A Dopamine/D1 Receptor/Protein Kinase A/Dopamine- and cAMP-Regulated Phosphoprotein (M_r 32 kDa)/Protein Phosphatase-1 Pathway Regulates Dephosphorylation of the NMDA Receptor

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We have investigated the mechanism by which activation of dopamine (DA) receptors regulates the glutamate sensitivity of medium spiny neurons of the nucleus accumbens. Our results demonstrate that DA regulates the phosphorylation state of the NR1 subunit of NMDA-type glutamate receptors. The effect of DA was mimicked by SKF82526, a D1-type DA receptor agonist, and by forskolin, an activator of cAMP-dependent protein kinase (PKA), and was blocked by H-89, a PKA inhibitor. These data indicate that DA increases NR1 phosphorylation through a PKA-dependent pathway. DA-induced phosphorylation of NR1 was blocked in mice bearing a targeted deletion of the gene for dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa (DARPP-32), a phosphoprotein that is a potent and selective inhibitor of protein phosphatase-1, indicating that the effect of PKA is mediated, in part, by regulation of the DARPP-32/protein phosphatase-1 cascade. In support of this interpretation, NR1 phosphorylation was increased by calyculin A, a protein phosphatase-1/2A inhibitor. A model is proposed in which the ability of DA to regulate NMDA receptor sensitivity is attributable to a synergistic action involving increased phosphorylation and decreased dephosphorylation of the NR1 subunit of the NMDA receptor.

Key words: D1 receptor; NMDA receptor; protein phosphatase-1; DARPP-32; protein kinase C; D2 receptor

DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa, is a cytosolic protein that is selectively enriched in dopaminoceptive brain neurons, including the medium spiny neurons of neostriatum and nucleus accumbens (Walaas et al., 1983; Ouimet et al., 1984). Dopamine (DA), by acting on the D1 class of DA receptors, stimulates adenylyl cyclase to increase cAMP formation and the activity of cAMP-dependent protein kinase (PKA), leading to the phosphorylation of DARPP-32 on a single threonine residue, Thr^{34}, converting it into a potent inhibitor of protein phosphatase-1 (PP-1) (Hemmings et al., 1984). Conversely, glutamate, by acting on NMDA receptors, increases calcium influx and increases the activity of the calcium/calmodulin-dependent protein phosphatase calcineurin, which dephosphorylates DARPP-32, thereby inactivating it (King et al., 1984; Nishi et al., 1997). The control of PP-1 activity by DARPP-32 is likely to have a significant role in the regulation of neuronal excitability (Fienberg et al., 1998). For example, the functions of calcium channels (Surmeier et al., 1995), voltage-dependent sodium channels (Surmeier et al., 1992; Schiffman et al., 1995), and Na^+,K^+-ATPase (Aperia et al., 1991) are regulated directly or indirectly by PP-1.

In mammals, D1 receptors enhance the currents evoked by NMDA agonists (Cepeda et al., 1993; Harvey and Lacey, 1997; Hernandez-Lopez et al., 1997; Cepeda and Levine, 1998). This effect of DA is mediated by a PKA-dependent pathway (Colwell and Levine, 1995; Blank et al., 1997), which most likely involves the phosphorylation and activation of DARPP-32 (Blank et al., 1997). The mechanism by which the DA/D1 receptor/PKA/DARPP-32 pathway is able to control the activity of the NMDA receptor has not been elucidated. However, NMDA receptors, which are heteromeric complexes composed of two families of receptor subunits NR1 and NR2, are substrates for phosphorylation by serine/threonine protein kinases (Hollmann and Heinemann, 1992). In purified systems, the NR1 subunit, which is required for a functional NMDA receptor, is phosphorylated by PKA (Tingley et al., 1993; Leonard and Hell, 1997). We report here that a DA/D1 receptor/PKA/DARPP-32/PP-1 pathway, by regulating the rate of dephosphorylation of NR1, provides an additional and essential molecular mechanism for the control by DA of NMDA receptor function in the nucleus accumbens.

MATERIALS AND METHODS

Preparation, radioactive labeling, and treatment of nucleus accumbens slices. Male Sprague Dawley rats (150–200 gm) were killed by decapitation. The brain was rapidly removed from the skull and transferred to an ice-cold surface where it was blocked and then mounted to the cutting surface of a Vibratome (TPI). Coronal sections (500 μm) of the brain were cut and pooled in 10 ml of ice-cold, oxygenated phosphate-free Krebs' bicarbonate buffer containing the following components (in mM): 125 NaCl, 4 KCl, 26 $NaHCO_3$, 1.5 $CaCl_2$, 1.5 MgSO, and 10 glucose, pH 7.4. Slices of nucleus accumbens (Pellegrino et al., 1979) were dissected from these coronal sections under a dissecting microscope. The slices were pooled in a dish of cold buffer and then transferred individually to 4 ml polypropylene centrifuge tubes containing 2 ml of fresh buffer at 4°C. The Krebs' bicarbonate buffer was then replaced with fresh solution. The tubes were connected to an oxygenation manifold supplying a 95% O_2/5% CO_2 mix and maintained in a 30°C water bath. After 15 min the buffer was replaced with fresh, oxygenated Krebs' bicarbonate buffer

