Dephosphorylation of Ser-137 in DARPP-32 by protein phosphatases 2A and 2C: different roles *in vitro* and in striatonigral neurons

Frédéric DESDOUITS*, Julio C. SICILIANO*†, Angus C. NAIRN‡, Paul GREENGARD‡ and Jean-Antoine GIRAULT*[1]

*INSERM U114, Chaire de Neuropharmacologie, Collège de France, Paris, France, †Departamento de Histologia, Facultad de Medicina, Montevideo, Uruguay, and ‡Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, U.S.A.

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, $M_r = 32000$) is highly expressed in striatonigral neurons in which its phosphorylation is regulated by several neurotransmitters including dopamine and glutamate. DARPP-32 becomes a potent inhibitor of protein phosphatase 1 when it is phosphorylated on Thr-34 by cAMP- or cGMP-dependent protein kinases. DARPP-32 is also phosphorylated on Ser-137 by protein kinase CK1 (CK1), *in vitro* and *in vivo*. This phosphorylation has an important regulatory role since it inhibits the dephosphorylation of Thr-34 by calcineurin *in vitro* and in striatonigral neurons. Here, we show that DARPP-32 phosphorylated by CK1 is a substrate *in vitro* for protein phosphatases 2A and 2C, but not protein phosphatase 1 or calcineurin. However, in substantia nigra slices, dephosphorylation of Ser-137 was markedly sensitive to decreased temperature, and not detectably affected by the presence of okadaic acid under conditions in which dephosphorylation of Thr-34 by protein phosphatase 2A was inhibited. These results suggest that, in neurons, phospho-Ser-137-DARPP-32 is dephosphorylated by protein phosphatase 2C, but not 2A. Thus, DARPP-32 appears to be a component of a regulatory cascade of phosphatases in which dephosphorylation of Ser-136 by protein phosphatase 2C facilitates dephosphorylation of Thr-34 by calcineurin, removing the cyclic nucleotide-induced inhibition of protein phosphatase 1.

INTRODUCTION

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, $M_r = 32000$) is enriched in a number of specific neuronal and non-neuronal cells, including striatonigral neurons (reviewed in [1]). Many, but not all, of these cells express the D1 dopamine receptors and DARPP-32 is thought to mediate some of the actions of dopamine downstream of this receptor [2]. DARPP-32 is phosphorylated on Thr-34 *in vitro* and in intact cells by cAMP-dependent protein kinase (PKA) [2,3] and cGMP-dependent protein kinase (PKG) [4,5]. Phosphorylation of DARPP-32 on Thr-34 converts the protein from a low affinity (IC50 $\sim 1\ \mu$M) into a high affinity ($IC_{50} \sim 0.5$ nM) inhibitor of protein phosphatase 1 (PP1) [3,6]. DARPP-32 phosphorylated on Thr-34 is dephosphorylated by protein phosphatases 2A (PP2A) and calcineurin (Ca^{2+}/calmodulin-activated protein phosphatase; protein phosphatase 2B) [3].

Phosphorylation of Thr-34 is regulated by several neurotransmitter pathways. Stimulation of dopamine D1 receptors (2), norepinephrine β receptors [5], VIP receptors [5] and γ-aminobutyric acid (GABA)-A receptors [7], as well as increases in nitric oxide [8], enhance phosphorylation of Thr-34. In contrast, stimulation of glutamate *N*-methyl-D-aspartate receptors [9], CCK receptors [10] or depolarization (F. Desdouits, J. C. Siliano and J.-A. Girault, unpublished work) decrease this phosphorylation. Physiological targets for regulation via the DARPP-32/PP1 cascade include Ca^{2+} channels [11], Na^+ channels [11a], and Na^+, K^+-ATPase [12].

DARPP-32 is also phosphorylated *in vivo* on Ser-137 by protein kinase CK1 (CK1, casein kinase 1) [13] and on Ser-102 by protein kinase CK2 (CK2, casein kinase 2) [14]. Phosphorylation of DARPP-32 by CK1 and CK2 modulates the phosphorylation state of Thr-34. Phosphorylation by CK2 increases the V_{max} of phosphorylation of DARPP-32 by PKA but not PKG [14]. Phosphorylation of Ser-137 in DARPP-32 by CK1 inhibits the dephosphorylation of Thr-34 by calcineurin but not PP2A [15]. Notably, phosphorylation on Ser-137 by CK1 increases the electrophoretic mobility of DARPP-32 in polyacrylamide gels in the presence of SDS [13]. This shift in mobility is unusual since phosphorylation tends generally to increase the apparent size of proteins in SDS/PAGE, and allows the monitoring of Ser-137 phosphorylation in intact cells. The stoichiometry of phosphorylation of striatal DARPP-32 at Ser-137 is higher in nerve terminals in the substantia nigra than in the somatodendritic region in the striatum [13]. Thus, the phosphorylation by CK1 is likely to reduce or prevent calcineurin-induced dephosphorylation of Thr-34 in nerve terminals [15].

