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The α and γ1 isoforms of protein phosphatase 1 are highly and specifically concentrated in dendritic spines

(basal ganglia/DARPP-32/dopamine/protein kinase)

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ABSTRACT **Protein phosphatase 1 (PP1) is a highly conserved enzyme that has been implicated in diverse biological processes in the brain as well as in nonneuronal tissues. The present study used light and electron microscopic immunocytochemistry to characterize the distribution of two PP1 isoforms, PP1α and PP1γ$_1$, in the rat neostriatum. Both isoforms are heterogeneously distributed in brain with the highest immunoreactivity being found in the neostriatum and hippocampal formation. Further, both isoforms are highly and specifically concentrated in dendritic spines. Weak immunoreactivity is present in dendrites, axons, and some axon terminals. Immunoreactivity for PP1α is also present in the perikaryal cytoplasm and nuclei of most medium- and large-sized neostriatal neurons. The specific localization of PP1 in dendritic spines is consistent with a central role for this enzyme in signal transduction. The data support the concept that, in the course of evolution, spines developed as specialized signal transduction organelles enabling neurons to integrate diverse inputs from multiple afferent nerve terminals.**

Increasing evidence shows that signal transduction processes involve the activation/inactivation of effector proteins by phosphorylation and dephosphorylation (1). Protein phosphatase 1 (PP1) is one of the most highly conserved proteins in eukaryotes; the yeast enzyme shows 80–90% identity with its mammalian counterparts (2). It is likely that this high degree of sequence conservation is due at least in part to its fundamental role in a variety of cellular processes. Thus, PP1 has been implicated in the control of such diverse processes as glycogen metabolism, protein synthesis, and neuronal metabolism (2). In neurons, PP1 has also been implicated in long-term depression and synaptic plasticity (3–5).

Biochemical studies have indicated that the catalytic subunit of PP1 is normally found in association with "regulatory" subunits that in turn modulate PP1 activity directly and/or by targeting PP1 to specific subcellular locations (6). Two well-characterized examples of such regulatory subunits are inhibitor 2 (7) and the G subunit (8).

Most studies of PP1 have concentrated on the skeletal muscle protein and remarkably little is known about PP1 in brain. Recently we have identified neuronal isoforms of PP1 and described the distribution of their mRNA by "*in situ*" hybridization (9). PP1α and PP1γ$_1$ were found to be more highly expressed in brain than in other tissues by immunoblotting and to be specifically enriched in the medium-sized spiny neurons of the striatum (9). These neurons are also highly enriched for DARPP-32 (dopamine and cAMP-regulated phosphoprotein, M_r 32 kDa) (10, 11), which, in its phosphorylated but not dephosphorylated form, is a potent inhibitor of PP1 (12).

Several first messenger pathways [dopaminergic, glutamatergic, and GABAergic (GABA = γ-aminobutyric acid)] achieve part of their effects in striatal medium-sized spiny neurons through regulation of the state of phosphorylation of DARPP-32 (13–15). The convergence of major neurotransmitter pathways on DARPP-32, and the subsequent regulation of PP1 activity by DARPP-32, suggests a critical role for PP1 in mediating the actions of these neurotransmitters. Implicit in this model is the idea that PP1 must be located in the vicinity of the receptors for these neurotransmitters. In the case of the medium-sized spiny neurons of the neostriatum, these receptors are in the spines (16–18). We report here that two isoforms of PP1, namely PP1α and PP1γ$_1$, are not only present in spines but are also specifically concentrated in these structures. Moreover, immunoreactivity for these isoforms of PP1 is heterogeneously distributed in brain, with the greatest intensity of immunostaining in the caudatoputamen and hippocampal formation.