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containing 2.0 mCi of [^{32}P]orthophosphoric acid (DuPont NEN, Boston, MA) (specific activity 8500–9120 Ci/mmol), and the tissue was preincubated for 60 min. The radioactive buffer was then removed, and tissue sections were rinsed twice with 2 ml of fresh buffer. The tissue was incubated for 30 sec to 60 min in the absence or presence of test substances, as indicated. At the end of the incubation, the buffer was rapidly aspirated, and the tissue slices were immediately frozen in liquid nitrogen and stored at −80°C until assayed.

In some experiments nucleus accumbens slices were prepared, as described above for rat brain, from wild-type C57BL/6 mice (8–10 weeks of age) and mice that lack the gene for DARPP-32 (Fienberg et al., 1998). DARPP-32 mutant mice and their wild-type controls were generated from the offspring of heterozygous mating pairs. All mice were age-matched, and only males were used.

Immunoprecipitation and analysis of [^{32}P]phosphate-labeled NR1. [^{32}P]phosphate-labeled tissue slices were sonicated in 150 μl of 1% SDS containing NaF (50 mM) and 1 mM EGTA added as phosphatase inhibitors, and a mixture of protease inhibitors, including 25 mM benzamidine, 100 μM phenylmethylsulfoxide, 20 μg/ml chymostatin, 20 μg/ml pepstatin A, 5 μg/ml leupeptin, and 5 μg/ml antipain (Peptide International). To this homogenate was added 5 vol of Buffer A, composed of 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumin (BSA), and the mixture of phosphatase and protease inhibitors described above. Aliquots of the homogenate (10 μl) were retained for the determination of total [^{32}P]phosphate incorporation into trichloroacetic acid (TCA)-precipitated protein. A 10 mg aliquot of pre-swollen protein A-Sepharose CL-4B (Pharmacia Biotech) was added to each sample, and the mixture was agitated for 30 min at 4°C. The Sepharose beads were pelleted by centrifugation for 15 sec at 2000 rpm in a tabletop microcentrifuge. The supernatant was transferred to tubes containing 3 μg of a monoclonal antibody (54.1; PharMingen, San Diego, CA) generated against the peptide sequence representing the intracellular loop linking the putative transmembrane regions III and IV (amino acids 660–811) of the NMDA receptor subunit NR1. The samples were mixed for 2 hr at 4°C and then for an additional hour with 25 μl of a rabbit anti-mouse antibody (1 mg/ml) (Organon Teknika Cappel, Durham, NC). The homogenates were transferred to fresh 1.5 ml Eppendorf tubes containing 10 mg of pre-swollen protein A-Sepharose CL-4B beads and mixed for 1 hr at 4°C. The beads were pelleted by centrifugation and washed once with 1 ml of Buffer A: three times with 1 ml of a buffer containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.2% BSA; three times with a buffer containing 20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.5% Triton X-100, and 0.2% BSA; and once with 1 ml of a buffer containing 50 mM Tris/HCl, pH 8.0. After the final wash the beads were resuspended in 50 μl of a SDS-PAGE sample buffer composed of 50 mM Tris/HCl, pH 6.7, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.01% bromophenol blue. The tubes were vortexed vigorously, and the beads were centrifuged. The recovered proteins were separated on 7.5% acrylamide gels (Laemmli, 1970). The gels were dried, and [^{32}P]phosphate incorporation into NR1 was quantified using a PhosphorImager 400B and ImageQuant software from Molecular Dynamics (Sunnyvale, CA). Values for [^{32}P]phosphate content were corrected for the total [^{32}P]phosphate incorporated into TCA-precipitable protein.

RESULTS

Phosphorylation of NR1 in the nucleus accumbens by activators of PKA and PKC

Immunoprecipitation of [^{32}P]phosphate-labeled protein from rat nucleus accumbens homogenates, using a monoclonal antibody generated against NR1, yielded a single prominent protein band on SDS-PAGE gels with an apparent M_r of 116 kDa. A low level of basal phosphorylation was detected in untreated [^{32}P]phosphate-labeled slices. Forskolin, which by activating adenylyl cyclase stimulates PKA, and phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C (PKC), each increased the phosphorylation state of this protein band (Fig. 1). Results comparable to these were obtained in experiments using rat neostriatal slices (data not shown).

To verify the identity of the 116 kDa protein, nucleus accumbens slices were treated with forskolin (10 μM) plus PDBu (5 μM).

