Amplification of dopaminergic signaling by a positive feedback loop

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Dopamine and cAMP-regulated phosphoprotein of M_r 32,000 (DARPP-32) plays an obligatory role in most of the actions of dopamine. In resting neostriatal slices, cyclin-dependent kinase 5 (Cdk5) phosphorylates DARPP-32 at Thr-75, thereby reducing the efficacy of dopaminergic signaling. We report here that dopamine, in slices, and acute cocaine, in whole animals, decreases the state of phosphorylation of striatal DARPP-32 at Thr-75 and thereby removes this inhibitory constraint. This effect of dopamine is achieved through dopamine D1 receptor-mediated activation of cAMP-dependent protein kinase (PKA). The activated PKA, by decreasing the state of phosphorylation of DARPP-32-Thr-75, deinhibits itself. Dopamine D2 receptor stimulation has the opposite effect. The ability of activated PKA to reduce the state of phosphorylation of DARPP-32-Thr-75 is apparently attributable to increased protein phosphatase-2A activity, with Cdk5 being unaffected. Together, these results indicate that via positive feedback mechanisms, Cdk5 signaling and PKA signaling are mutually antagonistic.

Dopamine and cAMP-regulated phosphoprotein of M_r 32,000 (DARPP-32) is a cytosolic protein that is selectively enriched in neostriatal medium spiny neurons (1, 2). When DARPP-32 is phosphorylated by cAMP-dependent protein kinase (PKA) on a single threonine residue, Thr-34, it is converted into a potent inhibitor of protein phosphatase-1 (PP-1) (3). Dopamine and numerous other neurotransmitters have been shown to regulate the phosphorylation/dephosphorylation of DARPP-32 at Thr-34 in neostriatum, thereby altering the activity of PP-1 and regulating the phosphorylation state and activity of many downstream physiological effectors, including various neurotransmitter receptors and voltage-gated ion channels (4). Mice lacking DARPP-32 exhibit profound deficits in their molecular, electrophysiological, and behavioral responses to dopamine, drugs of abuse, and antipsychotic medication, demonstrating the importance of the DARPP-32/PP-1 signaling cascade in mediating the actions of dopamine, agents that affect dopamine signaling, and other neurotransmitters that act on neostriatal neurons (4, 5).

We have recently reported that DARPP-32 is phosphorylated by cyclin-dependent kinase 5 (Cdk5), both *in vitro* and in neostriatal neurons (6). *In vitro*, phospho-Thr-75 DARPP-32 inhibits PKA by a competitive mechanism. *In vivo*, reduction of phospho-Thr-75 DARPP-32 in neostriatal slices, either by the Cdk5 inhibitor roscovitine or by the use of genetically altered mice ($p35^{-/-}$ mice), results in increased biochemical and physiological responses to dopamine (6). These results demonstrated that PKA activity in the neostriatum is regulated by the state of phosphorylation of DARPP-32 at Thr-75. Thus, DARPP-32 is a bifunctional signal transduction molecule that controls the activities of PP-1 and PKA through the phosphorylation of Thr-34 and Thr-75, respectively.

Apart from the effect of Cdk5, nothing has been known about the signaling mechanisms involved in the regulation of the state of phosphorylation of DARPP-32 at Thr-75. We report here that dopamine, through the activation of PKA, increases the activity of PP-2A, leading to the dephosphorylation of phospho-Thr-75 DARPP-32. In this way, activated PKA attenuates its own inhibition. Thus, this positive feedback loop will result in amplification of signaling through the dopamine/PKA/Thr-34-DARPP-32/PP-1 signal transduction cascade.

Materials and Methods

Preparation and Incubation of Striatal Slices. Neostriatal slices were prepared from male C57BL/6 mice (6–8 weeks old) as described (7). Slices were treated with drugs as specified in each experiment, and drugs were obtained from the sources as described (6–8). After drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at −80°C until assayed.

For studies of the effects of D1 agonists, slices were incubated for a total of 15 min. The D1 antagonist SCH23390 (1 μM) was added at 0 min, and a D1 agonist, SKF81297 (1 μM), was added at 10 min of incubation. For studies of the effects of D2 agonists, slices were incubated for a total of 20 min. The D2 antagonist raclopride (1 μM) was added at 0 min, and a D2 agonist, quinpirole (1 μM), was added at 10 min of incubation. For the studies of the effects of dopamine shown in Fig. 1*c*, slices were preincubated with SCH23390 (1 μM) for 10 min and incubated for an additional 10 min with dopamine (100 μM) plus nomifensine (10 μM) in the presence of SCH23390. Alternatively, slices were preincubated with raclopride (1 μM) for 10 min and incubated for an additional 6 min with dopamine (100 μM) plus nomifensine (10 μM) in the presence of raclopride.

Whole Animal Studies. Mice were intraperitoneally injected with vehicle (0.9% NaCl) or vehicle containing cocaine·HCl (20 mg/kg). Animals were killed at 30 min postinjection by focused microwave irradiation (4.5–5 kW for 1.4 s), using a small animal microwave (Muromachi Kikai, Tokyo, Japan). Brains were rapidly removed, and the neostriatum was dissected and stored at −80°C until it was assayed for phosphoprotein levels.