The physiological implications of phosphorylation of Ser-137 prompted us to identify the protein phosphatase(s) responsible for its dephosphorylation. Here, we show, using both purified phosphatases and brain homogenates, that DARPP-32 phosphorylated on Ser-137 is a substrate for PP2A and PP2C, but not for PP1 or calcineurin. We also demonstrate that, in nigral slices, Ser-137 is dephosphorylated by an okadaic acid-resistant protein phosphatase, presumably PP2C. The results suggest that DARPP-32 is a physiological substrate for PP2C in neurons and provide evidence for a phosphatase cascade sequentially involving PP2C, calcineurin and PP1.

EXPERIMENTAL PROCEDURES

Materials

Wild-type or mutated recombinant rat DARPP-32 was produced and purified as described [16]. Bovine DARPP-32 was purified from calf caudate nucleus [17]. PKA from rabbit skeletal muscle or calf heart [18], CK1 [19] and CK2 from calf thymus [14], PP1 and PP2A from rabbit muscle [20] and PP2C from rat liver [21], were purified as described. Calcineurin was purchased from

Abbreviations used: DARPP-32, dopamine- and cAMP-regulated phosphoprotein, $M_r = 32000$; CK1, protein kinase CK1/casein kinase 1; CK2, protein kinase CK2/casein kinase 2; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2C, protein phosphatase 2C; PP1c, catalytic subunit of PP1; PP2Ac, catalytic subunit of PP2A.

[1] To whom correspondence should be addressed.

Sigma. DARPP-32 antibodies and phosphorylation-state specific antibodies have been characterized previously [5,22]. Phosphorylase a and phospho-casein were prepared as described [20,21]. Radioactive ATP and autoradiographic films were from Du Pont-New England Nuclear. Rats were purchased from Charles River (France). Acrylamide was from National Diagnostic. Okadaic acid and calyculin A were purchased from R.B.I.

In vitro phosphorylation and dephosphorylation

In vitro phosphorylation by PKA, CK2 or CK1 was carried out in the presence of [γ-^{32}P]ATP (500–2000 c.p.m./pmol), as described [13,14]. Purified phosphatases were assayed in 50 mM Tris/HCl, 15 mM 2-mercaptoethanol, and 1 mg/ml BSA, as described [14], in the presence of 100 μM $CaCl_2$ and 1 μM calmodulin in the case of calcineurin, or 10 mM $MgCl_2$ in the case of PP2C. Purified PP2C was assayed in the presence of 300 nM okadaic acid to inhibit traces of contaminating PP2A. Reactions were started by the addition of substrate, and stopped by adding 150 μl of 20 % (w/v) trichloroacetic acid. After a further addition of 150 μl of 6 mg/ml bovine serum albumin, samples were centrifugated for 5 min at room temperature at 17000 ***g***, and the amount of ^{32}P in the supernatant and the pellet was determined by measurement of Čerenkov radiation. Under initial rate conditions, release of phosphate was linear with time and corresponded to < 15 % of the phosphate incorporated into the substrate. For measurement of phosphatase activities, striata and substantia nigra were homogenized in 10 mM Tris/HCl, pH 7.5, 2 mM EGTA, 10 mM β-mercaptoethanol, 20 μg/ml leupeptin, 20 μg/ml trasylol, 2 μg/ml pepstatin A and 0.5 mM PMSF, with 20 strokes of a Teflon pestle in a Potter Elvejhem glass homogenizer which had been precooled on ice. Since brain homogenates contained EGTA, 2.5 mM $CaCl_2$ and 1 μM calmodulin were added for the measurement of calcineurin activity in the homogenate. Phosphatase activities in brain homogenates were assayed, as described for purified enzymes, using ~ 4 μg of protein.

Analysis of DARPP-32 phosphorylation in nigral slices

Rat substantia nigra slices were processed as described [15]. Incubations were stopped by removing the medium and slices were quickly frozen in liquid nitrogen. Tissues were sonicated in boiling 1 % SDS in water (w/v) and subjected to SDS/PAGE. DARPP-32 was analysed by immunoblotting, with either a monoclonal antibody which reacts only with DARPP-32 phosphorylated on Thr-34 [5], or a mixture of two monoclonal antibodies (C24-5a and C24-6a) which react with DARPP-32 independently of its state of phosphorylation [22]. Immunoreactivity was detected using horseradish peroxidase-coupled donkey-anti-mouse secondary IgG antibody and a chemiluminescence method (ECL, Amersham). The intensity of immunoreactive bands on the films was quantified by computer-assisted densitometric measurement.