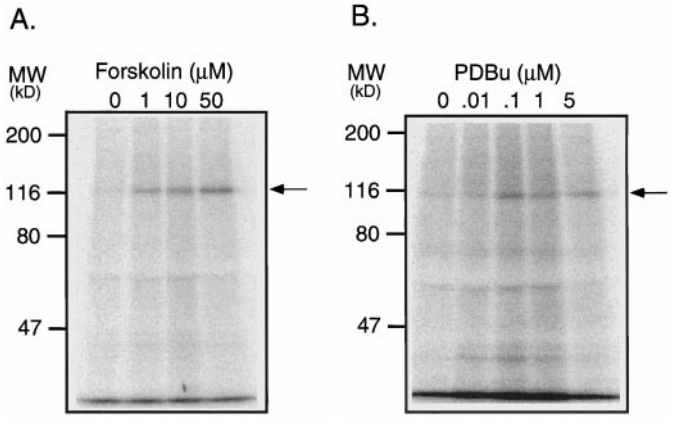

Figure 1. Effect of forskolin or PDBu on the phosphorylation of the NR1 subunit of the NMDA receptor. Nucleus accumbens slices, which had been prelabeled with [^{32}P]phosphate for 60 min, were incubated for 5 min with various concentrations of either forskolin (*A*) or PDBu (*B*). Autoradiogram shows incorporation of [^{32}P]phosphate into NR1. *Arrow* indicates the protein band corresponding to NR1.

Table 1. Effects of D2 receptor agonist and D2 receptor antagonist on D1-stimulated increase in NR1 phosphorylation in nucleus accumbens slices

	[^{32}P]phosphate-labeled NR1 (%)	
Treatment	(−)Raclopride	(+)Raclopride
Control	50 ± 4	44 ± 19
D1 agonist	100	88 ± 6
D1 + D2	49 ± 4*	98 ± 16
D2 agonist	43 ± 7	

Nucleus accumbens slices were prelabeled with [^{32}P]phosphate. Slices were incubated for a total of 15 min. Raclopride (1 μM) was added at 0 min, and D1 agonist (SKF81297, 1 μM) and D2 agonist (quinpirole, 1 μM) were added at 10 min. [^{32}P]phosphate incorporation into NR1 was determined as described in Materials and Methods. Data were calculated as percentage of radioactivity in slices incubated with D1 agonist and represent means ± SEM ($^*p < 0.05$, compared with D1 agonist alone, Mann-Whitney *U* test; $n = 4$–10 experiments).

Immunoprecipitation of NR1 from these slices was abolished by preadsorption of the NR1-specific monoclonal antibody with 10 μg/ml of a hexahistidine fusion protein containing the region between the third and fourth transmembrane domains of NR1 (residues 660–811) (data not shown).

Phosphorylation of NR1 by dopamine receptor agonists

Treatment of nucleus accumbens slices with DA (100 μM) in the presence of nomifensine (10 μM), an inhibitor of DA reuptake, increased [^{32}P]phosphate content of immunoprecipitated NR1 (Fig. 2). The [^{32}P]phosphate content of NR1 was increased about threefold within 1 min of DA addition. NR1 phosphorylation decreased to near basal levels by 10 min of incubation.

DA receptors have been divided into D1 and D2 subclasses (for review, see Sibley and Monsma, 1992). SKF82526 (1 μM) (Fig. 3) and SKF81297 (1 μM) (Table 1), agonists at D1-type DA receptors, each increased [^{32}P]phosphate incorporation into NR1 by two- to fourfold within 5 min of incubation and maintained phosphorylation of NR1 for at least 10 min. In contrast, the D2-type receptor agonist quinpirole alone had no significant effect on the state of phosphorylation of NR1 under the same conditions (Fig. 3).

Activation of D2-type DA receptors blocks D1-stimulated in-

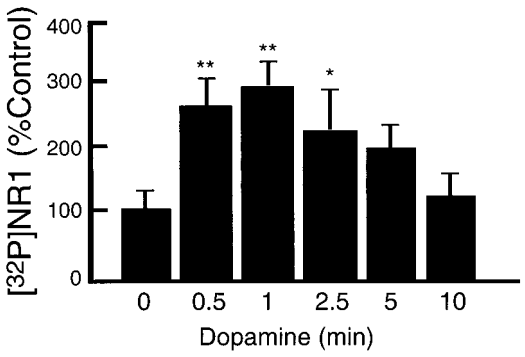

Figure 2. Effect of DA on the phosphorylation of NR1. Nucleus accumbens slices, which had been prelabeled with [^{32}P]phosphate for 60 min, were incubated for 0.5–10 min with dopamine (100 μM) plus nomifensine (10 μM), a dopamine uptake inhibitor. [^{32}P]phosphate incorporation into NR1 was determined as described in Materials and Methods. The data were calculated as percentage of radioactivity in control slices and represent means ± SEM for five (0–5 min) or three (10 min) experiments (different from control; $^*p < 0.05$, $^{**}p < 0.01$; Student's *t* test).

