKC Phosphorylation of NMDA Receptors—[3H]MK-801 binds specifically and with high affinity to NMDA receptors in membrane fractions as well as after solubilization with deoxycholate (48, 49). The Kp for [3H]MK-801 binding to NMDA receptors in rat brain membranes is 2.6 ± 0.7 nM, and Bmax is 2.2 pmol/mg protein (49). However, it is unclear whether [3H]MK-801 labels all NMDA receptors when solubilized. The following strategy was, therefore, developed to measure the amount of NMDA receptor in solubilized fractions. First, we determined the number of [3H]MK-801 binding sites in a rat brain membrane fraction that had been prepared as described by McKernan et al. (49). The membrane fractions were incubated with 100 nM [3H]MK-801. At this concentration, [3H]MK-801 binding to NMDA receptors is saturated (48, 49), but MK-801 binding to nicotinic acetylcholine receptors (50) is negligible because the Kp for the latter interaction (7 μM) is nearly 2 orders of magnitude higher than the [3H]MK-801 concentration used in our experiments. Nonspecific binding was obtained in the presence of a 100-fold excess of unlabeled MK-801 and subtracted from the total amount of [3H]MK-801 binding. Similar to earlier studies (48, 49), our membrane fractions specifically bound 1.3 ± 0.3 pmol [3H]MK-801/mg of protein (average of seven experiments ± S.E.). To determine the amount of NMDA receptors precipitated for phosphorylation by αR1, part of the precipitates were analyzed by immunoblotting. The amount of NMDA receptor as detected by ECL using αR1 as primary antibody was compared by densitometry with those of membrane fractions loaded onto the same gel. Membrane protein of 25, 50, 100, and 200 μg had been applied to control for linearity of the ECL signals. The results show that typically 30–60 fmol of [3H]MK-801 binding sites were present during the in vitro phosphorylations. Incorporation of [32P]phosphate into NMDA receptors by PKA and PKC was determined after SDS-PAGE and autoradiography by scintillation counting of NR1- and NR2-containing gel pieces.

Assuming that one NMDA receptor complex binds one [3H]MK-801 molecule, these experiments indicate a molar ratio of about 1 for the incorporation of [32P]phosphate per receptor
complex into NR1 by PKA as well as by PKC (Table I). The molar ratio of \(^{32}P\)phosphate incorporation into NR2 subunits by PKA was 0.27, and the ratio for incorporation by PKC was 1.48. The latter ratio may reflect the fact that one NMDA receptor complex probably contains several NR1 and NR2 subunits. In addition, each subunit may possess more than one phosphorylation site. Taken together, these data indicate that the \textit{in vitro} phosphorylation efficiency by PKA and PKC is high, suggesting that the NMDA receptor is a good substrate for both kinases \textit{in vitro} and possibly \textit{in vivo}. If one NMDA receptor binds more than one \(^{3}H\)MK-801 molecule, the \(^{32}P\) incorporation rate per receptor would be even higher. Similar considerations are valid if a fraction of \(^{3}H\)MK-801 associated during the binding assay to a receptor different from the NMDA receptor (e.g., nicotinic acetylcholine receptors; see above).

\textbf{In Vivo Phosphorylation of PKA and PKC Sites—}Recent electrophysiological studies indicate that NMDA receptor currents are down-regulated during stimulation of synaptic transmission and that this effect is reversed by PKA (39). Accordingly, either NMDA receptors (by themselves or associated) or other regulatory, proteins are tonically phosphorylated by PKA before stimulation of synaptic transmission. To test if NMDA receptors are constitutively phosphorylated in the rat central nervous system \textit{in vivo}, rat brains were cooled down to 0–4 °C immediately after decapitation, prepared from the skull, and cut in half. One half was solubilized in the absence and the other half in the presence of phosphatase inhibitors (see “Experimental Procedures” for more details). We expected that phosphorylated NMDA receptors would be dephosphorylated by endogenous phosphatases when no phosphatase inhibitors were present. For example, the PKA site of class C L-type Ca\(^{2+}\) channels is completely dephosphorylated during isolation in the absence, but not in the presence, of phosphatase inhibitors (53).\(^2\) After dephosphorylation, the number of phosphorylation sites available for \textit{in vitro} phosphorylation (in this context