RESULTS

Dephosphorylation of DARPP-32 by purified protein phosphatases

The activity of catalytic subunits of PP1 (PP1c), or PP2A (PP2Ac), calcineurin, and PP2C was examined on purified recombinant rat DARPP-32 phosphorylated by PKA, CK2 or CK1 (Table 1). We compared the activities of these phosphatases towards two reference substrates (phosphorylase a and casein). Recombinant rat DARPP-32 phosphorylated on Thr-34 by PKA was a good substrate for PP2Ac and calcineurin, whereas, when it was phosphorylated by CK2, it was only significantly dephosphorylated by PP1c and PP2Ac. DARPP-32 phosphorylated by CK1 was an excellent substrate for PP2Ac but it was also significantly dephosphorylated by PP2C (Table 1). In contrast, DARPP-32 phosphorylated by CK1 was a poor substrate for PP1c and calcineurin.

Table 1 Dephosphorylation of phospho-DARPP-32 by purified protein phosphatases

The activities of purified PP1c, PP2Ac, calcineurin and PP2C were measured under initial rate conditions, using reference substrates (phosphorylase a, i.e. phosphorylated by phosphorylase kinase, and casein phosphorylated by PKA) and DARPP-32 phosphorylated by PKA, CK2 or CK1. Phospho-substrates were used at a final concentration of 1 μM, with the exception of casein (PKA) which was used at 4 μM. Amounts of each phosphatase used were chosen to have a similar activity on their respective preferred substrate. Data are expressed as mean $\pm$ S.D. of two separate experiments performed in triplicate. N.A., no significant activity detected.

	Phosphatase activity (fmol/min)			
Substrate	PP1c	PP2Ac	Calcineurin	PP2C
Phosphorylase a	157 $\pm$ 15	91 $\pm$ 8	N.A.	N.A.
Casein (PKA)	N.A.	260 $\pm$ 17	18 $\pm$ 3	189 $\pm$ 5
DARPP-32 (PKA)	16 $\pm$ 2	130 $\pm$ 6	110 $\pm$ 6	12 $\pm$ 1
DARPP-32 (CK2)	36 $\pm$ 4	46 $\pm$ 2	14 $\pm$ 2	11 $\pm$ 1
DARPP-32 (CK1)	13 $\pm$ 1	136 $\pm$ 7	11 $\pm$ 1	41 $\pm$ 3

	Ser-137	Ser-189
1. Rat DARPP-32	...EEED**S**QAEV...	...RATQ**S**EPGE...
2. Rat S137A-DARPP-32	...EEEDAQAEV...	...RATQ**S**EPGE...
3. Bovine DARPP-32	...EEED**S**QAEV...	...DPALNEPGE...

Figure 1 Amino acid sequences surrounding serines 137 and 189 in DARPP-32

The sequence surrounding the two residues phosphorylated by CK1 in rat DARPP-32 *in vitro* (Ser-137 and Ser-189, bold underlined), and the corresponding regions in mutated S137A-DARPP-32 and bovine DARPP-32, are shown. Only Ser-137 appears to be phosphorylated in intact cells [13].

Table 2 Dephosphorylation of Ser-137 and Ser-189 in DARPP-32 by purified protein phosphatases

Recombinant wild-type rat DARPP-32, recombinant mutated rat S137A-DARPP-32, and bovine DARPP-32 (see Figure 1 for the phosphorylation sites sequences) were phosphorylated by CK1 to a stoichiometry of 1.90, 0.88 and 0.95, respectively. The phosphorylated proteins were repurified as described in [13], and incubated at a final concentration of 0.5 μM in the presence of either purified PP2Ac or PP2C. Data are expressed as mean $\pm$ S.E.M. of three separate experiments. Statistical analysis was done by analysis of variance ($F(2,6) = 146.2$; $P < 0.0001$ and $F(2,6) = 5.7$; $P = 0.041$ for PP2C and PP2A data, respectively) followed by Fisher's Protected Least Significant Difference test ($^*P < 0.05$ and $^{**}P < 0.01$ comparing the dephosphorylation rate of S137A-DARPP-32 to either recombinant DARPP-32 or bovine DARPP-32).

