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

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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, phosphorylation of 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 ~ 1 \( \mu \)M) into a high affinity (IC50 ~ 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 \( \beta \) receptors [5], VIP receptors [5] and \( \gamma \)-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. Desdoits, 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 \( V_{\text{max}} \) 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.

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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 Diagnost. 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 $[^{32}P]ATP$ (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 mM okadaic acid to inhibit traces of contaminating PP2A. Reactions were started by the addition of substrate, and stopped by adding 150 µl of 20% (v/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, pH 7.5, 2 mM EGTA, 10 mM β-mercaptoethanol, 20 µg/ml leupeptin, 20 µg/ml trasylool, 2 µg/ml pepstatin A and 0.5 mM PMSF, with 20 strokes of a Teflon pestle in a Potter Elvehjem 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 phosphorlated on Thr-34 [5], or a mixture of two monoclonal antibodies (C24-5a and C24-6a) which react 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 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 phosphorlated 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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PP1c</th>
<th>PP2Ac</th>
<th>Calcineurin</th>
<th>PP2C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase a</td>
<td>157 ± 15</td>
<td>91 ± 8</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Casein (PKA)</td>
<td>N.A.</td>
<td>260 ± 17</td>
<td>18 ± 3</td>
<td>189 ± 5</td>
</tr>
<tr>
<td>DARPP-32 (PKA)</td>
<td>16 ± 2</td>
<td>130 ± 6</td>
<td>110 ± 6</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>DARPP-32 (CK2)</td>
<td>36 ± 4</td>
<td>46 ± 2</td>
<td>14 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>DARPP-32 (CK1)</td>
<td>13 ± 1</td>
<td>136 ± 7</td>
<td>11 ± 1</td>
<td>41 ± 3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphatase activity (fmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat DARPP-32</td>
<td>63 ± 14</td>
</tr>
<tr>
<td>Rat S137A-DARPP-32</td>
<td>25 ± 1*</td>
</tr>
<tr>
<td>Bovine DARPP-32</td>
<td>57 ± 6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphatase activity (fmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2Ac</td>
<td>146 ± 4</td>
</tr>
<tr>
<td>PP2C</td>
<td>62 ± 4**</td>
</tr>
</tbody>
</table>

![Figure 1 Amino acid sequences surrounding serines 137 and 189 in DARPP-32](image)

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].

### 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.
Dephosphorylation of DARPP-32 by PP2A and PP2C

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 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.

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). The lower band corresponding to DARPP-32 phosphorylated on Ser-137 is indicated (P-Ser-137 DARPP-32).
Phosphorylation of DARPP-32 on Ser-137 was estimated by the electrophoretic mobility-shift induced by the phosphate of endogenous DARPP-32 on 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 Mg²⁺ (mean ± S.E.M., three experiments). The slight (15%) Mg²⁺-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 migrating as the lower band of the doublet (P-Ser-137 DARPP-32) reflects the stoichiometry of phosphorylation of DARPP-32. 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 ± S.E.M. of three experiments; analysis of variance F(3,8) = 85.7; P < 0.0001). 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 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.

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 mM okadaic acid. The slices were then incubated for a further 10 min at either 4 °C (lanes 1 and 2) 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 ± 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 mM 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 (IC₅₀ ~ 0.1 nM), okadaic acid (IC₅₀ ~ 1 nM) and cantharidine (IC₅₀ ~ 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 inhibited the dephosphorylation 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, dephosphorylates 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 ± 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 Mg²⁺ (mean ± S.E.M., three experiments). The slight (15%) Mg²⁺-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.

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 Ca²⁺ 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 Ca2+ pathway [39]. Our results provide strong evidence that DARPP-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

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**REFERENCES**