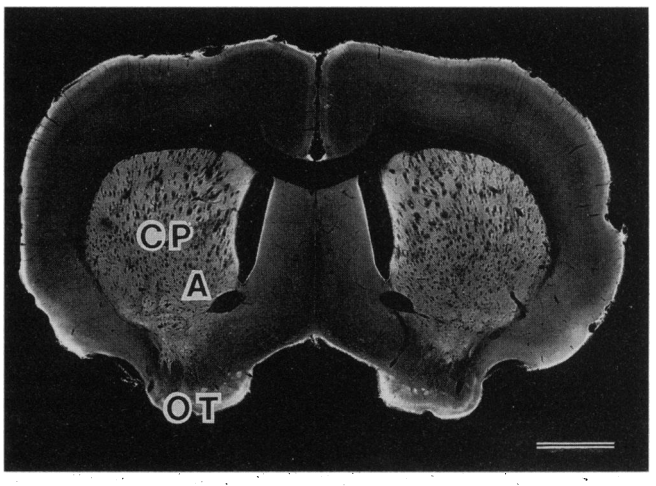

FIG. 1. Low-power photograph of immunoreactivity for PP1γ$_1$ in a coronal section of the forebrain. This photograph was produced by placing a microscope slide in an enlarger and exposing it onto photographic paper. Hence, immunoreaction product appears white. Immunoreactivity is strongest in the caudatoputamen (CP), nucleus accumbens (A), and olfactory tubercle (OT). The white edge around the section is artifact. (Bar = 100 μm.)

METHODS

Six male Sprague–Dawley rats were transcardially perfused with 100 ml of phosphate buffer (0.1 M, pH 7.4) followed by 4% formaldehyde (freshly depolymerized from paraformaldehyde in the same buffer) under deep sodium pentothal anesthesia. After 1 hr postfixation *in situ*, brains were removed and sectioned at 50–100 μm on a vibratome. The sections were then processed through the following series of incubations: primary antibody (1:5000, overnight); biotinylated secondary

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Abbreviations: PP1, protein phosphatase 1; PSD, postsynaptic density.

antibody (Vector Laboratories, according to their instructions, 1 hr); and Vector avidin-biotin complex (according to their instructions, 1 hr). All incubations were preceded by 3 × 15 min washes in phosphate-buffered saline (PBS; 0.01 M sodium phosphate/0.15 M sodium chloride, pH 7.5) and all antibodies were diluted in PBS. The sections were incubated with 3,3′-diaminobenzidine (15 mg/50 ml of buffer) and H_2O_2 (15 μl of a 30% solution) for 2 min, washed, mounted on substrated microscope slides (coated with gelatin/chrome alum), dehydrated, and coverslipped.

For electron microscopic immunocytochemistry, tissue was processed as described above except for the following changes. No buffer prerinse was used for perfusion, and 0.1% glutaraldehyde was added to the fixative. Prior to incubation in primary antibody, sections were incubated with 1% sodium borohydride for 30 min and subsequently rinsed for 1 hr in phosphate buffer. Following chromogen development in 3,3′-diaminobenzidine, sections were osmicated (2% osmium tetroxide in phosphate buffer) for 30 min, dehydrated, embedded in Epon, and resectioned on an ultratome at a thickness of 80 nm. Ultrathin sections were photographed at an electron microscope without additional contrast with uranyl acetate or lead citrate.

The specificity of the antibodies for PP1 has been demonstrated by immunoblotting, as reported earlier (9). Controls used in the present study included the substitution of primary antibody preabsorbed batchwise with purified peptide antigen for primary antibody. This substitution, as well as omission of first antibody, second antibody, and/or avidin-biotin complex, produced no immunostaining.

RESULTS AND DISCUSSION

Immunoreactivity for PP1α and PP1γ_1 was present in all brain regions, but the highest level of immunostaining was present in the neostriatum (Figs. 1 and 2*A*) and hippocampal formation. These data are consistent with biochemical studies showing similar regional differences in the concentration of PP1 and of its mRNA (9).

Immunoreaction product for both isoforms was contained in numerous small (1 μm or less in diameter) puncta in the neuropil (Fig. 2 *B* and *D*). The regional differences in staining intensity noted above reflected the strength of immunoreactivity within individual puncta; those in the caudatoputamen were much more intensely immunoreactive than puncta, for