	Phosphatase activity (fmol/min)	
Substrate	PP2Ac	PP2C
Rat DARPP-32	63 $\pm$ 14	146 $\pm$ 4
Rat S137A-DARPP-32	25 $\pm$ 1*	62 $\pm$ 4**
Bovine DARPP-32	57 $\pm$ 6	150 $\pm$ 5

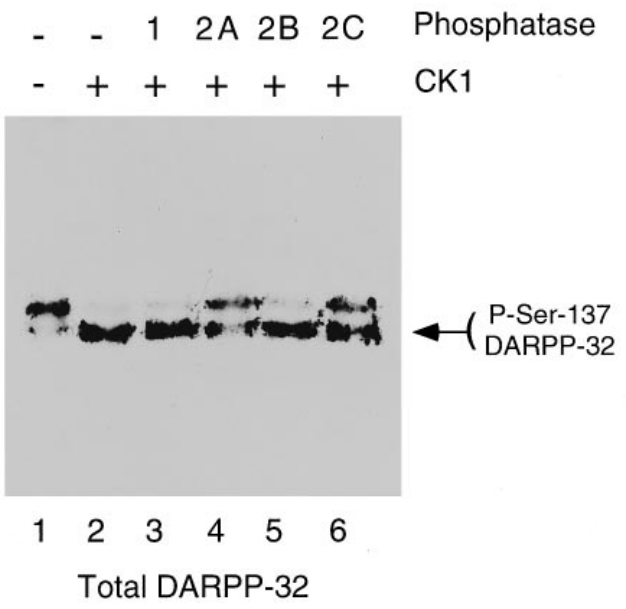


Figure 2 Dephosphorylation of Ser-137 in DARPP-32 by purified protein phosphatases

Recombinant rat DARPP-32 (lane 1) was phosphorylated by CK1 (lane 2) and repurified. The phosphorylation of Ser-137 was assessed by monitoring the downward shift induced in DARPP-32 migration in SDS/PAGE (compare lanes 1 and 2). Phosphorylated DARPP-32 was then incubated in the presence of equivalent amounts (see Table 1) of purified PP1c (lane 3), PP2Ac (lane 4), calcineurin (2B, lane 5) or PP2C (lane 6). Samples were analysed by immunoblotting with monoclonal antibodies which react with DARPP-32 independently of its state of phosphorylation. Dephosphorylation of Ser-137 by PP2Ac (lane 4) and PP2C (lane 6) reversed the shift in migration of DARPP-32. The lower band corresponding to DARPP-32 phosphorylated by CK1 on Ser-137 is indicated (P-Ser-137 DARPP-32). Note that the small amount of recombinant DARPP-32 migrating as the lower band in the absence of phosphorylation of CK1 (lane 1) corresponds to a breakdown product.

Recombinant DARPP-32 is phosphorylated *in vitro* by purified CK1 on two major sites, Ser-137 and Ser-189, of which only the former appears to be physiological [13]. In experiments in which the initial rates of dephosphorylation were assessed by measuring the release of phosphate, the dephosphorylation of the two sites could not be distinguished. To determine the relative contribution of the two sites, we compared the activity of PP2Ac and PP2C on wild-type rat DARPP-32, mutated rat DARPP-32 containing an alanine at position 137 instead of a serine (S137A-DARPP-32), and bovine DARPP-32, in which Ser-189 is naturally changed to an asparagine whereas Ser-137 is conserved (see Figure 1). The initial rates of dephosphorylation of bovine DARPP-32 and wild-type recombinant rat DARPP-32 by PP2A were identical, whereas this rate was significantly less when S137A-DARPP-32 was used as a substrate (Table 2). Similar results were obtained when PP2C activity was studied on the three forms of DARPP-32 (Table 2). These experiments demonstrate that the absence of phospho-Ser-189 has no effect on the rates of dephosphorylation of DARPP-32 by PP2A or PP2C, whereas the absence of phospho-Ser-137 significantly decreases these rates. Thus, phospho-Ser-137 is a better substrate for both phosphatases than phospho-Ser-189. Finally, we took advantage of the fact that the shift in electrophoretic mobility of DARPP-32 is due only to phosphorylation of Ser-137 by CK1 [13] to examine specifically the protein phosphatase acting on this residue. This mobility shift was reversed by either PP2Ac or PP2C, but not by PP1c or calcineurin (Figure 2), demonstrating that both PP2Ac and PP2C dephosphorylate Ser-137 *in vitro*.

