Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2682–2685, March 1995
Neurobiology

Dopamine- and cAMP-regulated phosphoprotein DARPP-32: Phosphorylation of Ser-137 by casein kinase I inhibits dephosphorylation of Thr-34 by calcineurin

(multisite phosphorylation/protein phosphatase/basal ganglia/glutamate)

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Contributed by Paul Greengard, December 20, 1994

ABSTRACT **Although protein phosphatases appear to be highly controlled in intact cells, relatively little is known about the physiological regulation of their activity. DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32,000, is phosphorylated *in vitro* by casein kinase I, casein kinase II, and cAMP-dependent protein kinase on sites phosphorylated *in vivo*. DARPP-32 phosphorylated on Thr-34 by cAMP-dependent protein kinase is a potent inhibitor of protein phosphatase 1 and an excellent substrate for calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. Here we provide evidence, using both purified proteins and brain slices, that phosphorylation of DARPP-32 on Ser-137 by casein kinase I inhibits the dephosphorylation of Thr-34 by calcineurin. This inhibition occurs only when phospho-Ser-137 and phospho-Thr-34 are located on the same DARPP-32 molecule and is not dependent on the mode of activation of calcineurin. The results demonstrate that the inhibition is due to a modification in the properties of the substrate which alters its dephosphorylation rate. Thus, casein kinase I may play a physiological role in striatonigral neurons as a modulator of the regulation of protein phosphatase 1 via DARPP-32.**

When DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32,000, is phosphorylated on Thr-34 by cAMP-dependent protein kinase (PKA), it is converted into a potent inhibitor of protein phosphatase 1 catalytic subunit (PP1c) (1). Thr-34 is dephosphorylated by calcineurin (calcium/calmodulin-dependent protein phosphatase; protein phosphatase 2B) and protein phosphatase 2A (1, 2). In intact neurons of the neostriatum, dopamine, through the D_1-type receptor, activates PKA and increases the phosphorylation of DARPP-32 on Thr-34 (reviewed in ref. 3). Conversely, glutamate, through *N*-methyl-D-aspartate receptor channels, activates calcineurin and induces the dephosphorylation of DARPP-32 on Thr-34 (4). DARPP-32 is also phosphorylated *in vivo* on serine residues which are phosphorylated *in vitro* by casein kinase I and casein kinase II (5, 6). Phosphorylation of bovine DARPP-32 by casein kinase II converts the protein into a better substrate for PKA (5). Phosphorylation, *in vitro* and *in vivo*, of rat DARPP-32 on Ser-137 by casein kinase I induces an unusual shift in the migration of the protein in SDS/PAGE which could be due to a conformational change persisting in the presence of SDS (6). This increase in electrophoretic mobility provides a convenient means to identify DARPP-32 phosphorylated by casein kinase I *in vivo* and *in vitro*. Phosphorylation of Ser-137 has no effect on the ability of DARPP-32 to serve as a substrate for PKA and casein kinase II (F.D. and J.-A.G., unpublished data), nor does it alter the ability of PKA-phosphorylated DARPP-32 to inhibit PP1c (6). Here we report that phosphorylation of DARPP-32 on Ser-137 by casein kinase I inhibits the dephosphorylation of Thr-34 by calcineurin.

MATERIALS AND METHODS

Materials. Recombinant rat DARPP-32 was produced in *Escherichia coli* and purified as described (7). PKA from rabbit skeletal muscle or calf heart (8) and casein kinase I from calf thymus (9) were purified as described. Casein phosphorylated by PKA was prepared as described (10). Rats were purchased from Charles River; [γ-^{32}P]ATP from DuPont/NEN; acrylamide from National Diagnostics; nitrocellulose membranes (0.2-μm pore size) from Schleicher & Schuell; autoradiographic films from Kodak and Amersham; phenylmethanesulfonyl fluoride (PMSF) from Boehringer Mannheim; and trypsin, calcineurin, calmodulin and 8-bromo-cAMP from Sigma.