\(^2\) J. W. Hell, unpublished results.
NMDA receptors were solubilized with deoxycholate and immunoprecipitated with α1. The amount of [3H]MK-801 binding sites present in these samples was determined by immunoblotting of part of these samples, using as standards membrane fractions for which the amount of [3H]MK-801 binding sites had been measured by binding assays. The rest of the samples were phosphorylated by PKA or PKC, NR1 and NR2 subunits separated by SDS-PAGE, and [32P]phosphate incorporation into NR1 and NR2 subunits determined by scintillation counting of excised gel pieces. Given are the average values of the ratio of bound [32P]phosphate to [3H]MK-801 binding sites present during the phosphorylations from three experiments ± S.E.

| Kinase | NR1 | NR2 |
|-------|-----|-----|
| PKA   | 1.07 ± 0.05 | 0.27 ± 0.05 |
| PKC   | 1.17 ± 0.10 | 1.48 ± 0.07 |

**FIG. 5. In vivo phosphorylation of NMDA receptors.** NMDA receptors were solubilized with deoxycholate and immunoprecipitated with α1 in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of phosphatase inhibitors, back-phosphorylated with PKA (lanes 1 and 2) or PKC (lanes 3 and 4), and subjected to SDS-PAGE and autoradiography. Note the increase in 32P incorporation when NMDA receptors were isolated without phosphatase inhibitors. Marker proteins are indicated on the left.

referred to as “back-phosphorylation”) will be increased, and subsequent in vitro phosphorylation using [γ-32P]ATP will lead to a larger incorporation of [32P]phosphate. Solubilized NMDA receptors were immunoprecipitated, phosphorylated with PKA or PKC, and analyzed by SDS-PAGE. [32P]phosphate incorporation into NR1 as well as NR2 subunits by both PKA and PKC during the in vitro phosphorylation was clearly higher when NMDA receptors were prepared without phosphatase inhibitors (Fig. 5). Similar results were obtained in three different experiments. To ensure that identical amounts of NMDA receptors were immunoprecipitated and present during the in vitro phosphorylation step, one-third of each precipitate was used for immunoblotting. Probing with α1 or antibodies against NR2 subunits demonstrated that exactly the same amounts of NR1 and NR2 subunits were present in samples prepared with and without phosphatase inhibitors (data not shown).

Expression of NMDA receptors in adult rats is highest in brain cortex, hippocampus, and cerebellum (12, 13). These brain areas were processed separately, and 32P incorporation into NMDA receptors during in vitro phosphorylation was quantified after SDS-PAGE by scintillation counting of excised gel pieces containing NR1 and NR2 subunits. [32P]phosphate incorporation into NR1 by PKA and into NR2 subunits by PKC was significantly higher in all three brain regions when samples were prepared without phosphatase inhibitors (Fig. 6). The same results were obtained for PKA phosphorylation of NR2 subunits and PKC phosphorylation of NR1 in cortical and hippocampal preparations (Fig. 6). These results suggest that the in vitro PKA as well as the in vitro PKC phosphorylation sites had been phosphorylated in vivo before homogenization of the tissue. During solubilization and immunoprecipitation, a significant portion of these phosphorylation sites had obviously been dephosphorylated when no phosphatase inhibitors were utilized, thereby becoming available for the in vitro phosphorylation with [32P]phosphate. However, the differences in back-phosphorylation with PKA or PKC of cerebellar NR1 or NR2 subunits, respectively, obtained in the absence and presence of phosphatase inhibitors, were not significant (Fig. 6), although they were highly significant for NR2 and NR1 subunits within the very same samples back-phosphorylated with PKA and PKC, respectively, ruling out sample-to-sample variation in the amount of NMDA receptors or phosphatase activity. The latter results may reflect a difference in NMDA receptor composition in the cerebellum when compared with cortex and hippocampus. In fact, in contrast to NR2B, NR2C is strongly expressed in the mature cerebellum but hardly detectable in cortical structures (12, 13). NR2C may not be tonically phosphorylated by PKA and may also influence the phosphorylation of NR1 by PKC.