Dephosphorylation of DARPP-32 phosphorylated by CK1 in striatal and nigral homogenates

Protein phosphatases exist in various forms due to their association with multiple regulatory and targeting subunits which may modify their physiological specificity [23,24]. Therefore, we characterized the native phosphatase activities present in striatal homogenates capable of dephosphorylating DARPP-32 phosphorylated by CK1, using several inhibitors. These inhibitors included okadaic acid and cantharidin, which inhibit PP2A with

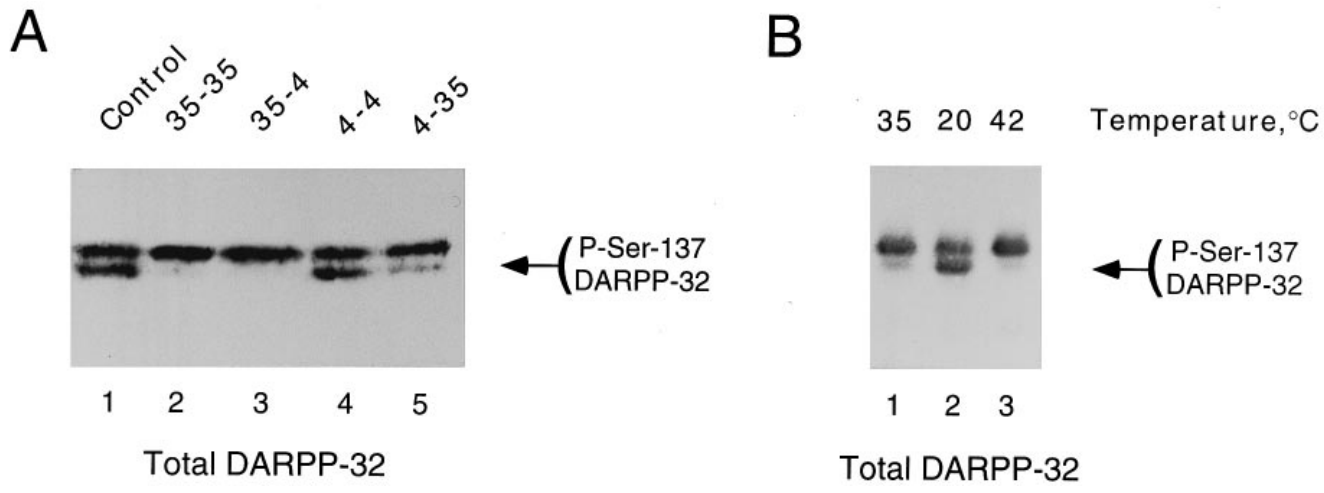


Figure 3 Effect of temperature on the dephosphorylation of DARPP-32 Ser-137 in nigral slices

(**A**) Nigral slices were homogenized immediately after dissection (lane 1) or incubated for 45 min at 35 °C (lanes 2 and 3) or 4 °C (lanes 4 and 5) and then for 10 min at 35 °C (lanes 2 and 5) or 4 °C (lanes 3 and 4). (**B**) Nigral slices were incubated for 45 min at either 35 °C (lanes 1), 20 °C (lane 2) or 42 °C (lane 3). Endogenous DARPP-32 was analysed by immunoblotting with monoclonal antibodies reacting with DARPP-32 independently of its state of phosphorylation (Total DARPP-32). (**A**) and (**B**) are representative of three separate experiments giving similar results. The lower band corresponding to DARPP-32 phosphorylated on Ser-137 is indicated (P-Ser-137 DARPP-32).

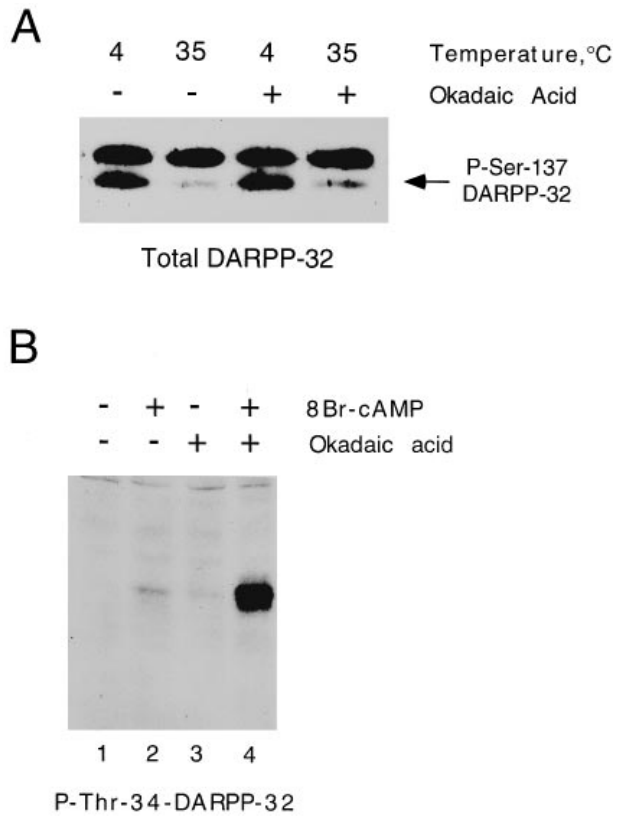


Figure 4 Effect of okadaic acid on the dephosphorylation of DARPP-32 Ser-137 and Thr-34 in nigral slices