Analysis of DARPP-32 Phosphorylation in Nigral Slices. Rat substantia nigra slices (11) were preincubated in RPMI 1640 medium for 1 hr at 25°C in the presence of 1 μM okadaic acid. 8-Bromo-cAMP (1 mM) or vehicle was added for a further 10 min. Stimulation was stopped by removing the medium, and slices were quickly frozen in liquid nitrogen. Tissues were homogenized in boiling 1% (wt/vol) SDS in water by sonication and subjected to SDS/PAGE (11). DARPP-32 was analyzed by sequential immunoblotting, first with a monoclonal antibody which reacts only with DARPP-32 phosphorylated on Thr-34 (12), and then with a mixture of two monoclonal antibodies (C24-5a and C24-6a) which react with DARPP-32 independent of its state of phosphorylation (13). Immunoreactivity was detected with an enhanced chemiluminescence method (Amersham) using a horseradish peroxidase-coupled donkey anti-mouse secondary IgG antibody. The relative amounts of immunoreactive bands were quantified by computer-assisted densitometric measurement of the films. Recombinant DARPP-32 phosphorylated *in vitro* was used as a standard to estimate the stoichiometry of phosphorylation of DARPP-32 in nigral slices.

***In Vitro* Phosphorylation and Dephosphorylation.** *In vitro* phosphorylation by PKA or casein kinase I was carried out as described (6, 7). DARPP-32 was phosphorylated by PKA and by casein kinase I to a stoichiometry of $\approx$1 and $\approx$2 mol/mol, respectively, except when indicated. Stoichiometry of phosphorylation was followed by adding radioactive [γ-^{32}P]ATP. For study of calcineurin action, phosphorylation was carried out with high-specific-radioactivity ATP (500–2000 cpm/

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Abbreviations: PKA, cAMP-dependent protein kinase; PP1c, protein phosphatase 1 catalytic subunit; PMSF, phenylmethanesulfonyl fluoride.

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pmol). When DARPP-32 was phosphorylated with "nonradioactive ATP" a trace amount of [γ-^{32}P]ATP was used to verify that the phosphorylation reaction had gone to completion (10–50 cpm/pmol).

Calcineurin assays were carried out in 50 mM Tris·HCl, pH 7.0/15 mM 2-mercaptoethanol/0.1% bovine serum albumin, as described (5), and in the presence or the absence of Ca^{2+}, calmodulin, and Mn^{2+} in a final volume of 30 μl for 10 min at 30°C. Reactions were started by the addition of substrate and were stopped by adding 150 μl of 20% (wt/vol) trichloroacetic acid. After a further addition of 150 μl of 0.6% bovine serum albumin, samples were centrifuged for 5 min at room temperature at 17,000 × *g*, and Cerenkov radiation in the supernatant and the pellet was measured.

Activation of Calcineurin by Partial Proteolysis. Calcineurin (1 μg) was incubated in the presence or absence of 0.1 μg of trypsin for 30 min at 37°C in 50 mM Tris·HCl, pH 7.4/0.1% (vol/vol) 2-mercaptoethanol. The reaction was stopped by adding 1 mM PMSF, which had no effect on calcineurin activity (data not shown). As a control, calcineurin was also incubated with trypsin which had been inactivated with 1 mM PMSF prior to incubation. Proteolysis of calcineurin was monitored by subjecting the incubated samples to SDS/PAGE and Coomassie staining of the gels. Under these conditions, calcineurin A was proteolyzed into three major bands (53, 46, and 42 kDa), whereas calcineurin B was not detectably proteolyzed.

