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Nitric oxide/cGMP pathway stimulates phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, in the substantia nigra

(protein phosphorylation)

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ABSTRACT **DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of M_r 32,000, has been shown to be phosphorylated on threonine-34, both *in vitro* with high efficiency by cAMP-dependent and cGMP-dependent protein kinases and *in vivo* by dopamine acting through cAMP-dependent protein kinase. In the present study, we investigated the nitric oxide (NO)/cGMP pathway for its ability to regulate the state of phosphorylation of DARPP-32 in slices of rat substantia nigra. DARPP-32 was phosphorylated on threonine-34 in these slices by sodium nitroprusside (SNP), an NO donor. The effect of SNP was abolished by preincubation of the slices with hemoglobin, indicating that the effect of SNP was due to released NO. The same concentration of SNP produced a 4-fold elevation of the cGMP level but did not alter the level of cAMP. The effect of SNP on DARPP-32 phosphorylation was mimicked by low concentrations of 8-bromo-cGMP and 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate, activators of cGMP-dependent protein kinase, but not by low concentrations of 8-bromo-cAMP, an activator of cAMP-dependent protein kinase. The data indicate a physiological role for the NO/cGMP pathway in the regulation of DARPP-32 phosphorylation in nerve terminals of striatonigral neurons. The results provide further evidence that the state of phosphorylation of DARPP-32 represents an important mechanism for integration of information arriving at striatonigral neurons via a variety of neuronal pathways.**

DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of M_r 32,000 on SDS/polyacrylamide gels, is a cytosolic phosphoprotein that is highly enriched in the medium-sized spiny neurons of the striatum and in the striatonigral nerve terminals in the substantia nigra (1–3). Previous studies have shown that dopamine, by increasing levels of cAMP and activating cAMP-dependent protein kinase, stimulates the phosphorylation of DARPP-32 in these brain regions (4). Phosphorylation of a single threonine residue, in position 34, by cAMP-dependent protein kinase, converts DARPP-32 into a potent inhibitor of protein phosphatase 1 (5). Kinetic studies of purified DARPP-32 indicate that this threonine residue is also an excellent substrate for phosphorylation by cGMP-dependent protein kinase *in vitro* (6). Those results raised the possibility that the physiological actions of DARPP-32 may be regulated through its phosphorylation by either cAMP-dependent protein kinase or cGMP-dependent protein kinase. However, no signal transduction pathway in the brain has yet been demonstrated to regulate the state of phosphorylation of DARPP-32 through the activation of cGMP-dependent protein kinase.

Atrial natriuretic peptide, a hormone that activates a membrane-bound receptor with a guanylyl cyclase intracellular domain (7), was recently shown to produce a prominent elevation in cGMP level and DARPP-32 phosphorylation in the choroid plexus (8). The striatum and the substantia nigra are devoid of atrial natriuretic peptide receptors (9, 10). However, the medium-sized spiny neurons of striatum and their terminals in the substantia nigra contain high concentrations of soluble guanylyl cyclase (11), cGMP (11, 12), and cGMP-dependent protein kinase (13). The recent discovery of nitric oxide (NO) as an intercellular messenger that activates soluble guanylyl cyclase (for review, see refs. 14–17) and hence cGMP-dependent protein kinase (18), together with the histochemical demonstration of NO synthase-containing nerve fibers in the substantia nigra (19), prompted us to examine the NO/cGMP pathway for a possible role in the regulation of DARPP-32 phosphorylation.

MATERIALS AND METHODS

Materials. Sodium nitroprusside (SNP), L-arginine, 8-bromo-cAMP, 8-bromo-cGMP, RPMI 1640 balanced salt solution (RPMI medium), bovine Hb, and sodium dithionite were obtained from Sigma. 8-(4-Chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP) was purchased from BioLog Life Science Institute (Bremen, Germany), bicinchoninic acid reagent and goat anti-mouse horseradish peroxidase-linked antibody were from Pierce, Bradford protein assay reagent was obtained from Bio-Rad, nitrocellulose filters (pore size, 0.2 μm) were from Schleicher & Schuell, forskolin was purchased from Calbiochem, and *N*-methyl-D-aspartic acid (NMDA) was from Tocris (Essex, England). cAMP and cGMP RIA kits and enhanced chemiluminescence detection kits were obtained from Amersham.

Hb was prepared by adding a 10-fold molar excess of sodium dithionite to a 1 mM solution of commercial Hb, 75% of which is methemoglobin. Sodium dithionite was removed by dialysis against nanopure distilled water. The freshly prepared Hb solution was divided into aliquots, frozen at −20°C, and stored for up to 14 days (20).