**DISCUSSION**

**In Vivo Phosphorylation of NMDA Receptors by PKA and PKC—**Although electrophysiological studies indicate that NMDA receptor activity is regulated by the action of norepinephrine, and more directly by PKA (38, 39), PKA phosphorylation of NMDA receptors has not yet been reported. Our results demonstrate direct phosphorylation of NR1, NR2A, and NR2B. This conclusion is based on double immunoprecipitation protocols involving the dissociation of NMDA receptor complexes after specific immunoprecipitation and reprecipitation of NR1, NR2A, and NR2B with NP-40 or antibodies against NR2 subunits demonstrated that exactly the same amounts of NR1 and NR2 subunits were present in samples back-phosphorylated with PKA (left) or PKC (right), and subjected to SDS-PAGE. 32P incorporation into NR1 (top) or NR2 subunits (bottom) was quantified by PhosphorImager analysis (Molecular Dynamics). The values for each pair of samples were normalized by setting the value of each inhibitor-absent sample equal to 1000. Each value represents the normalized average from three experiments ± S.E. The same amounts of NR1 and NR2 subunits were present for inhibitor-present and inhibitor-absent samples as confirmed by immunoblotting (not shown).

An increasing number of electrophysiological investigations show that PKC regulates NMDA receptor activity (11, 29–37; see Introduction). Earlier investigations indicated that NR1 is phosphorylated upon stimulation of PKC in cortical cultures and in HEK 293 cells transfected with NR1 (16). In that study NR1 had been isolated by a single immunoprecipitation step. Employing single and double immunoprecipitation protocols, we unequivocally identify NR1 as a PKC substrate. We extended these investigations to demonstrate that both NR2A and NR2B are phosphorylated by PKC.

**TABLE I**

Stoichiometry of [32P]phosphate incorporation into NMDA receptor subunits by PKA and PKC

| Kinase | NR1 | NR2 |
|-------|-----|-----|
| PKA   | 1.07 ± 0.05 | 0.27 ± 0.05 |
| PKC   | 1.17 ± 0.10 | 1.48 ± 0.07 |

**FIG. 6. Quantification of the in vivo phosphorylation of NMDA receptors.** Hippocampus (Hi), cortex (Cx), and cerebellum (Cb) were dissected from rat brains and NMDA receptors solubilized with deoxycholate and immunoprecipitated with α1 in the absence (−) or presence (+) of phosphatase inhibitors, back-phosphorylated with PKA (left) or PKC (right), and subjected to SDS-PAGE. 32P incorporation into NR1 (top) or NR2 subunits (bottom) was quantified by PhosphorImager analysis (Molecular Dynamics). The values for each pair of samples were normalized by setting the value of each inhibitor-absent sample equal to 1000. Each value represents the normalized average from three experiments ± S.E. The same amounts of NR1 and NR2 subunits were present for inhibitor-present and inhibitor-absent samples as confirmed by immunoblotting (not shown).
NMHD receptors were phosphorylated by PKA and PKC isoforms to a stoichiometry greater than 1 mol of phosphate incorporated per mol of 3HMK-801 binding sites, although the concentration of ATP during the in vitro phosphorylation reaction (10 μM) was lower than under normal physiological conditions (around 1 mM). The phosphorylation stoichiometries, therefore, suggest that NMHD receptor subunits are efficient substrates for PKA and PKC and may readily be phosphorylated under physiological conditions. Two-dimensional phosphopeptide mapping of NMHD receptor subunits that had been immunoprecipitated twice before tryptic digestion to minimize the risk of contaminating phosphoproteins demonstrated that the main phosphorylation sites of PKA and PKC are substantially different for NR1, NR2A, and NR2B under our conditions (Fig. 4). Furthermore, [32P]phosphate incorporation by PKA and PKC was additive, supporting this conclusion.