RESULTS AND DISCUSSION

Stoichiometry of Phosphorylation of DARPP-32 on Thr-34 in Striatonigral Neurons Is Increased by Phosphorylation on Ser-137. DARPP-32 in substantia nigra slices migrates as a doublet in SDS/PAGE (Fig. 1). The lower band of the doublet corresponds specifically to [phospho-Ser-137]DARPP-32 (6). After incubation of slices with 8-bromo-cAMP, the lower band had a stoichiometry of phosphorylation on Thr-34 about 3 times greater than that of the upper band (Fig. 1). This effect could be the consequence of either facilitation of phosphorylation of the lower band by PKA or inhibition of its dephosphorylation by a protein phosphatase. [phospho-Thr-34]DARPP-32 is dephosphorylated *in vitro* by protein phosphatase 2A and calcineurin. The higher stoichiometry of phosphorylation of Thr-34 in the lower band of DARPP-32 in nigral slices was observed in the presence of okadaic acid, a potent inhibitor of protein phosphatase 2A but a weak inhibitor of calcineurin (14), suggesting that if a phosphatase is involved in the differential phosphorylation of the two bands, it is probably calcineurin.

Phosphorylation of Ser-137 on Purified DARPP-32 Inhibits the Dephosphorylation of Thr-34 by Calcineurin. Phosphorylation of DARPP-32 by casein kinase I did not alter its ability to serve as a substrate for PKA *in vitro* (Fig. 2). Thus, the differential effect of 8-bromo-cAMP observed on the two bands of DARPP-32 in nigral slices is likely to be due to the modification of the activity of a protein phosphatase. Phosphorylation of DARPP-32 by casein kinase I had no inhibitory effect on the ability of protein phosphatase 2A to dephosphorylate Thr-34 (data not shown). In contrast, calcineurin dephosphorylated phospho-Thr-34 in the lower band (phosphorylated on Ser-137) less efficiently than in the upper band of the doublet *in vitro* (Fig. 2). Furthermore, phosphorylation of DARPP-32 by casein kinase I greatly reduced the activity of calcineurin on phospho-Thr-34 studied under initial rate conditions (Table 1). *In vitro*, casein kinase I phosphorylates other residues in DARPP-32 in addition to Ser-137 (6). However, the effect of casein kinase I phosphorylation on dephosphorylation of DARPP-32 by calcineurin was not observed with a Ser-137 → Ala mutant of DARPP-32, indicating that the inhibitory effect was due to phosphorylation of Ser-137 (Table 1).

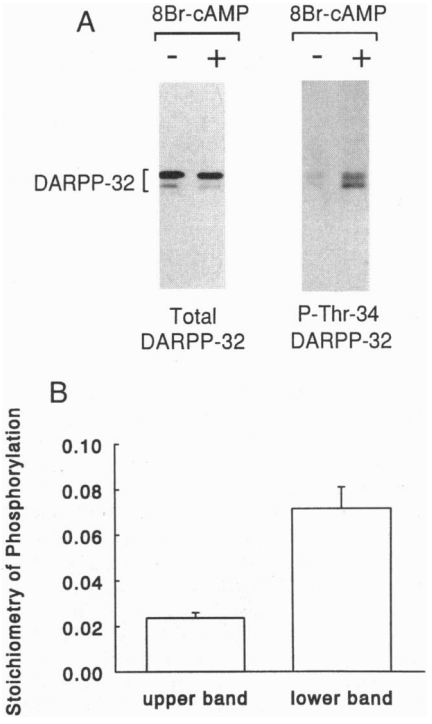

FIG. 1. Phosphorylation of upper and lower bands of DARPP-32 doublet in substantia nigra slices in response to 8-bromo-cAMP. Rat nigral slices were incubated for 10 min in the absence (−) or presence (+) of 1 mM 8-bromo-cAMP (8Br-cAMP). Incubation medium contained 1 μM okadaic acid. (*A*) DARPP-32 was analyzed by immunoblotting with monoclonal antibodies reacting with DARPP-32 independent of its state of phosphorylation (total DARPP-32). The same membranes were incubated with monoclonal antibody which reacts only with DARPP-32 phosphorylated on Thr-34 (P-Thr-34 DARPP-32). (*B*) Stoichiometry of phosphorylation of the two bands in nigral slices treated with 8-bromo-cAMP, measured by densitometric comparison with purified DARPP-32 phosphorylated *in vitro* and loaded on the same gels. Data are means and SEM of four independent experiments; $P < 0.005$ by paired Student's *t* test.