(**A**) Nigral slices were incubated for 45 min at 4 °C in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 μM okadaic acid. The slices were then incubated for a further 10 min at either 4 °C (lanes 1 and 3) or 35 °C (lanes 2 and 4). The proportion of endogenous DARPP-32 migrating as the lower band of the doublet (P-Ser-137 DARPP-32) reflects the stoichiometry of phosphorylation of Ser-137. The stoichiometry was: absence of okadaic acid at 4 °C, 42 ± 2; 35 °C: 18 ± 3; presence of okadaic acid at 4 °C, 41 ± 2; 35 °C, 19 ± 4 (means $\pm$ S.E.M. of three experiments; analysis of variance $F(3,8) = 85.7$; $P < 0.0001$). (**B**) Nigral slices were incubated for 45 min at 35 °C in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 μM okadaic acid and for a further 10 min in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM 8Br-cAMP. DARPP-32 was analysed by immunoblotting with monoclonal antibodies reacting only with DARPP-32 phosphorylated by PKA on Thr-34 (P-Thr-34-DARPP-32) or with DARPP-32 independently of its state of phosphorylation (Total DARPP-32). (**B**) is representative of three separate experiments giving similar results.

a better potency than PP1, calyculin A which inhibits both with similar potency (see [25] for a review), and phospho-DARPP-32 which inhibits only PP1. Under basal conditions in striatal homogenates, dephosphorylation of purified recombinant DARPP-32 phosphorylated by CK1 was inhibited by calyculin A ($IC_{50} \sim 0.1$ nM), okadaic acid ($IC_{50} \sim 1$ nM) and cantharidine ($IC_{50} \sim 500$ nM). In contrast, the addition of 100 nM phospho-Thr-34-DARPP-32, a potent inhibitor of PP1 [3] had no effect on dephosphorylation of CK1-phosphorylated DARPP-32, whereas it inhibitied the dephosphorylaton of phosphorylase a (a substrate for PP1 and PP2A) by approx. 60 % under the same conditions (results not shown). This pharmacological profile suggested that PP2A, but not PP1, dephosphorylated phospho-Ser-137, under these conditions.

In nigral homogenates the stoichiometry of phosphorylation of endogenous DARPP-32 on Ser-137 was 0.39 ± 0.01 mol phosphate/mol protein (mean $\pm$ S.E.M. from three experiments), as estimated by the electrophoretic mobility-shift induced by the phosphorylation of this residue. Following a 30 min incubation of the homogenates at 37 °C, the stoichiometry of phosphorylation of Ser-137 was 0.11 ± 0.01 in the absence of any addition, 0.39 ± 0.01 in the presence of 0.1 μM okadaic acid, and 0.33 ± 0.03 in the presence of okadaic acid and 10 mM Mg^{2+} (mean $\pm$ S.E.M., three experiments). The slight (15 %) Mg^{2+}-stimulated dephosphorylation of DARPP-32 in the presence of okadaic acid is likely to correspond to the activity of PP2C. Such a low contribution of PP2C to the total phosphatase activity in nigral homogenates is in agreement with previous reports [26–28] and does not preclude a significant role for this phosphatase in the dephosphorylation of DARPP-32 on Ser-137 in intact neurons.