Immunoblotting. Frozen tissue samples were sonicated in boiling 1% SDS. Equal amounts of protein (100 μg) were processed, using 12% acrylamide gels as described (7). Immunoblotting was carried out, using phosphorylation-state-specific antibodies raised against a DARPP-32 peptide containing phospho-Thr-34, the site phosphorylated by PKA (9), and a DARPP-32 peptide containing phospho-Thr-75, the site phosphorylated by Cdk5 (6). A monoclonal antibody generated against DARPP-32 (10), which is not phosphorylation state specific, was used to determine the total amount of DARPP-32. None of the experimental manipulations used in the present study altered the total amount

Abbreviations: DARPP-32, dopamine- and cAMP-regulated phosphoprotein of M_r 32,000; Cdk5, cyclin-dependent kinase 5; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; A.U., arbitrary units.

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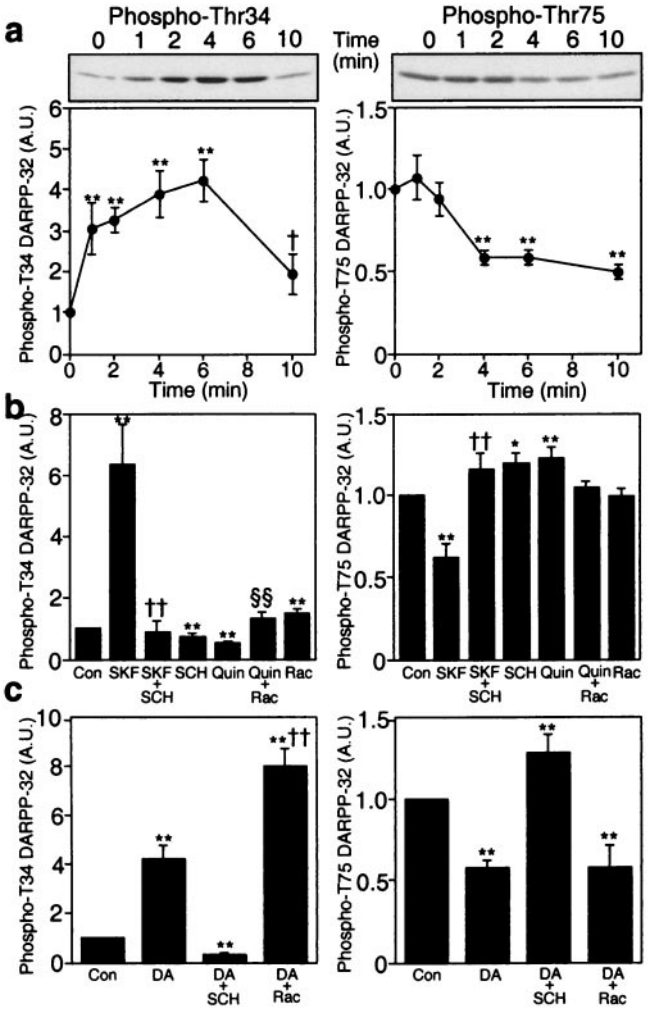

Fig. 1. Regulation of DARPP-32 phosphorylation at Thr-34 and Thr-75 by dopamine via D1- and D2-type receptors in neostriatum. (*a*) Effect of dopamine on the level of phospho-Thr-34 and phospho-Thr-75 DARPP-32. Slices were incubated with dopamine (100 μM) in the presence of a dopamine uptake inhibitor, nomifensine (10 μM), for the indicated times. Immunoblots are shown (*Upper*) for the detection of phospho-Thr-34 (*Left*) and phospho-Thr-75 (*Right*), using phosphorylation state-specific antibodies and the same nitrocellulose membrane. Quantitative results, normalized to values obtained with untreated slices, are shown below each blot. A.U., arbitrary units. Data represent means ± SEM for four experiments. **, $P < 0.01$ compared with 0 min; †, $P < 0.05$ compared with 6 min, Student's *t* test. (*b*) Regulation of DARPP-32 phosphorylation at Thr-34 (*Left*) and Thr-75 (*Right*) by dopamine D1 and D2 receptor agonists and antagonists. Data are shown for untreated neostriatal slices (Con) and slices treated with a D1 agonist, SKF81297 (SKF) (1 μM); a D1 antagonist, SCH23390 (SCH) (1 μM); a D2 agonist, quinpirole (Quin) (1 μM); and a D2 antagonist, raclopride (Rac) (1 μM), as indicated. Data represent means ± SEM for 5–15 experiments. *, $P < 0.05$, **, $P < 0.01$ compared with control; ††, $P < 0.01$ compared with SKF81297 alone; §§, $P < 0.01$ compared with quinpirole alone, Student's *t* test. (*c*) Effect of SCH23390 and raclopride on dopamine regulation of DARPP-32 phosphorylation on Thr-34 (*Left*) and Thr-75 (*Right*). Data are shown for treatment of neostriatal slices with dopamine (100 μM), SCH23390 (1 μM), and raclopride (1 μM), as indicated. Data represent means ± SEM for five experiments. **, $P < 0.01$ compared with control; ††, $P < 0.01$ compared with dopamine alone, Student's *t* test.

of DARPP-32. Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham Pharmacia). Phospho-DARPP-32 bands were quantified by densitometry, using National Institutes of Health IMAGE 1.61 software.