Phosphorylation of Ser-137 Inhibits the Calcineurin-Catalyzed Dephosphorylation of Phospho-Thr-34 by a Substrate-Directed Mechanism. DARPP-32 phosphorylated by casein kinase I is not a good substrate for calcineurin (<10% of the rate of dephosphorylation of Thr-34), whereas it is dephosphorylated efficiently by protein phosphatases 2A and 2C (F.D. and J.-A.G., unpublished work). Thus, it is unlikely that phospho-Ser-137 inhibits calcineurin action on phospho-Thr-34 by substrate competition. This was confirmed by mixing DARPP-32 phosphorylated by PKA with DARPP-32 phosphorylated by casein kinase I: the addition of an equimolar amount of casein kinase I-phosphorylated DARPP-32 to PKA-phosphorylated DARPP-32 did not affect the dephosphorylation of Thr-34 by calcineurin (Table 1). To further establish that the various phospho forms of DARPP-32 were not inhibitors of calcineurin activity, we studied their effects on the dephosphorylation of another substrate, casein phosphorylated by PKA. Nonphosphorylated DARPP-32 and DARPP-32 phosphorylated by casein kinase I, PKA, or both kinases, at a final concentration of 10 μM, did not inhibit the dephosphorylation of phosphocasein (200 μM), beyond a slight effect of phospho-Thr-34 attributable to substrate competition (data not shown).

Inhibition by phosphorylation of Ser-137 of the dephosphorylation of Thr-34 was observed only when the two residues were phosphorylated on the same molecule (Table 1). In support of the biological importance of Ser-137 in regulating the ability of DARPP-32 to serve as a substrate for calcineurin, the mutation

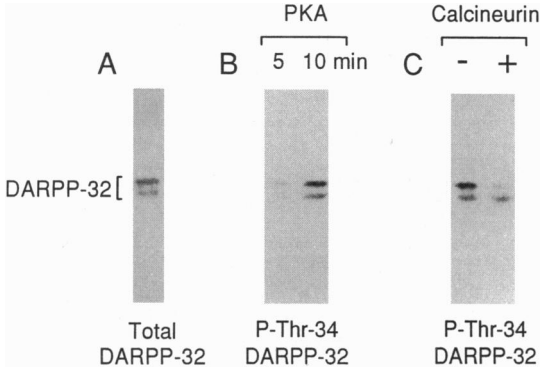

FIG. 2. Phosphorylation of Ser-137 does not alter phosphorylation of Thr-34 by PKA but decreases dephosphorylation of phospho-Thr-34 by calcineurin. (*A*) DARPP-32 was partially phosphorylated by casein kinase I (stoichiometry of phosphorylation of Ser-137 ≈ 0.4). This resulted in the migration of DARPP-32 as a doublet, revealed by immunoblotting with antibodies reacting with DARPP-32 independent of its state of phosphorylation (total DARPP-32). The partially phosphorylated DARPP-32 was repurified and used for the experiments shown in *B* and *C*. (*B*) DARPP-32 was incubated with PKA and ATP for 5 or 10 min, and phosphorylation of Thr-34 was assessed by immunoblotting with a [phospho-Thr-34]DARPP-32-specific antibody (P-Thr-34 DARPP-32). Phosphorylation of the two bands of the DARPP-32 doublet by PKA was similar. (*C*) Casein kinase I-phosphorylated DARPP-32 which had been stoichiometrically phosphorylated on Thr-34 by PKA, followed by heat inactivation of the PKA, was incubated in the absence (−) or presence (+) of calcineurin. DARPP-32 phosphorylated on Thr-34 was detected by immunoblotting with [phospho-Thr-34]DARPP-32-specific antibody. The lower band (phosphorylated on Ser-137) was less sensitive to the action of calcineurin than the upper band. Immunoblotting for the total protein showed that the proportion of the lower band (≈40%) was constant under all conditions (data not shown).

of this residue to an alanine reduced the rate of dephosphorylation by 25–30% (Table 1). These results indicate that phosphorylation of Ser-137 inhibits calcineurin action on phospho-Thr-34 via an intramolecular action within the DARPP-32 molecule. Kinetic studies revealed that the V_{max}, but not the apparent K_m, of the dephosphorylation of Thr-34 by calcineurin was affected by phosphorylation of Ser-137 (Table 2).