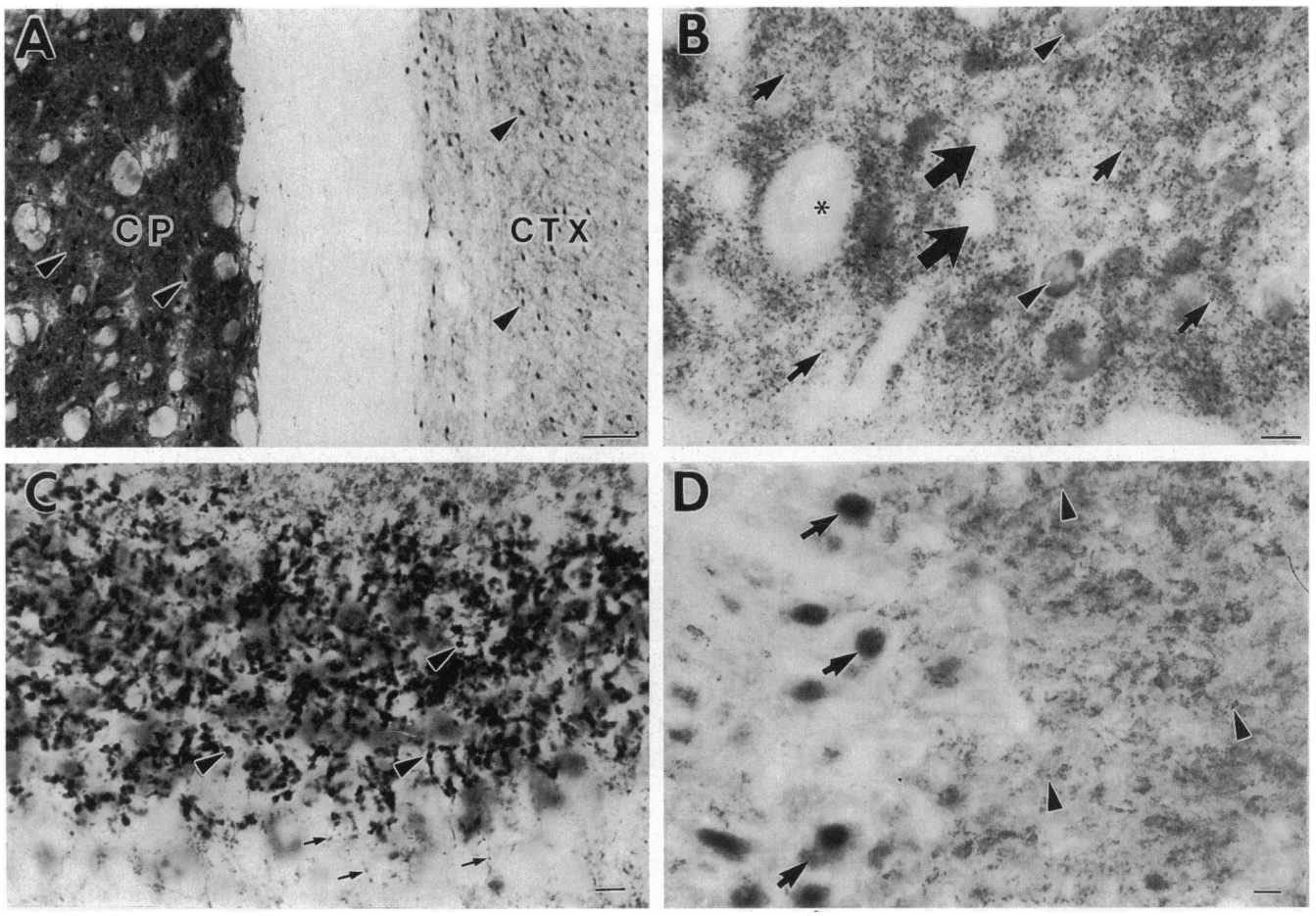

FIG. 2. Light microscopic photomicrographs illustrating the immunostaining patterns of PP1. (*A*) Immunoreactivity for PP1α is much stronger in the caudatoputamen (CP) than in the cerebral cortex (CTX) or subcortical white matter (the white strip between the caudatoputamen and the cortex). The arrowheads point to examples of immunoreactive neurons. The pattern of immunoreactivity for PP1γ_1 at this level of magnification is identical except that neither cell bodies nor neuronal nuclei are immunoreactive (not shown). (Bar = 100 μm.) (*B*) Immunoreactivity for PP1γ_1 in the caudatoputamen. Immunoreaction product is contained primarily in small (about 1 μm or less in diameter) puncta throughout the neuropil (small arrows). Very weak staining can be seen in some somata (arrowheads) but not in others (large arrows). The asterisk marks a fascicle of the internal capsule. (Bar = 10 μm.) (*C*) Immunoreactivity for synapsin I in the CA3 region of the hippocampus. The large puncta (arrowheads) represent the unusually large axon terminals (about 4 μm in diameter) of the mossy fibers, and the small puncta represent the more common small axon terminals (about 1 μm in diameter) found in most brain regions. This staining pattern is unlike that seen in *D*. (Bar = 10 μm.) (*D*) Immunoreactivity for PP1α in the CA3 region of the hippocampus. The puncta are very small, 1 μm or less in diameter (arrowheads), and form small clusters. These puncta clearly differ in size from the large puncta seen in *C*. The arrows point to examples of neurons in which most of the immunoreactivity is contained within nuclei. (Bar = 10 μm.)