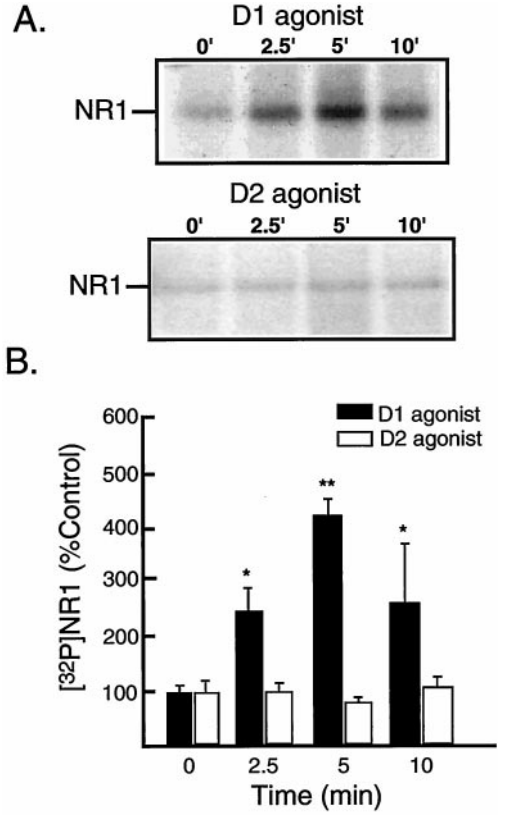

Figure 3. Effect of D1-type and D2-type dopamine agonists on the state of NR1 phosphorylation. Nucleus accumbens slices, which had been prelabeled with [^{32}P]phosphate for 60 min, were incubated for 2.5–10 min with a D1 agonist (SKF82526, 1 μM) or a D2 agonist (quinpirole, 1 μM), as shown in autoradiograms (*A*) and a bar graph (*B*). [^{32}P]phosphate incorporation into NR1 was determined as described in Materials and Methods. Data were calculated as percentage of radioactivity in control slices and represent means ± SEM for four experiments (different from control; $^*p < 0.05$, $^{**}p < 0.01$; Student's *t* test).

creases in DARPP-32 phosphorylation both by reducing PKA-dependent phosphorylation of DARPP-32 and by increasing dephosphorylation of DARPP-32 by calcineurin (Nishi et al., 1997). We examined whether D2 receptor activation would also modulate D1-stimulated phosphorylation of NR1. The ability of the D1-type receptor agonist SKF81297 to increase the phosphorylation of NR1 was abolished by the simultaneous presence of quinpirole (Table 1).

We also examined whether raclopride, a potent neuroleptic drug, might affect the phosphorylation state of NR1. Pretreatment of slices with raclopride inhibited the ability of quinpirole to decrease D1-stimulated NR1 phosphorylation (Table 1).

Table 2. Effect of protein kinase inhibitors on DA-stimulated phosphorylation of NR1 in nucleus accumbens slices

Treatment	[^{32}P]phosphate-labeled NR1 (%)
A.	
Control	26 ± 6
H-89	32 ± 5
Dopamine/nomifensine	100
H-89 + Dopamine/nomifensine	35 ± 4*
B.	
Control	47 ± 9
Calphostin	54 ± 12
Dopamine/nomifensine	100
Calphostin C+ Dopamine/nomifensine	108 ± 29
C.	
Control	29 ± 9
PDBu	100
Calphostin C + PDBu	26 ± 2**

Nucleus accumbens slices were prelabeled with [^{32}P]phosphate. Slices were incubated for a total of 20 min. A PKA inhibitor (H-89, 0.5 μM) or a PKC inhibitor (calphostin C, 1 μM) was added at 0 min, followed by addition of either PDBu (5 μM) at 15 min or DA (100 μM) plus nomifensine (10 μM) at 19 min. [^{32}P]phosphate incorporated into NR1 was determined as described in Materials and Methods. The data were calculated as percentage of radioactivity in stimulated slices and represent means ± SEM for three to five experiments ($^*p < 0.05$ compared with dopamine/nomifensine; $^{**}p < 0.05$ compared with PDBu; Student's *t* test).

The effect of protein kinase inhibitors on DA-induced NR1 phosphorylation

Selective protein kinase inhibitors were used to assess the relative involvement of intracellular signaling pathways involving PKA or PKC in the regulation by dopamine of the phosphorylation state of NR1. Preincubation of nucleus accumbens slices with H-89, an inhibitor of PKA (0.5 μM), had no significant effect on basal phosphorylation of NR1 but did abolish the increase in [^{32}P]phosphate content of NR1 induced by treatment with dopamine (Table 2A). In contrast, calphostin C (1 μM), an inhibitor of PKC, had no significant effect on either the basal or the DA-stimulated phosphorylation of NR1 in nucleus accumbens slices (Table 2B). Under these conditions, calphostin C treatment fully blocked phosphorylation of the receptor subunit in response to PDBu (5 μM) (Table 2C). These experiments indicate that the increase in [^{32}P]phosphate incorporation into NR1 induced by DA treatment involves activation of PKA but not PKC.