Protein Phosphatase Assays. Endogenous protein phosphatase activities toward ^{32}P-Thr-75 DARPP-32 were determined in striatal homogenate as described (7). Dephosphorylation reactions were initiated by the addition of ^{32}P-Thr-75 DARPP-32 (1 mg/ml). $CaCl_2$ (100 μM) plus calmodulin (1 μM) or $MgCl_2$ (10 mM) was added to the assay buffer to determine the activity of calcineurin or PP-2C, respectively.

Assay for Cdk5 Activity. Cdk5 immunoprecipitations and assays were performed as described (11). Immunoprecipitations used homogenates of neostriatal slices that were incubated in the absence or presence of forskolin (2 μM or 10 μM). Neostriatal slices were homogenized with a Dounce homogenizer in lysis buffer (150 mM NaCl/20 mM Tris·HCl/1 mM EDTA/0.5% Nonidet P-40/5 mM DTT/1 mM PMSF/2.5 mM benzamidine/20 μg/ml antipain/20 μg/ml leupeptin/5 μg/ml chymostatin/5 μg/ml pepstatin/50 mM NaF/5 mM Na_3VO_4, pH 7.4). Cdk5 was immunoprecipitated from neostriatal homogenates, using a Cdk5 antibody (C-8) conjugated to agarose beads (Santa Cruz Biotechnology). Immunoblot analysis revealed that Cdk5 and its cofactor, p35, were coimmunoprecipitated as a complex from neostriatal homogenates (not shown). The immunoprecipitated samples were resuspended and incubated with histone H1 (type III-S) (Sigma) (2 μg), in 30 μl of kinase assay buffer (50 mM Hepes/10 mM $MgCl_2$/1 mM DTT/50 μM ATP/5 μCi of [γ-^{32}P]ATP) for 5 min at 30°C. Histone H1 phosphorylation under these assay conditions was linear for up to 20 min. Reactions were terminated by adding 30 μl of sample buffer (250 mM Tris·HCl/40% glycerol/8% SDS/20% 2-mercaptoethanol, pH 6.8). Samples were boiled for 10 min, and proteins were separated by SDS/PAGE (12% acrylamide) and imaged with a PhosphorImager 400B and IMAGEQUANT software (Molecular Dynamics).

Results

The effect of dopamine on phosphorylation of DARPP-32 at the PKA site (Thr-34) and the Cdk5 site (Thr-75) was assessed in mouse neostriatal slices, using phosphorylation state-specific antibodies. Treatment of slices with dopamine (100 μM) in the presence of the dopamine uptake inhibitor nomifensine (10 μM) induced a rapid but transient increase in DARPP-32 phosphorylation at Thr-34 (Fig. 1*a*, *Left*), as previously described (8, 12). A near-maximal effect of dopamine was observed within 1 min of incubation and sustained for 4–6 min. The level of DARPP-32-Thr-34 phosphorylation returned to a near-basal level within 10 min of incubation. In contrast, treatment of slices with dopamine induced a decrease in DARPP-32 phosphorylation at Thr-75, which was not observed until 4 min and remained at 10 min of incubation (Fig. 1*a*, *Right*). Thus, phosphorylation of these distinct threonine residues on DARPP-32 is differentially regulated by dopamine.

Dopamine stimulates D1-type and D2-type receptors, both of which are expressed on neostriatal neurons. Therefore, the effects of each subclass of dopamine receptor on DARPP-32 phosphorylation were examined in neostriatal slices. A D1 agonist, SKF81297 (1 μM), increased the level of phospho-Thr-34 DARPP-32 and decreased the level of phospho-Thr-75 DARPP-32 (Fig. 1*b*). Treatment of slices with a D1 antagonist, SCH23390 (1 μM), decreased the basal level of phospho-Thr-34 and increased the basal level of phospho-Thr-75, suggesting a tonic activation of D1 receptors. The effects of SKF81297 on both Thr-34 and Thr-75 were abolished by pretreatment of slices with SCH23390.