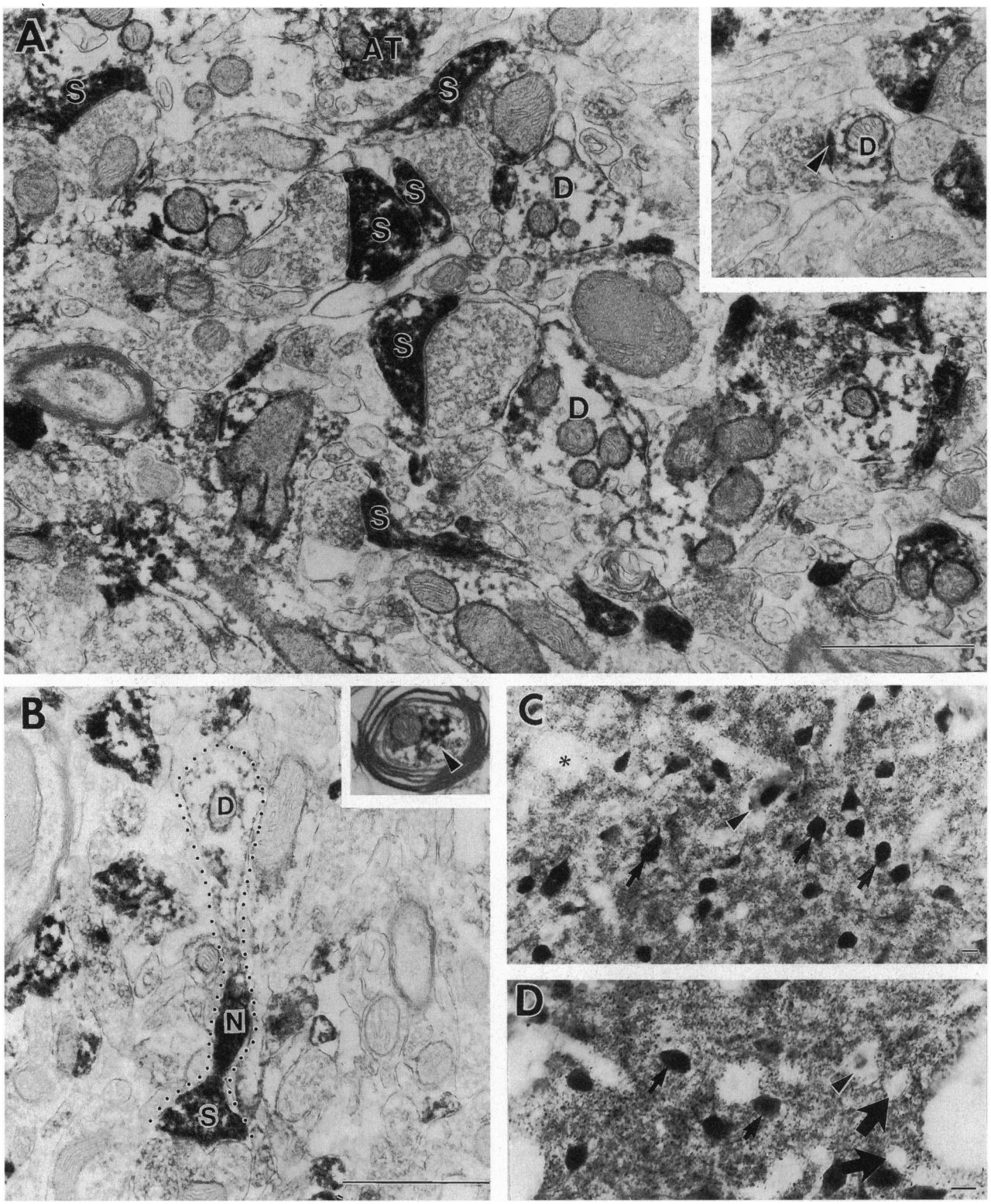

FIG. 3. Electron and light photomicrographs of PP1 immunoreactivity in the caudatoputamen. (*A* and *B*) Electron micrographs of immunoreactivity for PP1α in the caudatoputamen. (*A*) Robust immunoreactivity for PP1α is present in dendritic spine heads (S). Weak immunoreactivity is present in dendritic shafts (D). Some axon terminals are also immunoreactive (AT). (*Inset*) The postsynaptic density (PSD; arrowhead) in the dendrite (D) is labeled with immunoreaction product. Similar images are observed for PP1γ_1 (not shown). (Bar = 1 μm.) (*B*) The dots outline a spine head (S), its neck (N), and its parent dendritic shaft (D). Immunoreactivity is sharply reduced in the dendritic shaft. (*Inset*) Immunoreactivity within axons is associated with microtubules (arrowhead) but not with the axolemma. (Bar = 1 μm.) (*C* and *D*) Light photomicrographs of immunoreactivity for PP1α in the caudatoputamen. (*C*) Medium-sized (10–15 μm) neurons (arrows) including their nuclei show strong immunoreactivity. A large neuron (arrowhead) shows immunoreactivity mainly in its nucleus. Other large neurons had moderate immunoreactivity in the cytoplasm. The asterisk marks a fascicle of the internal capsule. (Bar = 10 μm.) (*D*) Some neurons are not immunoreactive for PP1α (large arrows). Immunoreactive neurons are labeled with small arrows. The arrowhead points to a neuron with an immunoreactive nucleus and unlabeled perikaryal cytoplasm. (Bar = 10 μm.)

example, in the cerebral cortex. The sizes of the puncta, however, showed little regional variation. This was notably true in the CA3 subregion of the hippocampus, where puncta immunostained for PP1 were much smaller than those immunostained for synapsin I (Fig. 2 *C* and *D*). The latter represent large (4 μm in diameter) mossy fiber terminals. The difference in immunostaining pattern between PP1 and synapsin I suggests that the small immunoreactive puncta seen at the light microscopic level represent PP1 contained in processes other than the axon terminals. Furthermore, whereas synapsin I immunolabels puncta that decorate somata and dendrites (19), this pattern of immunostaining is not seen for PP1.