Assay of Phospho-DARPP-32. A phosphorylation state-specific monoclonal antibody was raised against a DARPP-32 peptide containing phosphothreonine-34, the site phosphorylated by cAMP-dependent and cGMP-dependent protein kinases. This antibody, which has been characterized previously (8), was used to assay the amount of DARPP-32 phosphorylated at threonine-34.

Preparation of Nigral Slices. Female Sprague–Dawley rats (180–200 g) were stunned and decapitated. The brain was rapidly removed and immersed in ice-cold RPMI medium.

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Abbreviations: DARPP-32, dopamine- and cAMP-regulated phosphoprotein of M_r 32,000; SNP, sodium nitroprusside; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate; NMDA, *N*-methyl-D-aspartic acid.

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The midbrain was isolated by cutting the brainstem coronally between the mammillary body and the pons. The midbrain was laid on a flat glass surface on ice, and the substantia nigra was dissected out and further divided into five or six small slices. The nigral slices from each side were placed into separate tubes. The slices were preincubated in warm (30°C), oxygenated RPMI medium. After a 45-min preincubation, the solution was replaced with fresh RPMI medium containing test substances, and incubation was carried out for 10 min. At the end of the incubation, the medium was removed, and the tissues were rapidly frozen in liquid nitrogen and stored at −70°C until assayed.

Immunoblotting. Nigral slices were homogenized by sonication in boiling 1% (wt/vol) SDS. The protein concentration was determined with a bicinchoninic acid-based assay (21), using bovine serum albumin as a standard. Aliquots of the homogenates containing equal amounts of protein (180–250 μg) were removed, and 0.25 vol of five times concentrated stop solution was added [final concentrations: 3% SDS, 62 mM Tris·HCl (pH 6.8), 0.3 M 2-mercaptoethanol, 5% (vol/vol) glycerol, and traces of bromophenol blue]. Proteins were separated by SDS/15% PAGE (22). After electrophoresis, proteins in the gel were transferred to nitrocellulose filters (23). Immunoblotting was performed at room temperature using 4% (wt/vol) nonfat dry milk as blocking agent. After blocking for 2 hr, the nitrocellulose filters were incubated with the monoclonal antibody specific for phospho-DARPP-32 (1:100) for 2 hr, washed, and incubated with a goat anti-mouse horseradish peroxidase antibody (1:3000) for 30 min. The enhanced chemiluminescence Western blotting detection method was used to reveal antibody binding. The filters were dried, wrapped in Saran Wrap, and exposed to x-ray film.

The phospho-DARPP-32 bands on the x-ray films were quantitated by using a Bio-Rad model 620 video densitometer and Bio-Rad one-dimensional MOLECULAR ANALYST software. Results were calculated as a percentage of the control.

cGMP and cAMP RIA. Preparation and treatment of nigral slices was the same as for the phosphorylation experiments, except that the slices were sonicated in 4 mM Tris/EDTA buffer instead of in boiling SDS. An aliquot was taken for protein concentration measurement using the Bradford method (24). The remainder of the solution was then heated for 3 min in a boiling water bath to denature the protein. After centrifugation to precipitate protein, the supernatant was divided and used for cGMP and cAMP assays according to the Amersham protocols.

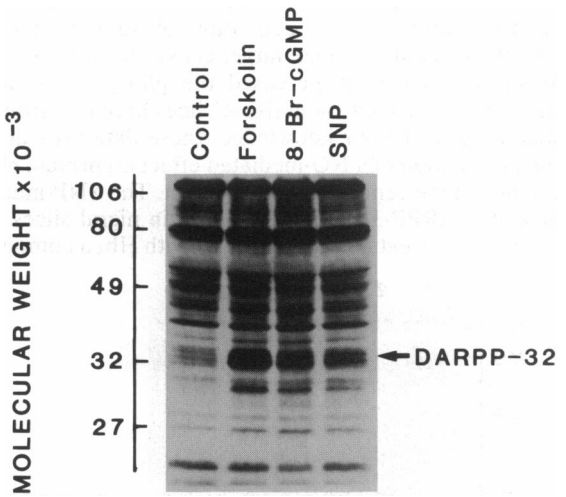

FIG. 1. Effects of forskolin (10 μM), 8-Br-cGMP (4 mM), and SNP (1 mM) on DARPP-32 phosphorylation in slices of rat substantia nigra. Slices were preincubated in oxygenated RPMI medium at 30°C for 45 min. The medium was replaced by fresh solution with or without the indicated test substance, and incubation was carried for an additional 10 min. The tissues were frozen in liquid nitrogen and stored at −70°C until assayed. Proteins were separated by SDS/PAGE and transferred to a nitrocellulose filter. Phosphorylated DARPP-32 was detected using a phosphorylation state-specific antibody. Other experimental details were as described in *Materials and Methods*.