In Vivo Phosphorylation of NMHD Receptors—To test the physiological relevance of the in vitro PKA and PKC phosphorylation sites, we asked whether these sites are phosphorylated in vivo. NMHD receptors were immunoprecipitated in the presence and absence of phosphatase inhibitors and subjected to in vitro phosphorylation by PKA and PKC in the presence of γ-[32P]ATP. At least three scenarios are immediately obvious for this kind of back-phosphorylation strategy: 1) NMHD receptors are not phosphorylated at the sites probed by back-phosphorylation with PKA or PKC; 2) NMHD receptors are phosphorylated in vivo, but not efficiently dephosphorylated by endogenous phosphatases; 3) NMHD receptors are phosphorylated in vivo and effectively dephosphorylated after homogenization in phosphatase inhibitor-free buffer. No difference in [32P]phosphate incorporation into NMHD receptors prepared in the absence and presence of phosphatase inhibitors would have been expected in the first two scenarios. However, the difference in [32P]phosphate incorporation was quite striking. Most samples obtained in the presence of phosphatase inhibitors showed 40–70% less [32P]phosphate incorporation than those isolated in the absence of those inhibitors (Fig. 6). These results suggest that a large portion of the in vitro PKA and PKC phosphorylation sites had been phosphorylated in vivo before homogenization of the tissue.

A phosphatase inhibitor-induced decrease in back-phosphorylation of NR1 as well as NR2 subunits by PKA and PKC was consistently observed in hippocampal and cortical samples. However, back-phosphorylation by PKA of NR2 subunits isolated from the cerebellum was only slightly decreased when phosphatase inhibitors were present during the preparation (Fig. 6). This result cannot be explained by a variability in the amount of NMHD receptor present during the back-phosphorylation or in the efficacy of the back-phosphorylation reaction itself, because back-phosphorylation of NR1 was strongly reduced by phosphatase inhibitors in the very same samples. These observations may rather reflect differences in the subunit composition of NMHD receptors. NR2C is nearly exclusively expressed in the cerebellum of adult rats (11–13). It may substitute for NR2A and NR2B in many cerebellar NMHD receptor complexes and may not be extensively phosphorylated under normal conditions by PKA. In fact, when NR1 is coexpressed with NR2C in Xenopus oocytes, the resulting NMHD receptor is not modulated by PKC stimulation with phorbol esters, although NR1 when coexpressed with NR2A or NR2B in the same system is potentiated by phorbol esters (37).

Similarly, phosphatase inhibitors had only a slight effect on the back-phosphorylation by PKC of NR1 in NMHD receptor complexes isolated from the cerebellum. NR2 back-phosphorylation by PKC, however, was strongly reduced by these inhibitors in the same samples (Fig. 6). NR2C, which is specifically expressed in the cerebellum (Refs. 11–13; see above) may prevent phosphorylation of PKC sites in NR1 despite the fact that NR2C itself may be phosphorylated by PKC in vivo. Moreover, a number of splice variants exist for NR1 that exhibit differential distribution in the rat brain (54) and may be phosphorylated to different degrees in vivo (16). It is therefore possible that splicing isoforms of NR1 that are not significantly phosphorylated by PKC prevail in the cerebellum.

PKA and PKC appear to target different sites during in vitro phosphorylation as demonstrated by two-dimensional phosphopeptide mapping (Fig. 4), and both kinases are equally efficient in phosphorylating NR1 in vitro (Table I). Taken together, these findings suggest that the PKA sites had been phosphorylated by a PKA-like activity rather than by PKC isoforms in the intact brain and vice versa. However, it is not possible to rule out that some cross-phosphorylation between PKA and PKC sites occurs in vivo or that other arginine/lysine-guided serine/threonine protein kinases, such as Ca2+- and calmodulin-dependent protein kinase II and cyclic GMP-dependent protein kinase, may have contributed to the phosphorylation in vivo.

We did not investigate the phosphorylation of NR2C and NR2D. Coexpression of NR1 with NR2C or NR2D resulted in the expression of NMHD receptors that are not responsive to PKC stimulation by phorbol esters (37). In addition, expression of NR2D is generally very low and that of NR2C is only clearly detectable in the cerebellum in adult rats (11–13).