A D2 receptor agonist, quinpirole (1 μM), decreased the level of phospho-Thr-34 DARPP-32 and slightly but significantly increased the level of phospho-Thr-75 DARPP-32 (Fig. 1*b*). Treatment of slices with a D2 antagonist, raclopride (1 μM), increased the basal level of phospho-Thr-34 DARPP-32 but did

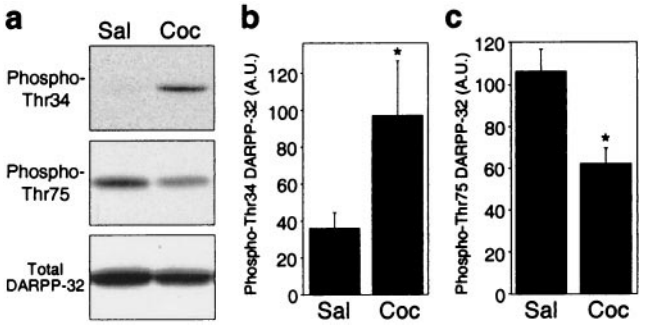

Fig. 2. Regulation by acute cocaine of DARPP-32 phosphorylation at Thr-34 and Thr-75. Data are shown for mice treated with saline (Sal) or cocaine (Coc). (*a*) Representative immunoblots for detection of each phosphorylation site and for total DARPP-32 are shown. (*b* and *c*) The amounts of phospho-Thr-34 DARPP-32 (*b*) and phospho-Thr-75 DARPP-32 (*c*) were quantified by densitometry. Data represent means ± SEM for five to six mice per group. *, $P < 0.05$ compared with saline-injected mice, Student's *t* test.

not affect the basal level of phospho-Thr-75 DARPP-32. The effects of quinpirole on both Thr-34 and Thr-75 were abolished by pretreatment of slices with raclopride.

We also compared the effects of a D1 antagonist and a D2 antagonist on the ability of dopamine to regulate phosphorylation of DARPP-32 on Thr-34 and Thr-75 (Fig. 1*c*). In the presence of SCH23390, dopamine decreased phospho-Thr-34 and increased phospho-Thr-75, effects attributable to the activation by dopamine of D2 receptors. Raclopride potentiated the effect of dopamine on phospho-Thr-34 (Fig. 1*c*) and enabled dopamine to maintain an elevated level of phospho-Thr-34 (data not shown), effects attributable to an unopposed activation of D1 receptors.

Cocaine, a major drug of abuse, is thought to exert its effects primarily by blocking transporter-mediated reuptake of dopamine at axonal terminals. The effect of cocaine on the phosphorylation of DARPP-32 at the PKA and Cdk5 sites in neostriatum was assessed in whole animals. A single i.p. injection of cocaine (20 mg/kg) resulted in an increase in phospho-Thr-34 DARPP-32 and a reduction in phospho-Thr-75 DARPP-32, whereas total levels of DARPP-32 were unchanged (Fig. 2). These results indicate that some of the physiological effects of acute cocaine administration are likely to be mediated through dopamine-dependent regulation of the phosphorylation state of DARPP-32 at two distinct sites controlling PP-1 and PKA activity in neostriatal medium spiny neurons.

To identify the mechanism by which D1 class receptors might regulate DARPP-32 phosphorylation at Thr-75, the effects of two PKA activators, forskolin and 8-bromo-cAMP, were examined. Treatment of slices with forskolin (2 μM or 10 μM) increased the level of phospho-Thr-34 DARPP-32 (Fig. 3 *a* and *b*). Concomitantly, treatment with forskolin decreased the level of phospho-Thr-75 DARPP-32 (Fig. 3 *a* and *c*). An inactive analog of forskolin, dideoxyforskolin (2 μM or 10 μM), did not affect the level of either phospho-Thr-34 DARPP-32 or phospho-Thr-75 DARPP-32. Treatment of slices with the cAMP analog 8-bromo-cAMP (5 mM) mimicked the effects of forskolin. These results indicate that activation of PKA reduces the level of phospho-Thr-75 DARPP-32.

The ability of PKA to cause the dephosphorylation of phospho-Thr-75 DARPP-32 might be explained either by an inhibition of Cdk5 activity or by an activation of a dephosphorylation pathway. To examine the possible role of Cdk5, the effect of forskolin on the level of phospho-Thr-75 DARPP-32 was compared in the absence and presence of a potent and selective Cdk5 inhibitor, roscovitine (Fig. 4*a*). Treatment of slices with roscovitine (10 and 50 μM) decreased the level of phospho-Thr-75 DARPP-32 to 65.4 ± 1.4% and 15.1 ± 5.0% of control, respectively. Treatment of slices with forskolin (2 μM) decreased the level of phospho-Thr-75 DARPP-32 by about 70% in the absence of roscovitine. Under conditions in which Cdk5 activity was inhibited by roscovitine, forskolin reduced the level of phospho-Thr-75 by a similar percentage independent of the level of Cdk5 activity, arguing against the possibility that PKA inhibits Cdk5 activity.

Further evidence that PKA does not inhibit Cdk5 activity was obtained in experiments in which Cdk5 activity was measured in homogenates of neostriatal slices that had been treated in the absence or presence of forskolin (Fig. 4*b*). Histone H1 kinase activity was measured in Cdk5 immunoprecipitates (but not with the use of control IgG), and this activity was abolished by the addition of roscovitine (10 μM). ^{32}P incorporation into histone H1 was similar whether Cdk5 was immunoprecipitated from untreated or forskolin-treated slices (2 μM or 10 μM). In addition, the amount of p35, which is a short-lived protein degraded by proteasomes (13), was not affected by the treatment of slices with forskolin (data not shown). Together, these data indicate that Cdk5 activity is not modulated by PKA.