The role of the DARPP-32/protein phosphatase-1 pathway in the regulation of NR1 phosphorylation

It seemed possible that the ability of the DA/D1 receptor/PKA pathway to increase the state of phosphorylation of NR1 might be attributable to direct phosphorylation of NR1 by PKA and/or to a decreased dephosphorylation of NR1 mediated by the PKA/DARPP-32/PP-1 pathway (see Fig. 7). One way to evaluate the potential role of the DARPP-32/PP-1 pathway in the regulation of NR1 phosphorylation was to examine the effects of protein

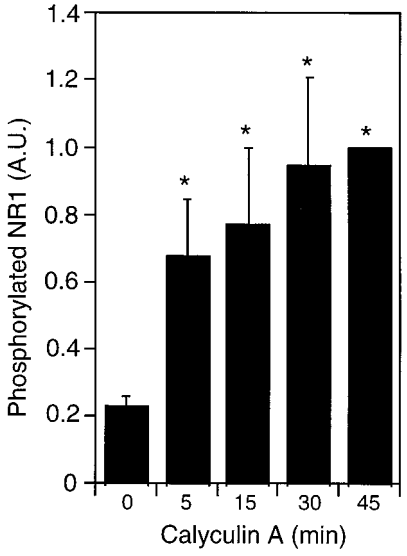

Figure 4. Effect of calyculin A, a protein phosphatase-1/2A inhibitor, on the phosphorylation state of NR1. Nucleus accumbens slices, which had been prelabeled with [^{32}P]phosphate, were incubated for 5–45 min in the absence or presence of calyculin A (200 nM). [^{32}P]phosphate-labeled NR1 was analyzed as described in Materials and Methods. Data were normalized to radioactivity in slices incubated for 45 min with calyculin A and represent means ± SEM for four experiments (different from control; $^{*}p < 0.05$; Mann–Whitney *U* test). Incubation of slices in the absence of calyculin A for 45 min had no effect on the amount of [^{32}P]phosphate-labeled NR1.

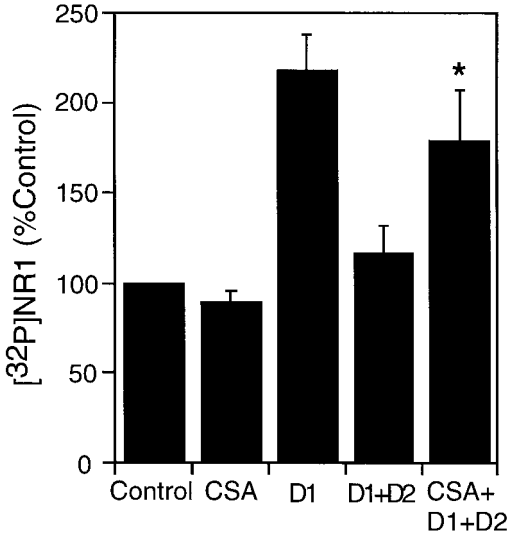

Figure 5. Effect of cyclosporin A, a calcineurin inhibitor, on the phosphorylation state of NR1. Nucleus accumbens slices were prelabeled with [^{32}P]phosphate for 60 min. Slices were incubated for a total of 60 min in the absence (control) or presence of cyclosporin A (*CSA*) (5 μM). At 55 min, a D1 agonist (SKF81297, 1 μM) and/or or a D2 agonist (quinpirole, 1 μM) was added. [^{32}P]phosphate incorporation into NR1 was determined as described in Materials and Methods. Data were calculated as percentage of radioactivity in control slices and represent means ± SEM for four experiments (different from *D1+D2* alone; $^{*}p < 0.05$; Student's *t* test). CSA had no effect on the SKF81297-stimulated phosphorylation of NR1.

phosphatase inhibitors on NR1 phosphorylation. Treatment of rat nucleus accumbens slices with calyculin A, a potent PP-1/2A inhibitor, caused a severalfold increase in NR1 phosphorylation (Fig. 4). These experiments indicate a role for PP-1 or -2A in controlling the state of phosphorylation of NR1 in the nucleus accumbens.

Activation of calcineurin by calcium has been shown in purified systems and in tissue slices to promote dephosphorylation and inactivation of DARPP-32 (King et al., 1984; Halpain et al., 1990; Nishi et al., 1997). As one way of evaluating the possible role of DARPP-32 in the regulation of the state of phosphorylation of NR1, we tested the ability of cyclosporin A, a potent inhibitor of calcineurin, to affect NR1 phosphorylation. Pretreatment of slices with cyclosporin A blocked the ability of D2 receptor activation to decrease the D1 receptor-stimulated phosphorylation of NR1 (Fig. 5), consistent with a role for DARPP-32 in the regulation of NR1 phosphorylation.