Table 1. Dephosphorylation of phospho-Thr-34 by calcineurin: Effect of Ser-137 phosphorylation

Substrate	Relative calcineurin activity
1. DARPP-32 (PKA)	100 ± 4 (n = 8)
2. DARPP-32 (PKA, casein kinase I)	32 ± 4** (n = 8)
3. S137A-DARPP-32 (PKA)	73 ± 4* (n = 5)
4. S137A-DARPP-32 (PKA, casein kinase I)	70 ± 9* (n = 5)
5. DARPP-32 (PKA) + DARPP-32 (casein kinase I)	93 ± 4 (n = 3)

Substrates 1–4: DARPP-32 or S137A-DARPP-32 (in which Ser-137 is changed to alanine) was incubated with nonradioactive ATP in the presence or absence of casein kinase I and then phosphorylated on Thr-34 in the presence of PKA plus [γ-^{32}P]ATP. Substrate 5: DARPP-32 phosphorylated by casein kinase I alone and nonradioactive ATP was mixed with an equimolar amount of DARPP-32 phosphorylated by PKA alone plus [γ-^{32}P]ATP. The dephosphorylation of phospho-DARPP-32 (1 μM) by bovine brain calcineurin (1 ng/μl) was measured under initial rate conditions, in the presence of 100 μM Ca^{2+} and 1 μM calmodulin. In each experiment, assays were carried out in triplicate. Calcineurin activity is expressed relative to that observed for DARPP-32 phosphorylated by PKA alone, which had a mean value of 4.1 nmol·min^{-1}·mg^{-1} under these conditions. Results are means ± SEM for n experiments. Statistical analysis was done by ANOVA ($F3,22$ = 25.2, P = 0.00002) followed by a Scheffé test (*, $P < 0.02$, **, $P < 0.001$). Similar results were obtained with rabbit skeletal muscle calcineurin (data not shown).

Table 2. Kinetic analysis of phospho-Thr-34 dephosphorylation by calcineurin

Substrate	K_m, μM	V_{max}, nmol·min^{-1}·mg^{-1}
DARPP-32 (PKA)	3.8 ± 0.5	30.6 ± 3.3
DARPP-32 (PKA, casein kinase I)	4.0 ± 0.4	12.0 ± 1.3

Substrates were as described in the legend to Table 1. Assays were carried out in the presence of 100 μM Ca^{2+} and 1 μM calmodulin. DARPP-32 concentration was varied from 0.5 to 5 μM. Data were calculated from linear regression of Lineweaver–Burk plots of the release of phosphate and are means ± SEM of three experiments. A paired Student's t test of the V_{max} data was performed: $P < 0.02$.

Effect of Phospho-Ser-137 on the Dephosphorylation of Thr-34 Is Independent of the Mode of Activation of Calcineurin. Calcineurin is a heterodimer composed of a catalytic subunit, calcineurin A, and a Ca^{2+}-binding regulatory subunit, calcineurin B. Calcineurin A can also bind calmodulin, which activates the enzyme by increasing the V_{max} of dephosphorylation (reviewed in ref. 15). We therefore investigated whether phosphorylation of Ser-137 on DARPP-32 inhibited calcineurin activity by modification of the interaction of the enzyme with calmodulin. Inhibition of dephosphorylation of Thr-34 by phosphorylation of Ser-137 was identical in the absence or presence of 1 μM calmodulin (Table 3). The inhibition was also observed when calcineurin was activated by Mn^{2+} (Table 3), which is believed to interact directly with calcineurin A and not with calmodulin or calcineurin B (15).