We next investigated the possibility that PKA might reduce phospho-Thr-75 by the activation of a protein phosphatase. To

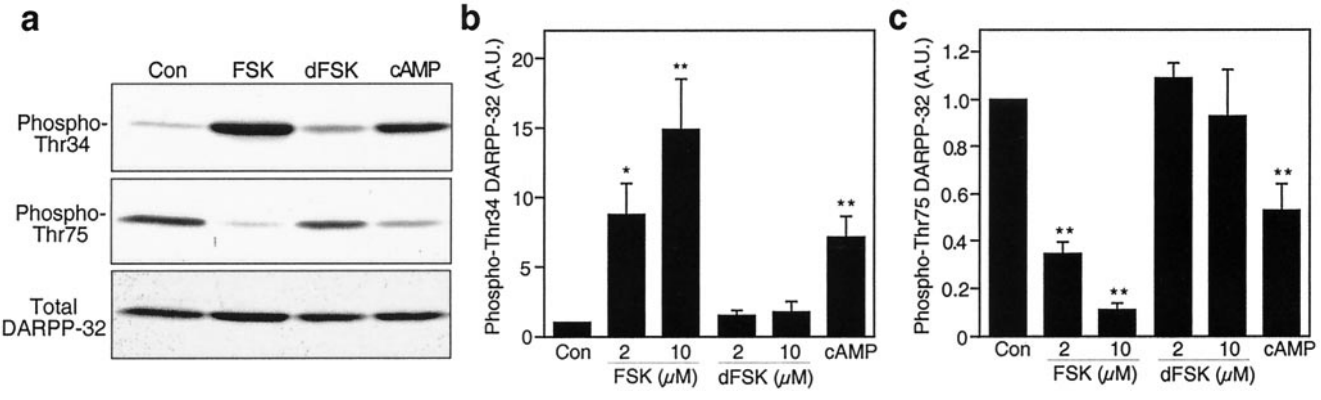

Fig. 3. Effect of PKA activation on DARPP-32 phosphorylation at Thr-34 and Thr-75 in neostriatum. (*a*) Immunoblots showing the levels of phospho-Thr-34, phospho-Thr-75, and total DARPP-32 in response to treatment with 10 μM forskolin (FSK), 10 μM 1,9-dideoxyforskolin (dFSK) (an inactive analog of forskolin), or 5 mM 8-bromo-cAMP (cAMP) for 5 min, detected on the same nitrocellulose membrane. (*b* and *c*) The amounts of phospho-Thr-34 DARPP-32 (*b*) and phospho-Thr-75 DARPP-32 (*c*) were quantified by densitometry, and the data were normalized to values obtained with untreated slices. Data represent means ± SEM for 3–10 experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control, Student's *t* test.

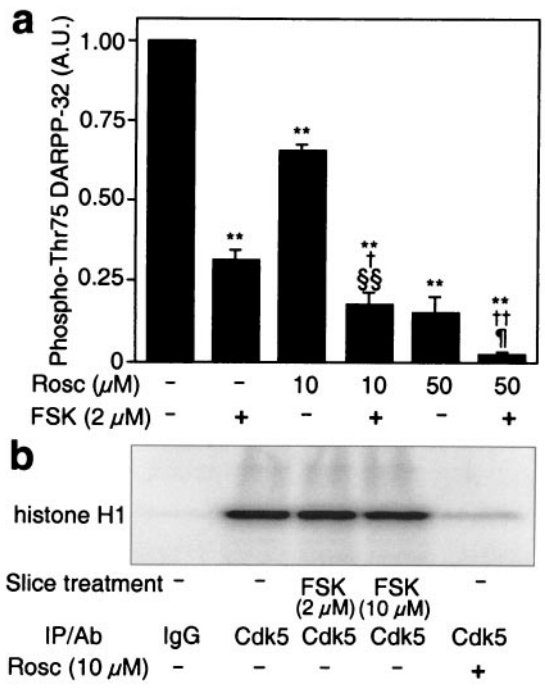

Fig. 4. PKA activation reduces phospho-Thr-75 DARPP-32 even when Cdk5 activity is inhibited. (*a*) Slices were incubated in the absence or presence of roscovitine (Rosc) (10 μM or 50 μM) for 60 min, followed by the addition of forskolin (FSK) (2 μM) for 5 min. The amounts of phospho-Thr-75 DARPP-32 were quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± SEM for 4–15 experiments. **, $P < 0.01$ compared with no addition; †, $P < 0.05$, ††, $P < 0.01$ compared with forskolin alone; §§, $P < 0.01$ compared with roscovitine (10 μM) alone; ¶, $P < 0.05$ compared with roscovitine (50 μM) alone, Student's *t* test. (*b*) Homogenates of neostriatal slices treated with forskolin at the indicated concentrations were subjected to immunoprecipitation (IP) with either a Cdk5 antibody (C-8) or a normal rabbit IgG. The immunoprecipitates, containing Cdk5 and its cofactor p35, were used to phosphorylate histone H1 in the absence or presence of roscovitine. Results similar to those shown here were obtained in two other experiments.