Table 1. Effect of test substances on phosphorylation of DARPP-32 in slices of substantia nigra

Test substance	DARPP-32 phosphorylation, % control
SNP (1 mM)	269 ± 38 (13)
Hb (20 μM)	104 ± 13 (5)
SNP (1 mM) plus Hb (20 μM)	105 ± 14 (5)
8-Br-cGMP (1 mM)	658 ± 55 (5)
8-Br-cGMP (0.1 mM)	409 ± 87 (8)
8-pCPT-cGMP (1 mM)	703 ± 91 (4)
8-pCPT-cGMP (0.1 mM)	361 ± 63 (4)
8-Br-cAMP (1 mM)	784 ± 64 (4)
8-Br-cAMP (0.1 mM)	200 ± 38 (5)

Experimental procedures were as described in *Materials and Methods* and in the legend to Fig. 1. Data represent mean ± SEM for the number of experiments indicated in parentheses.

RESULTS

Effect of SNP on Phosphorylation of DARPP-32. SNP increased phosphorylation of DARPP-32 on threonine-34 in slices of substantia nigra (Fig. 1). The level of phospho-DARPP-32 was 2- to 3-fold higher in SNP-treated slices as compared with control slices (Table 1). In the same series of experiments in which SNP was examined for a possible effect on DARPP-32 phosphorylation, forskolin, which increases intracellular cAMP and activates cAMP-dependent protein kinase, also increased DARPP-32 phosphorylation, in agreement with previous results (25). 8-Br-cGMP, which activates cGMP-dependent protein kinase, also increased DARPP-32 phosphorylation (Fig. 1; see below).

The effect of various concentrations of SNP on DARPP-32 phosphorylation is shown in Fig. 2. A maximal effect of SNP was observed at a final concentration of 1 mM. The effect of incubating slices of substantia nigra with SNP is shown as a function of incubation time in Fig. 3. The effect of SNP was maximal at an incubation time of 10 min and declined thereafter.

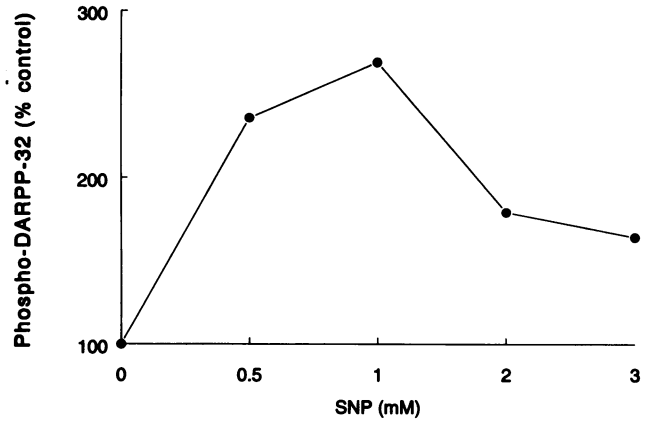

FIG. 2. Effect of various concentrations of SNP on DARPP-32 phosphorylation in slices of substantia nigra. Experimental procedures were as described in *Materials and Methods* and in the legend to Fig. 1.

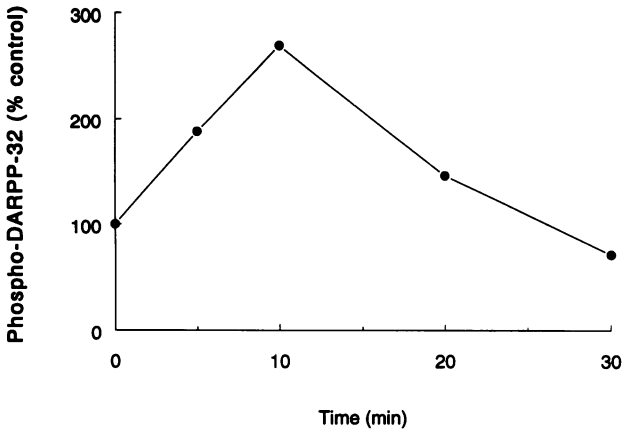

FIG. 3. Effect of SNP on DARPP-32 phosphorylation in slices of substantia nigra, as a function of incubation time. Experimental procedures were as described in *Materials and Methods* and in the legend to Fig. 1.