Dephosphorylation of Ser-137 in nigral slices

Phosphorylation of DARPP-32 on Ser-137 is high in rat substantia nigra or striatum, but is lost rapidly when the dissected tissue is preincubated at 30 °C before homogenization [13]. To study the mechanism of this dephosphorylation, we have used nigral slices incubated *in vitro*. The stoichiometry of phosphorylation of Ser-137, estimated by the amount of DARPP-32 with a fast electrophoretic migration (i.e. the lower band of the doublet), was unchanged after a 55 min incubation of the slices at 4 °C (Figure 3A, lanes 1 and 4). In contrast, Ser-137 was fully dephosphorylated after a 55 min incubation at 35 °C (Figure 3A, lane 2). The disappearance of the lower band, i.e. dephosphorylation of Ser-137, was highly sensitive to decreased temperature since it was prevented when slices were incubated at 20 °C, whereas it was still observed at 42 °C (Figure 3B). In principle, these observations could be explained either by an increase in phosphatase activity at 35 °C, or by a less likely increase in kinase activity at lower temperatures. To address this question, nigral slices were incubated for 45 min at 35 °C (lanes 2 and 3), or at 4 °C (lanes 4 and 5). The slices were then incubated for an additional 10 min at the same temperature (lanes 2 and 4) or shifted from 35 °C to 4 °C (lane 3) or from 4 °C to 35 °C (lane 5). The 10 min incubation at 35 °C resulted in an almost complete disappearance of the fast migrating form of DARPP-32, attesting of the dephosphorylation of Ser-137. In contrast, as expected, a 10 min incubation at 4 °C was unable to restore this phosphorylation.

We then tested which of the two phosphatases active on DARPP-32 *in vitro* was involved in this temperature-sensitive dephosphorylation in intact nerve terminals. The disappearance of the lower DARPP-32 band, reflecting dephosphorylation of Ser-137, was still observed in the presence of 1 μM okadaic acid, a potent inhibitor of PP2A, inactive on PP2C (Figure 4A). To verify that okadaic acid was active on PP2A in the nerve terminals containing DARPP-32, we monitored the state of phosphorylation of Thr-34 on DARPP-32, which is phosphorylated by PKA and dephosphorylated by PP2A and calcineurin (Table 1 and [3,4]). Under these experimental conditions, okadaic acid increased dramatically the effects of 8-Br-cAMP on Thr-34 phosphorylation (Figure 4B), showing that it was fully active. Thus, in striatonigral nerve terminals, phospho-Ser-137 in DARPP-32 is not dephosphorylated by PP2A, but by a temperature-sensitive, okadaic acid-resistant phosphatase, which is likely to be PP2C.

DISCUSSION

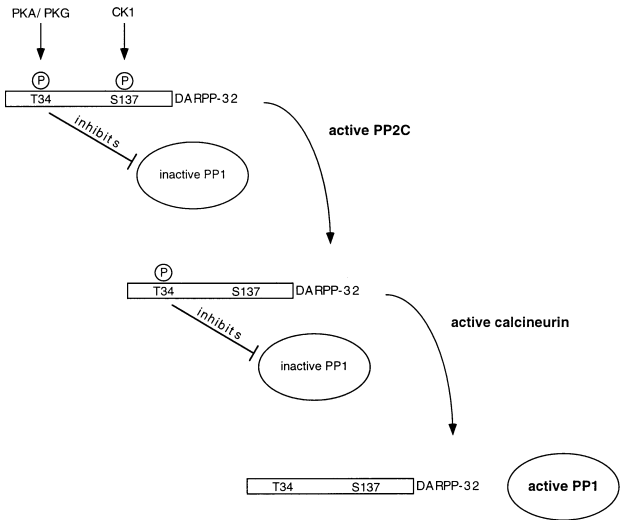
Although protein phosphatases play a critical role in neuronal physiology, including the regulation of ion channels [29] and of synaptic plasticity [30], relatively little is known about their regulation in neurons. Calcineurin is activated by Ca^{2+} influx in

response to depolarization or stimulation of glutamate receptors [9,31,32]. PP1c is regulated by protein–protein interactions with both targeting and inhibitory subunits [23]. Among those, DARPP-32 is the most thoroughly characterized inhibitor of PP1 in neurons [33]. PP2Ac is complexed to subunits, which regulate the substrate specificity and/or the localization of the enzyme [24]. In the case of PP2C, no regulation has been suggested due, in part, to the absence of identified specific substrates for this enzyme in intact cells. In the present study, we provide evidence that Ser-137 in DARPP-32 is dephosphorylated by PP2C in striatonigral neurons. In contrast, PP2A, which dephosphorylates both Thr-34 and Ser-137 *in vitro*, appears to be active only on Thr-34 in these neurons.

DARPP-32 phosphorylated by CK1 was efficiently dephosphorylated by purified PP2Ac. Other substrates of CK1, including large T antigen from SV40, p53 and acetyl CoA carboxylase, are also substrates for PP2A [34,35]. In the case of DARPP-32, PP2Ac dephosphorylates, *in vitro*, all the sites of phosphorylation that have been identified so far. This is in agreement with the relative lack of selectivity of free PP2Ac. However, native PP2A present in brain homogenates was also active on Ser-137, indicating that dephosphorylation of this residue did not result from an artifactual activity of the purified catalytic subunit.