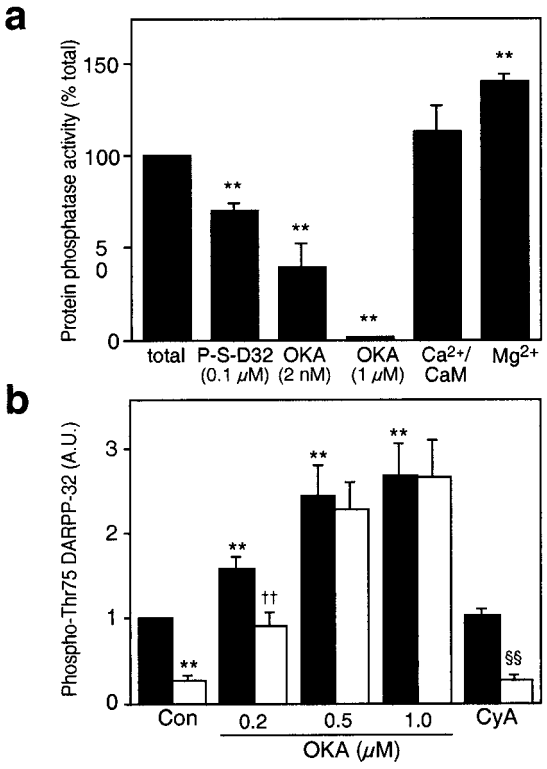

Fig. 5. Dephosphorylation of phospho-Thr-75 DARPP-32 *in vitro* (*a*) and in intact neostriatal neurons (*b*). (*a*) *In vitro* dephosphorylation of phospho-Thr-75 DARPP-32 by phosphatases in neostriatal homogenates in the presence of thio-phospho-Thr-34 DARPP-32 (P-S-D32, 0.1 μM) (a highly selective inhibitor of PP-1), 2 nM okadaic acid (OKA) (predominantly an inhibitor of PP-2A), 1 μM okadaic acid (an inhibitor of both PP-1 and PP2A), Ca^{2+}/calmodulin (CaM) (an activator of PP-2B), and Mg^{2+} (an activator of PP-2C). Data represent means ± SEM for four to six experiments. **, $P < 0.01$ compared with control, Student's *t* test. (*b*) Effects of phosphatase inhibition and PKA activation on phospho-Thr-75 DARPP-32 levels. Neostriatal slices were incubated in the presence of the indicated concentrations of okadaic acid or 10 μM cyclosporin A (CyA) for 60 min, followed by 5 min in the absence (closed bars) or presence (open bars) of 10 μM forskolin. The amount of phospho-Thr-75 DARPP-32 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Okadaic acid, but not cyclosporin A, abolished the ability of forskolin to reduce the level of phospho-Thr-75. Data represent means ± SEM for four to five experiments. **, $P < 0.01$ compared with no addition; ††, $P < 0.01$ compared with okadaic acid (200 nM) alone; §§, $P < 0.01$ compared with cyclosporin A alone, Student's *t* test.

identify the protein phosphatase(s) involved in the dephosphorylation of phospho-Thr-75 DARPP-32, phospho-Thr-75 DARPP-32 was incubated *in vitro* with mouse striatal homogenate as a source of protein phosphatase activity (Fig. 5*a*). Phospho-S-DARPP-32 (0.1 μM), a potent and selective inhibitor of PP-1 (14), reduced endogenous protein phosphatase activity by 30%. A low concentration of okadaic acid (2 nM), which was used to selectively inhibit PP-2A activity (15), reduced endogenous protein phosphatase activity by 60%. A high concentration of okadaic acid (1 μM), which inhibits both PP-2A and PP-1 activities (15), completely inhibited endogenous PP activity. These results indicate that PP-2A contributes 60–70% and PP-1, 30–40% of endogenous protein phosphatase activity toward phospho-Thr-75 DARPP-32. The addition of Ca^{2+} plus calmodulin (used to stimulate PP-2B, calcineurin) did not increase endogenous protein phosphatase activity. The addition of Mg^{2+} increased endogenous protein phosphatase activity by 40%, indicating that PP-2C can also dephosphorylate phospho-Thr-75 DARPP-32. These data indicate that, *in vitro*, PP-2A is the major protein phosphatase and that PP-1 and PP-2C contribute, to a lesser extent, to the dephosphorylation of phospho-Thr-75 DARPP-32.