Effect of Hb Pretreatment on SNP-Stimulated Phosphorylation of DARPP-32. To verify that the SNP effect was due to NO released into the incubation medium, Hb was added to the medium in the absence or presence of SNP. Preincubation of slices with 20 μM Hb for 10 min prior to addition of SNP abolished the SNP-stimulated phosphorylation of DARPP-32 (Fig. 4 and Table 1). In the absence of SNP, Hb had no effect on the level of phosphorylation of DARPP-32.

Effect of SNP on cGMP and cAMP Levels. One of the major biological effects of NO in a variety of tissues (17) is to stimulate soluble guanylyl cyclase, thereby increasing the formation of cGMP. As shown in Fig. 5, incubation of slices of substantia nigra with 1 mM SNP for 10 min increased the cGMP level ≈4-fold, from 0.28 ± 0.10 to 1.01 ± 0.15 pmol/mg of protein (mean ± SEM, $n = 6$; $P < 0.01$). These results are consistent with the results of an immunocytochemical study showing that SNP treatment greatly increased the cGMP content in nerve fibers of the substantia nigra (J. de Vente, personal communication). The basal level of cAMP in the nigral slices was much higher (22.2 ± 7.4 pmol/mg of protein; mean ± SEM, $n = 4$) than that of cGMP. SNP treatment had no significant effect on the level of cAMP (Fig. 5).

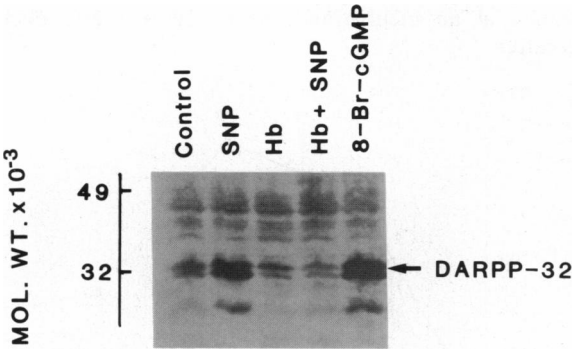

FIG. 4. Effect of preincubation with Hb on SNP-stimulated phosphorylation of DARPP-32 in slices of substantia nigra. Slices were preincubated in oxygenated RPMI medium at 30°C for 30 min. Hb was then added and preincubation was continued for an additional 10 min. After this 40-min preincubation period, SNP was added and incubation was carried out for 10 min. The test substances were present as indicated, at the following final concentrations: SNP, 1 mM; Hb, 20 μM; 8-Br-cGMP, 100 μM. Other experimental procedures were as described in *Materials and Methods* and in the legend to Fig. 1.

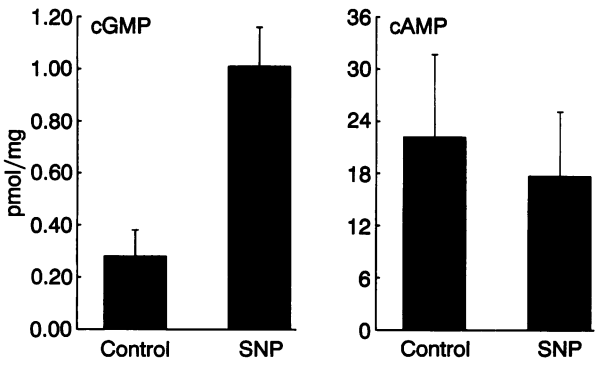

FIG. 5. Effect of SNP on cGMP and cAMP levels in slices of substantia nigra. Cyclic nucleotide levels were measured by RIA after incubation of slices in the presence of SNP (1 mM) for 10 min. (Difference between control and SNP-treated slices by Student's t test: cGMP, $n = 6$, $P < 0.01$; cAMP, $n = 4$, $P > 0.05$.) Experimental procedures were as described in *Materials and Methods* and in the legend to Fig. 1.

Effect of Cyclic Nucleotide Derivatives on Phosphorylation of DARPP-32. Evidence in support of the idea that SNP increased the phosphorylation of DARPP-32 by virtue of activating cGMP-dependent protein kinase was obtained in experiments utilizing 8-Br-cGMP and 8-pCPT-cGMP, two stable analogs of cGMP that selectively activate cGMP-dependent protein kinase (18, 26). At a concentration of 1 mM, each of these compounds markedly stimulated DARPP-32 phosphorylation, as did 8-Br-cAMP, an analog of cAMP that selectively activates cAMP-dependent protein kinase (Fig. 6 and Table 1). In contrast, at a concentration of 0.1 mM, each of the cGMP analogs caused a substantially greater increase in DARPP-32 phosphorylation than did 8-Br-cAMP.