The activation of calcineurin by calmodulin is thought to be the consequence of a change in the structure of the catalytic subunit, resulting in the neutralization of an autoinhibitory domain located in the carboxyl terminus of calcineurin A (15). Limited trypsin digestion of calcineurin in the absence of calmodulin leads to a truncated calcineurin A which still binds calcineurin B but not calmodulin and which is activated by Ca^{2+} alone (16). Trypsin-activated calcineurin was inhibited to the same extent as intact phosphatase by casein kinase I-phosphorylated DARPP-32 (Table 4).

Our results indicate that the inhibition by phosphorylation of Ser-137 of the dephosphorylation of phospho-Thr-34 on DARPP-32 is due to a modification of the substrate and not to a regulatory effect on calcineurin. This is of interest since the analysis of substrate determinants of calcineurin, using synthetic peptides, have suggested that its substrate specificity may depend on structural features in addition to the amino acid sequence immediately surrounding the phosphorylated

Table 3. Dephosphorylation of phospho-Thr-34 by calcineurin activated by Ca^{2+}, calmodulin, and Mn^{2+}: Effect of Ser-137 phosphorylation

Calcineurin activator(s)	Calcineurin activity, nmol·min^{-1}·mg^{-1}: DARPP-32 (PKA)	DARPP-32 (PKA, casein kinase I)	% of control
Ca^{2+}	1.16 ± 0.18	0.24 ± 0.02	21
Ca^{2+} + calmodulin	18.9 ± 3.9	5.0 ± 0.1	26
Ca^{2+} + Mn^{2+}	24.7 ± 2.6	5.2 ± 0.3	21
Ca^{2+} + Mn^{2+} + calmodulin	235 ± 44	51 ± 6	22

Calcineurin-catalyzed dephosphorylation of [phospho-Thr-34]DARPP-32 (1 μM), prepared as described in the legend to Table 1, was measured under initial rate conditions in the presence of 100 μM Ca^{2+}, 1 μM calmodulin, and 100 μM Mn^{2+}, as indicated. The concentration of calcineurin in the assay was 1.67 ng/μl in the absence and 0.067 ng/μl in the presence of Mn^{2+}, respectively. In each experiment, assays were carried out in triplicate. Results are means ± SD of the rate of dephosphorylation of DARPP-32 for three experiments.

Table 4. Dephosphorylation of phospho-Thr-34 by calcineurin activated by limited proteolysis: Effect of Ser-137 phosphorylation

Calcineurin activator	Calcineurin activity, nmol·min^{-1}·mg^{-1} DARPP-32 (PKA)	DARPP-32 (PKA, casein kinase I)	% of control
None	23.5	8.7	27
Trypsin	56.9	23.0	30
Inactivated trypsin	30.6	11.8	29

Calcineurin-catalyzed dephosphorylation of [phospho-Thr-34]DARPP-32 (1 μM), prepared as described in the legend to Table 1, was measured under initial rate conditions in the presence of 100 μM Ca^{2+}, 100 μM Mn^{2+}, and calcineurin at 0.067 ng/μl. Calcineurin was incubated in the presence or absence of active or inactivated trypsin. In each experiment, assays were carried out in triplicate. Results are the means of two experiments, which differed by <10%.

residue (17, 18). Inhibition of calcineurin activity on Thr-34 by phosphorylation of Ser-137 appears to provide a demonstration of a substrate-directed regulation of a protein phosphatase activity by phosphorylation.