We have reported that pretreatment of neostriatal slices with 200 nM okadaic acid inhibited PP-2A activity by 80% and PP-1 activity by 5% and that pretreatment with 1 μM okadaic acid inhibited PP-2A activity completely (IC_{50} of ≈100 nM) and PP-1 activity by about 30% (7). The role of PP-2A and PP-1 in the dephosphorylation of phospho-Thr-75 DARPP-32 was examined in neostriatal slices (Fig. 5*b*). Slices were incubated with various concentrations of okadaic acid for 60 min, and the state of DARPP-32 phosphorylation at Thr-75 was determined. Treatment of slices with okadaic acid increased the level of phospho-Thr-75 DARPP-32 maximally by 2.7 ± 0.4-fold at 1 μM with a half-maximal effect at ≈250 nM, implicating PP-2A in the dephosphorylation of phospho-Thr-75 DARPP-32 in intact cells. Experiments in which slices were treated with various concentrations of calyculin A also led to the conclusion that PP-2A plays the dominant role in the dephosphorylation of phospho-Thr-75 DARPP-32 (data not shown). In addition, treatment of slices for 60 min with cyclosporin A (10 μM), an inhibitor of calcineurin, did not affect the level of phospho-Thr-75 DARPP-32 (100.9 ± 7.4% of control) (Fig. 5*b*).

The possibility that PKA might activate PP-2A and thereby stimulate the dephosphorylation of phospho-Thr-75 was exam-

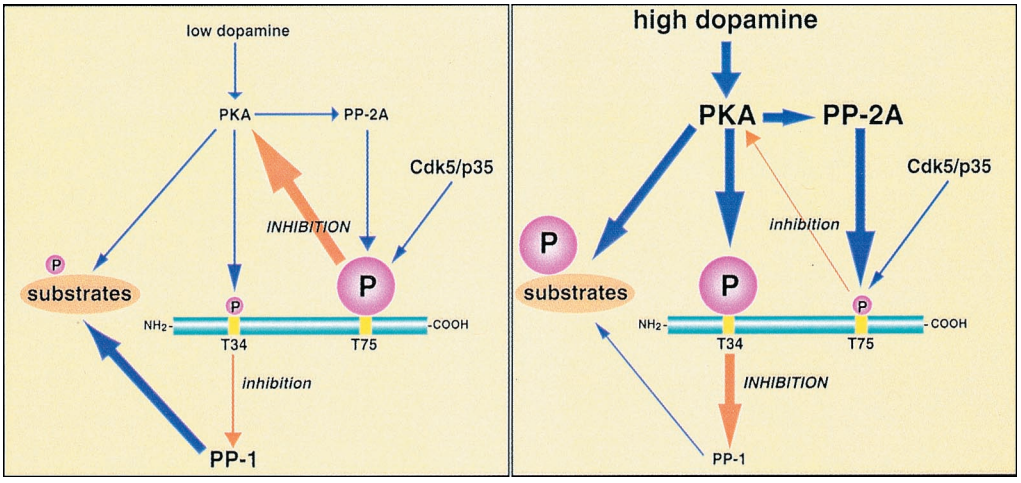

Fig. 6. Model illustrating the regulation of dopamine signaling by Cdk5/phospho-Thr-75 DARPP-32 and regulation of the Cdk5/phospho-Thr-75 DARPP-32 pathway by dopamine. The Cdk5 signaling and PKA signaling pathways, through a positive feedback loop, are mutually antagonistic. (*Left*) Under basal conditions, phosphorylation of DARPP-32 at Thr-75 by Cdk5 causes, successively, inhibition of PKA, inactivation of PP-2A, and decreased dephosphorylation of phospho-Thr-75 DARPP-32. Inhibition of PKA also results in decreased phosphorylation of DARPP-32-Thr-34 and, therefore, in activation of PP-1. Inhibition of PKA and activation of PP-1 synergistically reduce the phosphorylation of various substrates. (*Right*) Dopamine, by sequentially activating dopamine D1 receptors, PKA and PP-2A, reduces the level of phospho-Thr-75 DARPP-32. Dephosphorylation of DARPP-32 at Thr-75 by PP-2A removes the inhibition of PKA. Activation of PKA also results in increased phosphorylation of DARPP-32-Thr-34 and in inhibition of PP-1. Activation of PKA and inhibition of PP-1 synergistically increase the phosphorylation of various substrates.

ined in neostriatal slices (Fig. 5*b*). When slices were pretreated with okadaic acid, the inhibitory effect of forskolin on DARPP-32 phosphorylation at Thr-75 was partially blocked in the presence of 200 nM okadaic acid, largely abolished in the presence of 500 nM okadaic acid, and completely attenuated in the presence of 1 μM okadaic acid. Roscovitine (50 μM), unlike forskolin, retained its ability to decrease the level of phospho-Thr-75 DARPP-32 in the presence of okadaic acid (1 μM) (data not shown).

Discussion

It was shown previously that Cdk5 regulates the efficacy of dopaminergic signaling by phosphorylating DARPP-32 at Thr-75 (6). Because Thr-75-DARPP-32 is phosphorylated to a high stoichiometry under basal conditions, both in striatal slices and in whole animals, dopaminergic signaling is constrained by tonic activity of the Cdk5 signaling pathway. Inhibition of Cdk5 activity, either pharmacologically or genetically, reduces the level of phospho-Thr-75 DARPP-32 and the inhibition of PKA in neostriatal neurons, thereby increasing responsivity to dopamine signaling (6).