At the electron microscopic level, dendritic spine heads and spine necks were strongly immunoreactive (Fig. 3 *A* and *B*). Immunoreactivity was also observed in PSDs, but this immunoreactivity must be interpreted with caution, since immunoreaction product generated elsewhere in the spine could diffuse to the PSD and become fixed to it during osmication. This potential artifact is especially relevant in densely labeled spines and is manifest in this material by the partial coatings around mitochondria (Fig. 3*A*). In more lightly labeled dendritic shafts, however, PSDs were often more heavily immunolabeled than other elements in the dendrite (Fig. 3*A Inset*).

The presence of PP1 in PSDs, as visualized by immunocytochemistry, is in agreement with biochemical data indicating that PP1 is the major protein phosphatase present in PSDs. Shields *et al.* (20) identified PP1 as the major phosphatase present in synaptic junctions and showed that it dephosphorylated seven major endogenous substrate phosphoproteins for Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II). Further, inhibition of endogenous PP1 in an isolated PSD preparation allowed extensive phosphorylation of CaM kinase II, the major PSD protein (21).

The robust PP1 immunoreactivity in spine heads and spine necks accounts for the puncta seen at the light microscopic level. This immunoreactivity drops off dramatically at the junction between the spine neck and the parent dendritic shaft (Fig. 3*B*). Processes other than spines that could account for punctate immunostaining at the light microscopic level are dendritic shafts, axons, and axon terminals. These were usually weakly immunoreactive and many axon terminals were not immunolabeled. Thus, the α and γ_1 isoforms of PP1 not only are present in dendritic spines but are also highly and specifically concentrated in these structures.