To further evaluate the role of DARPP-32 in the regulation of NR1 phosphorylation, we examined the ability of the receptor to be phosphorylated in mice bearing a targeted deletion of the gene for DARPP-32. As in rat nucleus accumbens, a low level of basal [^{32}P]phosphate incorporation was observed in untreated slices of mouse nucleus accumbens. Incubation with DA (100 μM) plus nomifensine (10 μM) for 1 min induced approximately a three- to fourfold increase in [^{32}P]phosphate content of NR1 in accumbens slices prepared from wild-type C57BL/6 mice (Fig. 6*A*). Phosphorylation of the receptor increased to a maximal level within 1 min of DA treatment and remained high for >5 min, gradually decreasing toward basal levels after 10 min of drug exposure (data not shown). The DA-induced increase in NR1 phosphorylation was virtually abolished in nucleus accumbens slices from DARPP-32 mutant mice at 1 min (Fig. 6*A*) as well as at 2 and 5 min (data not shown) of DA treatment.

The inability of DA to stimulate phosphorylation of NR1 in mutant tissue could not be accounted for by a decrease in the number or sensitivity of D1 receptors, because the B_{max} and K_d for [^{3}H]SCH23390 binding were unaffected by DARPP-32 mutation (P. Svenningsson, A. A. Fienberg, and B. Fredholm, unpublished observations). This difference in phosphorylation of NR1 was also not caused by differences in labeling efficiency between mutant and wild-type tissue slices, because the total [^{32}P]phosphate incorporation into protein, as measured by TCA-precipitable counts, was unaffected by the DARPP-32 mutation (data not shown). The alterations in DA-stimulated NR1 phosphorylation were not caused by a difference in the total amount of receptor protein expressed in mutant tissue because the levels of NR1 protein detected by immunoblotting were comparable in wild-type and mutant mice. They were not caused by changes in the basal level of phosphorylation of NR1 (data not shown). Finally, these differences were not attributable to alterations in the level of PP-1 protein nor in the activity of either PKA or adenylyl cyclase in the DARPP-32 mutant mice (Fienberg et al., 1998).

The phosphorylation of NR1 in response to application of DA was compared with that observed in response to activation of either PKA by forskolin or PKC by PDBu. Treatment of slices with forskolin (1 μM) for 5 min increased NR1 phosphorylation significantly in tissue slices from wild-type mice but had a smaller effect on NR1 phosphorylation in slices from the DARPP-32 mutant mice (Fig. 6*B*). In contrast to the diminished increase in the [^{32}P]phosphate content of NR1 seen in mutant tissue in response to DA or forskolin, PDBu treatment for 15 min increased NR1 phosphorylation in both the wild-type and mutant tissue by four- to fivefold (Fig. 6*C*).