In the present study, we have demonstrated a reciprocal inhibitory signaling pathway: Dopamine reduces the state of phosphorylation of DARPP-32-Thr-75 and thereby opposes Cdk5-mediated signaling in neostriatal neurons (Fig. 6). Activation of dopamine D1-type receptors, both in neostriatal slices treated with pharmacological agents and in whole animals dosed acutely with cocaine, decreased the phosphorylation of DARPP-32 at Thr-75 by a mechanism involving the activation of PKA. Thus, dopamine/PKA signaling and Cdk5 signaling are mutually antagonistic. Moreover, activation of PKA and the resultant reduction of DARPP-32-Thr-75 phosphorylation and de-inhibition of PKA function as a positive feedback amplification mechanism for dopamine signaling.

The ability of the dopamine/D1 receptor/PKA cascade to reduce the state of phosphorylation of DARPP-32 at Thr-75 might be explained *a priori* either by inhibition of Cdk5 or by activation of a protein phosphatase. Characterization of protein phosphatases *in vitro* and in neostriatal slices revealed that PP-2A is the predominant PP in the dephosphorylation of phospho-Thr-75 DARPP-32. When PP-2A activity was inhibited by pretreatment of slices with okadaic acid, the effect of PKA activation on phospho-Thr-75 DARPP-32 was abolished. Under conditions of near-maximal inhibition of Cdk5 activity by roscovitine, PKA activation further reduced the phosphorylation of DARPP-32 at Thr-75. These results indicate that the effect of PKA on DARPP-32 phosphorylation at Thr-75 is likely to be mediated through the activation of PP-2A.

Activation of D2 receptors reduces the phosphorylation of Thr-34 both by inhibiting adenylyl cyclase, an effect mediated through G_i (16), and by activating PP-2B, an effect mediated through an increase in intracellular Ca^{2+} (8). A direct involvement of PP-2B in the regulation of phospho-Thr-75 DARPP-32 is unlikely, as phosphorylation of DARPP-32 at Thr-75 was stimulated, rather than inhibited, by D2 receptor activation and phospho-Thr-75 DARPP-32 is not a substrate for PP-2B. A reduction in cAMP level, PKA activity, and PP-2A activity seems the most likely explanation for the increase in Thr-75 phosphorylation by D2 receptor activation and provides a unique molecular mechanism for the well-established ability of D2 receptor activation to antagonize signaling via the dopamine/D1 receptor pathway.

The phosphatase PP-2A is important in neuronal function (17–19) and plays a central role in the dephosphorylation of phospho-Thr-75 DARPP-32 in neostriatal neurons. PP-2A consists of three subunits: a catalytic (C) subunit, a structural (A) subunit, and a regulatory (B-type) subunit. Regulatory subunits are subdivided into PR55 B, PR56 B′, PR72 B″, and PR61 families (20, 21). Usui *et al.* reported that the regulatory B′δ subunit (or B56δ), originally purified from erythrocytes and named the B″ (δ) subunit, is phosphorylated by PKA and that phosphorylated forms of B′δ stimulate the catalytic activity of PP-2A in a substrate-specific manner (22, 23). Immunoblot analysis using B′δ antibody revealed that the level of expression of the protein was slightly higher in striatum than in neocortex (A.N., unpublished observations), the region in the central nervous system in which the level of expression of B′δ was reported to be most abundant (24). B′δ contains two consensus phosphorylation site motifs for PKA, and one or the other of these sites is also conserved in other B-type subunits, for example

in B56γ3 (25). Preliminary studies indicate that PP-2A partially purified from brain is activated by purified PKA (F. Liu and S. Rakhilin, personal communication). It is possible that PKA also indirectly regulates the activity of PP-2A. It has been reported that insulin signaling results in a rapid inactivation of PP-2A by increased tyrosine phosphorylation of the catalytic subunit and that cAMP counteracts insulin's effect on PP-2A by decreasing tyrosine phosphorylation (26). Further studies are required to clarify the precise mechanism of PP-2A regulation by PKA.

We have shown here that dopamine controls DARPP-32 phosphorylation at the Cdk5 site through a mechanism involving the activation of PKA and probably of PP-2A, indicating that the modulation of protein phosphatase activity regulates the state of phosphorylation at the Cdk5 site. As a result, dopamine acts as a master switch controlling whether the dopaminoceptive cells reside in a Cdk5/PP-1-dominated state with low phosphorylation of numerous physiological effectors or in a PKA/PP-2A-dominated state with high phosphorylation of those effectors. Although a limited amount of information is currently available, Cdk5 activity might also be modulated by various neurotransmitters and second messengers, possibly through the phosphorylation of Cdk5 or its regulatory factors (27–29). Elucidation of regulatory factors, activating or inhibiting Cdk5, will also be important for gaining a clearer understanding of the complexities of dopamine signaling (see, e.g., ref. 30).

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