Regulation of PP1c by DARPP-32 in Striatonigral Neurons: Possible Physiological Implications. DARPP-32 is highly enriched in striatonigral neurons expressing high levels of D_1-type dopamine receptors positively coupled to adenylyl cyclase (see ref. 19 for a review of striatonigral connections). Thus, one action of dopamine on these neurons is to raise intracellular concentrations of cAMP and to activate PKA. In striatal slices, the addition of dopamine, D_1-type receptor agonists, or permeant cAMP analogs leads to the phosphorylation of DARPP-32 on Thr-34 (reviewed in ref. 20). The phosphorylation of Thr-34 is modulated *in vitro* by casein kinase II, which phosphorylates Ser-45 and Ser-102 on DARPP-32 and increases the rate of phosphorylation by PKA (5). The possible role of this modulation in intact cells is not known. The other main input of striatonigral neurons is from corticostriatal glutamatergic neurons (19). Activation of *N*-methyl-D-aspartate receptors by glutamate has been shown to induce the dephosphorylation of [phospho-Thr-34]DARPP-32 in striatonigral neurons, presumably by the activation of calcineurin (4). Here, we show that phosphorylation of Ser-137 on DARPP-32 by casein kinase I inhibits its dephosphorylation on Thr-34 by calcineurin *in vitro* and in intact cells. Thus, casein kinase I could play a role in regulating the activity of PP1c, by stabilizing [phospho-Thr-34]DARPP-32 in a way which would reinforce the cAMP signaling pathway (Fig. 3). These results suggest a role for casein kinase I in central nervous system neurons in the modulation of responsiveness to extracellular signals. Thus, it would be of great interest to identify the signaling pathways responsible for regulating the activity of casein kinase I in neurons.

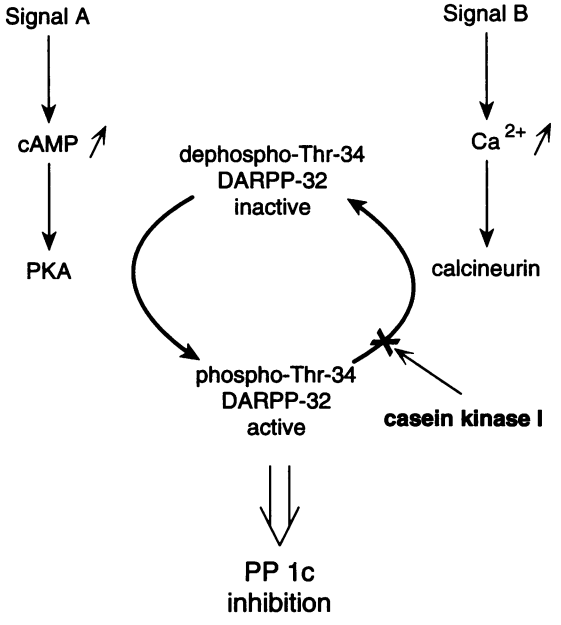

FIG. 3. A model for the physiological role of DARPP-32 phosphorylation by casein kinase I. Stimulation of DARPP-32 phosphorylation on Thr-34 via the cAMP/PKA pathway (signal A) can be triggered by D_1-type dopamine receptors (see ref. 3), vasoactive intestinal peptide receptors, or β-adrenergic receptors (12). Conversely, stimulation of DARPP-32 dephosphorylation on phospho-Thr-34 via the calcium/calcineurin pathway (signal B) can be triggered by *N*-methyl-D-aspartate-type glutamate receptors (4) or voltage-sensitive Ca^{2+} channels (unpublished results). The phosphorylation state of Thr-34 of DARPP-32 depends on the balance between the two opposing signaling pathways. Phosphorylation of Thr-34 converts DARPP-32 from an inactive molecule to a potent inhibitor of PP1c. Casein kinase I, by phosphorylating Ser-137 on DARPP-32, makes Thr-34 less sensitive to dephosphorylation by calcineurin. Through this mechanism, casein kinase I tilts the balance between dephospho- and phospho-Thr-34 in DARPP-32 in favor of the phosphorylated form. This facilitates the effectiveness of the cAMP pathway, and diminishes the effectiveness of the Ca^{2+} pathway, in regulating the inhibition of PP1c.

We thank Professor Jacques Glowinski for his constant support and encouragement. This work was supported, in part, by grants from the National Alliance for the Mentally Ill (Stanley Award) and from the National Parkinson Foundation (to J.-A.G.), the Fondation pour la Recherche Médicale (to J.C.S.), and from the National Institute of Mental Health (Grant MH40899 to P.G.). F.D. was a recipient of doctoral fellowships from the Direction des Recherches Etudes et Techniques du Ministère de la Défense and Rhône-Poulenc Rorer.

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