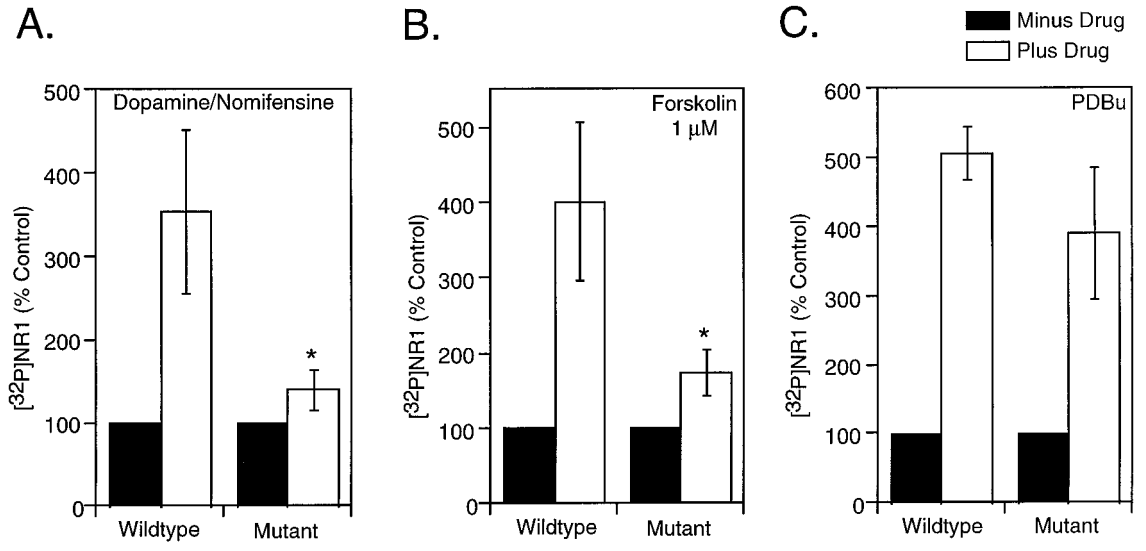

Figure 6. Effect of genetic deletion of DARPP-32 on the state of phosphorylation of NR1. Nucleus accumbens slices prepared from wild-type and DARPP-32 mutant mice were prelabeled with [^{32}P]phosphate for 60 min and then incubated in the absence or presence of DA (100 μM) plus nomifensine (10 μM) for 1 min (*A*), forskolin (1 μM) for 5 min (*B*), or PDBu (5 μM) for 15 min (*C*). [^{32}P]phosphate incorporation into NR1 was determined as described in Materials and Methods. Data were calculated as percentage of radioactivity in control slices and represent means ± SEM for three to six experiments (different from wild type; * $p < 0.05$; Student's *t* test).

DISCUSSION

The present results show that phosphorylation of the NMDA receptor subunit NR1 is regulated by a DA/D1 receptor/PKA/DARPP-32/PP-1 cascade in medium spiny neurons of the nucleus accumbens. Our data indicate that activation of DA receptors increases the state of phosphorylation of NR1 in these cells through a synergistic mechanism involving increased phosphorylation and decreased dephosphorylation of the NR1 receptor (Fig. 7). These data are consistent with evidence that NMDA receptor function is regulated by protein kinases and protein phosphatases.

The role of the DARPP-32/PP-1 cascade in regulating currents evoked by NMDA agonists has been studied in homomeric NR1 subunits expressed in *Xenopus* oocytes (Blank et al., 1997). In oocytes injected with rat neostriatal poly(A$^+$) mRNA, activation of PKA by forskolin potentiated NMDA responses. Coinjection of oocytes with neostriatal mRNA and antisense oligodeoxynucleotides directed against DARPP-32 reduced the PKA enhancement of NMDA responses. NMDA responses recorded from oocytes injected with rat hippocampal poly(A$^+$) mRNA were not affected by stimulation of PKA, indicating that the amount of inhibitor-1, a compound similar in structure and function to DARPP-32, which is present in hippocampus, is not sufficiently abundant to mediate the effect of forskolin on NMDA responses. However, when oocytes were coinjected with hippocampal poly(A$^+$) mRNA plus complementary RNA coding for DARPP-32 (resulting in a greatly elevated level of this PP-1 inhibitor), NMDA responses were potentiated after stimulation with PKA. These data suggest that the DARPP-32/PP-1 cascade plays a major role in regulating responses to NMDA agonists and are supported by other studies showing that drugs that inhibit the activity of PP-1/-2A also enhance NMDA responses in *Xenopus* oocytes (Blank et al., 1997) and in neurons (Wang et al., 1994).

It is plausible to hypothesize that phosphorylation/dephosphorylation of the NMDA receptor contributes to the mechanism by which activation of DA receptors regulates the glutamate

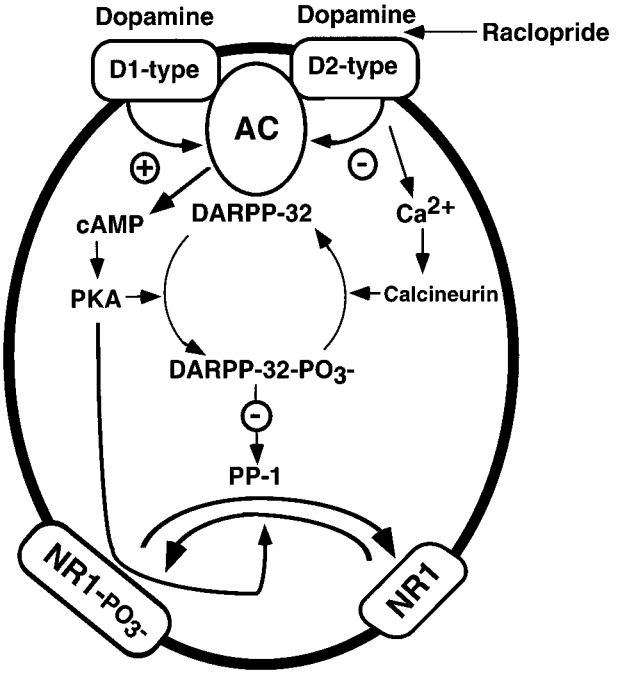

Figure 7. Proposed mechanism for regulation by dopamine of the state of phosphorylation of NR1. In this model, dopamine, by activation of D1-type DA receptors, increases adenylyl cyclase activity, leading to formation of cAMP and activation of PKA. Activated PKA phosphorylates both NR1 and DARPP-32. Phosphorylated DARPP-32 inhibits dephosphorylation of NR1 by PP-1. The activation of D2-type DA receptors, which is blocked by raclopride, (1) inhibits cAMP formation, leading to a decreased phosphorylation both of NR1 and of DARPP-32, and (2) increases calcium-dependent activation of calcineurin (Nishi et al., 1997), leading to increased dephosphorylation of DARPP-32. *D1-type*, D1-type dopamine receptor; *D2-type*, D2-type dopamine receptor; *AC*, adenylyl cyclase; *PKA*, cAMP-dependent protein kinase; *DARPP-32*, dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa; *PP-1*, protein phosphatase-1; *NR1*, NMDA receptor subunit NR1.