DISCUSSION

Recent studies indicate that the NO/cGMP pathway is an important intercellular signaling mechanism in a wide variety of tissues in the periphery as well as in the central nervous system (14–17). Protein phosphorylation is one plausible mechanism through which activation of soluble guanylyl cyclase by NO could regulate target cells. The present study demonstrated that SNP increased the phosphorylation of DARPP-32 on threonine-34 in tissue slices prepared from rat substantia nigra. To our knowledge, these data provide the first demonstration of a NO-mediated effect on protein phosphorylation in the central nervous system. The SNP-induced increase in DARPP-32 phosphorylation in nigral slices was abolished by pretreatment of the slices with Hb, a compound

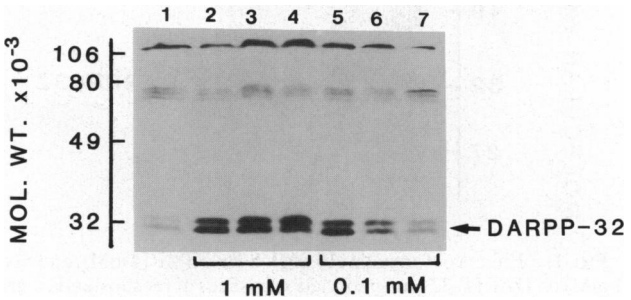

FIG. 6. Comparison of the effects of cGMP and cAMP derivatives on phosphorylation of DARPP-32 in slices of substantia nigra. 8-Br-cGMP (lanes 2 and 5), 8-pCPT-cGMP (lanes 3 and 6), and 8-Br-cAMP (lanes 4 and 7) were present in final concentrations of 1 mM or 0.1 mM as indicated. Lane 1, control. Other experimental procedures were as described in *Materials and Methods* or in the legend to Fig. 1.

that, by complexing with NO, prevents the activation of guanylyl cyclase by NO. The hypothesis that NO regulates phosphorylation of DARPP-32 by increasing the activity of cGMP-dependent protein kinase is strengthened by the observation that SNP treatment increased cGMP levels. The possible involvement of cGMP-dependent protein kinase in the SNP-stimulated phosphorylation of DARPP-32 is also supported by experiments with the cGMP analogs 8-Br-cGMP and 8-pCPT-cGMP, showing that activation of cGMP-dependent protein kinase alone is sufficient to increase the phosphorylation state of DARPP-32.

The present results provide evidence that the NO/cGMP pathway regulates the phosphorylation of DARPP-32 in nerve terminals of striatonigral neurons. However, the identity of the cell type(s) that participates in endogenous formation of NO is unclear. Many nerve fibers in the substantia nigra express NO synthase as shown by NADPH diaphorase histochemistry (19, 27, 28). Cholinergic fibers innervating the substantia nigra and originating in the laterodorsal pontine tegmentum (28–31) have been shown to coexpress NO synthase (28) and are a plausible candidate for the site of NO formation.

The nature of the transmitter(s) that activates NO synthase in nerve fibers in the substantia nigra also remains to be clarified. Previous studies using slices of rat cerebellum, a brain region with many NO synthase-containing neurones and fibers (16, 19, 32), showed that L-arginine, the NO precursor (33), did not elevate cGMP levels when treated alone but did potentiate the cGMP-elevating effect of the glutamatergic agonist NMDA (34). In the present study, no effect of L-arginine alone or L-arginine plus NMDA on DARPP-32 phosphorylation was observed in slices of substantia nigra. These results with the substantia nigra suggest that NMDA-receptive neurons may not be involved or that the amount of NO synthesis was below the threshold for the activation of the cGMP/cGMP-dependent protein kinase pathway under the experimental conditions tested.

The results of the present study support the concept that DARPP-32 represents a final common pathway through which many nerve cells and neurotransmitters exert their actions on medium-sized spiny neurons. For instance, dopamine, acting on D_1 receptors, elevates intracellular cAMP, leading to activation of cAMP-dependent protein kinase and phosphorylation of DARPP-32 (4). Glutamate, acting on NMDA receptors, elevates intracellular calcium, leading to activation of calcium/calmodulin-dependent phosphatase and dephosphorylation of DARPP-32 (25). γ-Aminobutyric acid, acting on type A γ-aminobutyric acid receptors, phosphorylates DARPP-32 through an unknown intracellular signal transduction mechanism (35). The present results indicate that NO, an intercellular messenger, elevates intracellular cGMP, leading to activation of cGMP-dependent protein kinase and phosphorylation of DARPP-32. The state of phosphorylation of DARPP-32 appears to provide an important mechanism for integration of information converging on striatonigral neurons from a variety of neuronal pathways.

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