Purified PP2C was also active on DARPP-32 phosphorylated by CK1. PP2C is present at lower levels than other types of serine/threonine protein phosphatases in brain [26]. In particular, the expression level of PP2C mRNA is low and diffuse in the striatum, suggesting that striatonigral neurons express low amounts of this enzyme [36]. This may explain why the activity of PP2C on DARPP-32 phosphorylated by CK1 was low compared with PP2A activity in nigral homogenates. Moreover, in tissue homogenates, the proteins from striatonigral terminals are diluted by those from other cell types. In contrast with what could be expected from the high activity of PP2A on DARPP-32 phosphorylated by CK1 *in vitro*, dephosphorylation of Ser-137 was fully resistant to okadaic acid in nigral slices. This was not due to a lack of activity of this compound in striatonigral terminals, since okadaic acid exerted a strong effect on dephosphorylation of Thr-34 under the same experimental conditions. Since, to our knowledge, PP2C is the only cytoplasmic regulatory serine/threonine phosphatase identified in mammals, fully resistant to okadaic acid [25,37], this phosphatase, which is active on DARPP-32 phosphorylated on Ser-137 *in vitro*, is the best candidate for dephosphorylating DARPP-32 in neurons. Calcineurin, which has a low sensitivity to okadaic acid, was inactive on phospho-Ser-137 *in vitro*. Nevertheless, in the absence of a specific inhibitor of PP2C, the identification of this enzyme in intact cells remains tentative. The marked temperature sensitivity of dephosphorylation of Ser-137, which was fully inhibited at 20 °C or less, provides some additional support for a role of PP2C, since purified PP2C exhibits an optimal temperature of 45 °C [38]. Thus Thr-34 and Ser-137, two important regulatory phosphorylation sites on DARPP-32, are dephosphorylated by distinct phosphatases in nigral termini of medium-sized spiny neurons. The contrast between the activity of PP2A on Thr-34 and Ser-137 *in vitro* and in striatonigral neurons demonstrates the high degree of substrate specificity of this enzyme in intact cells, the mechanism of which remains to be elucidated.

Very few neuronal substrates for PP2C have been identified so far. One is autophosphorylated CaM-kinase II, suggesting that PP2C may counteract the activation of this enzyme by the Ca^{2+} pathway [39]. Our results provide strong evidence that DARRP-32 phosphorylated on Ser-137 is a good and specific substrate for PP2C in neurons. The ease with which phosphorylation of Ser-137 can be monitored, using the shift in the electrophoretic mobility of DARPP-32, should facilitate further studies investigating the regulation of PP2C in intact cells. In addition, the present results, combined with previous work, indicate that DARPP-32 is a component of a network of dephosphorylation reactions leading to the reactivation of PP1 (Scheme 1). Protein kinase activation cascades have been described and appear to be a frequent mechanism for regulation of protein phosphorylation in cells in response to external or internal stimuli. Interestingly, these cascades represent powerful means for signal amplification and modulation [40]. In contrast, there are few known examples of phosphatase cascades. Reactivation of PP1 following dephosphorylation of DARPP-32 and/or inhibitor 1 by calcineurin in response to increases in cytosolic free Ca^{2+} is one of them. In the nervous system this reaction has been postulated to play an important role in hippocampal long-term depression, a well-studied model of synaptic plasticity [30]. In the case of DARPP-32, our results suggest an additional modulatory role for PP2C which, by increasing the sensitivity of Thr-34 to calcineurin, would facilitate the suppression by Ca^{2+} signals of cAMP- or cGMP-induced inhibition of PP1. Thus, DARPP-32 provides a very fine regulation of PP1 by multiple signal transduction pathways in striatonigral neurons.



Scheme 1 Phosphatase cascade involving DARPP-32 in striatonigral neurons

Following a stimulus which increases cAMP or cGMP, PKA or PKG, respectively, phosphorylate Thr-34 in DARPP-32, leading to a potent inhibition of protein phosphatase 1 (PP1). A large proportion of DARPP-32 is phosphorylated by CK1 on Ser-137, protecting phospho-Thr-34 from dephosphorylation by the Ca^{2+}-activated protein phosphatase calcineurin. PP2C dephosphorylates Ser-137, making phospho-Thr-34 more sensitive to calcineurin. Thus, PP2C would facilitate the reactivation of PP1 by making DARPP-32 more sensitive to calcineurin. PP2A (not indicated on this scheme) is able to shunt this cascade by dephosphorylating Thr-34 in a Ca^{2+}-independent manner, and independently of the phosphorylation state of Ser-137 (see the text for references).

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