sensitivity of medium spiny neurons. It seems likely that alterations in L-type calcium conductances are also involved in the mechanism by which PKA pathways involving DARPP-32 enhance currents evoked by NMDA agonists. It was shown by Surmeier et al. (1995) that L-type calcium conductances were increased by activation of a D1/PKA/PP-1/DARPP-32 cascade in medium spiny neurons. Moreover, the D1 receptor-mediated enhancement of NMDA-evoked currents in medium spiny neurons is dependent on enhancement of L-type calcium conductances (Hernandez-Lopez et al., 1997; Cepeda et al., 1998). Presumably, the NMDA-evoked currents depolarized the dendrites leading to activation of L-type calcium channels. According to this scenario, when calcium function was enhanced by D1 receptor stimulation, the larger magnitude of the depolarization was caused by activation of both NMDA receptors and calcium channels.

Preliminary results using wild-type and DARPP-32 mutant mice and phosphorylation state-specific antibodies against individual phosphorylation sites on NR1 indicate that the DA-mediated increases in NR1 phosphorylation demonstrated in the present study occur at the PKA site (serine^{897}) and at a PKC-dependent phosphorylation site (serine^{890}) (Snyder et al., 1998). These data imply that PKC-dependent phosphorylation sites that are substrates for PP-1 can be regulated by DA through activation of a D1 receptor/DARPP-32/PP-1 pathway.

The functional effect of phosphorylation of NR1 at the PKA-dependent site at serine^{897} (Tingley et al., 1997) has yet to be clarified. However, there is substantial evidence to indicate that PKC-dependent phosphorylation enhances the conductance of native NMDA receptors in spinal cord and hippocampal neurons (Chen and Huang, 1991, 1992). The specific role of NR1 phosphorylation in regulating various properties of the NMDA receptor has been examined using NR1 subunits expressed in fibroblasts. In these studies phosphorylation of NR1 by PKC (at serine^{889} and serine^{890}) disrupted the subcellular distribution of NR1 protein, suggesting that phosphorylation may play an important role in receptor clustering (Ehlers et al., 1995). Studies in HEK-293T cells indicate that calmodulin binds to sites in the COOH-terminal region of NR1 (Ehlers et al., 1996; Hisatsune et al., 1997) and that calmodulin binding to NR1 results in a fourfold decrease in NMDA channel open probability (Ehlers et al., 1996). PKC-dependent phosphorylation of NR1 (also in the COOH-terminal region) inhibits calmodulin binding, suggesting a possible role for NR1 phosphorylation in modulating the Ca^{2+}-dependent inactivation of the NMDA receptor (Hisatsune et al., 1997).

D1 and D2 subclasses of DA receptors exerted opposing effects on the phosphorylation state of NR1 in the nucleus accumbens. The activation of D2 receptors blocked a D1 receptor-mediated increase in NR1 phosphorylation, an effect that was prevented by cyclosporin A, an immunosuppressant drug that inhibits calcineurin (Fig. 7). These data are consistent with a previous report showing that D2 agonists activate calcineurin, leading to a decrease in the state of phosphorylation of DARPP-32 in these neurons (Nishi et al., 1997). The precise mechanism for the activation of calcineurin has not been elucidated. However, because these experiments were performed in tissue slices, these effects may involve activation of D2 receptors on medium spiny neurons and/or activation of D2 receptors on terminals of afferent projections to the nucleus accumbens (Nishi et al., 1997). The D2-mediated decrease in NR1 phosphorylation was also blocked by raclopride, a neuroleptic drug that is a D2 receptor antagonist. These data imply that typical antipsychotic drugs, which like raclopride block the binding of DA to D2 receptors, may achieve part of their clinical effect through the regulation of DA-stimulated phosphorylation of NR1.

Certain of the behavioral and biochemical manifestations of drug addiction have been attributed to interactions between D1-type dopamine receptors and NMDA-type glutamate receptors in the nucleus accumbens (Self and Nestler, 1995; for review, see Hyman, 1996; Koob and LeMoal, 1997). For example, the ability of amphetamine to induce behavioral sensitization (Wolf and Jeziorski, 1993) and to increase the expression of immediate early genes in rodents (Konradi et al., 1996) is dependent on stimulation of both D1 and NMDA receptors. The precise nature of the D1/NMDA interaction responsible for these effects is still not fully understood. The results of the present study provide a molecular mechanism by which the DA/D1 receptor pathway might interact with the NMDA receptor to produce some of these observed responses.

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