Physiological Relevance of NMHD Receptor Phosphorylation—Ca2+ influx through NMHD receptors is crucial for brain development (4, 5) and synaptic plasticity (6–8). The timing and probably the magnitude of Ca2+ influx are critical in determining which form of plasticity will occur. For example, homosynaptic LTP at the Schaffer collateral CA1 synapse in the hippocampus usually requires several high frequency tetani. In contrast, low frequency stimulations (1 Hz) over minutes lead to a lasting depression of signal transmission at this synapse (8). This phenomenon, which is known as long term depression, also requires Ca2+ influx. Since PKA and PKC increase the activity of NMHD receptors and thereby Ca2+ influx (11, 29–39), phosphorylation of NMHD receptors by these two kinases may promote the establishment of LTP. In this context it is noteworthy that various PKC inhibitors, including a PKC-specific pseudosubstrate peptide inhibitor, when injected postsynaptically prevent LTP in CA1 (6) and that inhibition of PKA by Rp-cyclic adenosine 3′,5′-monophosphorylthioate blocks the late phase of CA1 LTP (55). These effects may in part be due to the inhibition of tonic phosphorylation of the NMHD receptor by PKA and PKC. On the other hand, activation of phosphatases reduces the activity of NMHD receptors (40–42) and is necessary for long term depression (56, 57). Interestingly, NMHD receptor activity is reduced during a train of four stimuli in hippocampal cell cultures, and this down-regulation is prevented by noradrenergic stimulation of PKA (39). Noradrenergic input may, therefore, reverse this phosphorylation-sensitive depotentiation of NMHD receptors, thereby fostering the induction of LTP. Collectively, these observations suggest that the induction of NMHD receptor-dependent synaptic plasticity is fine-tuned by PKA and PKC activities that are opposed by phosphatase activity.

Another potential function of PKA and PKC phosphorylation of the NMHD receptors is to control their subcellular distribution. Certain splice variants of the NR1 subunit formed clusters when expressed in QT6 quail fibroblasts, and these clusters were disrupted upon treatment with PKC-stimulating phorbol esters (58). Phosphorylation of NMHD receptor subunits by PKC may mediate this PKC-induced disruption of receptor
clusters. NMDA receptor subunits bind with their COO−-terminal end to PSD-95/SAP90 (59), a protein specifically localized at postsynaptic sites (60). Coexpression of NMDA receptors and PSD-95/SAP90 in heterologous cell lines causes clustering of NMDA receptors that are evenly distributed in the plasma membrane when expressed alone (61). Several K+ channels also interact with and are clustered by PSD-95/SAP90 (62). One of the K+ channels binding to PSD-95/SAP90 is the inward rectifier Kir 2.3. PKA phosphorylation disrupts the interaction between this channel and PSD-95/SAP90 in vitro (63). It is therefore tempting to speculate that PKA or PKC phosphorylation controls the postsynaptic clustering of NMDA receptors by regulating their interaction with cytoskeletal structures.

Regulation of gene transcription by NMDA receptor-mediated activation of the transcription factors serum response factor and Elk-1 has recently been shown by Xia et al. (64). An increase of NMDA receptor activity caused by phosphorylation by PKA or PKC may therefore modulate the expression of genes under the control of serum response factor or Elk-1.

Ca2+-permeable glutamate receptors also play a critical role during neuropathological conditions. Overstimulation of glutamate receptors, especially the NMDA subtype, has been implicated in neuropathological conditions caused by ischemia as occurring during stroke, by status epilepticus, and by brain trauma (65–67). PKA and PKC phosphorylation of NMDA receptors may enhance NMDA receptor-induced neurotoxicity. Inhibiting PKA during or immediately after ischemic episodes, possibly by blocking noradrenergic receptors, may therefore be one strategy to alleviate subsequent neuronal damage.

In summary, our data demonstrate that NMDA receptors are substrates for PKA and PKC. Since NMDA receptor activity is modulated by PKA- and PKC-mediated phosphorylation in intact neurons, direct phosphorylation of NMDA receptors by PKA or PKC may modulate NMDA receptor-regulated neuronal functions including the induction of synaptic plasticity and gene expression.

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