Several neurotransmitters affect the physiological properties of striatal medium spiny neurons by regulating the phosphorylation state of DARPP-32 and thereby PP1 inhibition. Dopamine, by increasing intracellular cAMP levels and activating protein kinase A, causes the phosphorylation and activation of DARPP-32 (12, 13). Conversely, glutamate, by increasing intracellular Ca^{2+} levels and activating protein phosphatase 2B, causes the dephosphorylation and inactivation of DARPP-32 (14). Moreover, it has recently been found that GABA inhibits the dephosphorylation of DARPP-32 by protein phosphatase 2B (15). The convergence of these and several other neurotransmitter pathways on the DARPP-32/PP1 cascade indicates a critical role for PP1 in mediating the actions of these neurotransmitters. The localization of PP1 to dendritic spines is consistent with this concept. In the case of the DARPP-32-containing medium-sized spiny neurons of the neostriatum, the dopamine D_1 and the gluR1 glutamate receptors are, like PP1, specifically enriched in spines (16–18).

The striatal enrichment of PP1 is probably related to its role in dopaminergic signal transduction; dopamine, the dopamine D_1 receptor, and PP1 are all highly enriched in the neostriatum. PP1-immunoreactive spines are present, however, throughout the brain, consistent with the idea that PP1 is regulated by other neurotransmitter pathways as well. PP1 inhibitors other than DARPP-32 include inhibitor 1 (22), which is more broadly distributed than DARPP-32 and is enriched in the hippocampus (23), and inhibitor 2 (7). G substrate, another phosphatase inhibitor, is expressed specifically in the Purkinje cells of the cerebellum and seems to be related to inhibitor 1/DARPP-32 (24, 25).

Moderate immunoreactivity for PP1α was present in the neuronal cytoplasm of medium-sized (10–15 μm in diameter) and large (20–30 μm in diameter) neurons in the caudatoputamen (Figs. 2*A* and 3 *C* and *D*). Immunoreactivity for PP1 in neuronal cytoplasm has also been reported in human hippocampal neurons (26). Cell body immunoreactivity for the γ_1 isoform (Fig. 2*B*) was considerably weaker than that for the α isoform and was difficult to distinguish from background staining.

A small percentage of neurons was not immunoreactive for PP1α (Fig. 3*D*). The absence of immunoreactivity for PP1α in the somata of a minor subpopulation of medium-sized neostriatal neurons may simply reflect the absence of PP1α from certain somata or it may be attributable to the presence of PP1α at levels that are below the limits of detection with the current technique. It should be noted that neurons lacking detectable PP1α immunoreactivity in the somata may have detectable PP1α or PP1γ_1 in their spines. Future studies should determine whether the somata that do not immunostain for PP1α lack DARPP-32.

Nuclei were not immunoreactive for PP1γ_1 (Fig. 2*B*) but were strongly immunoreactive for PP1α in most neurons (Fig. 3 *C* and *D*). The presence of PP1α immunoreactivity in nuclei is consistent with biochemical findings in nonneuronal tissues. For example, the *bimG* gene of *Aspergillus* is required for completion of anaphase and encodes a homologue of mammalian PP1 (27). PP1 has been shown to be essential for the completion of chromosome disjoining in fission yeast (28). More recently, PP1 has been shown to act in opposition to yeast Ipl1 protein kinase in regulating chromosome segregation (29), and PP1 is also required for the completion of mitosis in *Drosophila* (30). Interestingly, the PP1 isoform involved in *Drosophila* is most closely related to mammalian PP1α (31).

PP1 specifically dephosphorylates the transcription factor CREB at Ser-133 and thus inhibits cAMP-dependent transcription, with PP1 being the major regulator of CREB activity in cAMP-responsive cells (32, 33). Further, PP1 involvement in the progression through the cell cycle and in retinoblastoma dephosphorylation has also been documented (34–37). Finally, PP1 activity was found to be highly enriched in the nucleus of rat liver cells (38), and nuclear-specific inhibitors of PP1 have recently been identified (39).

CONCLUDING REMARKS

There is evidence suggesting that dendritic spines are discrete biochemical compartments for Ca^{2+}-activated processes involved in synaptic plasticity (40, 41). An analysis of the spatiotemporal dynamics of stimulus-induced Ca^{2+} changes in spine heads, spine necks, and dendritic shafts led Koch and Zador (42) to make the interesting speculation that dendritic spines might provide biochemical compartmentalization for a number of other second messengers. Our results support and extend this concept.

Both the α and γ_1 isoforms of PP1 not only are present in dendritic spines but also are highly and specifically concentrated in these structures. The specific localization of PP1 in dendritic spines is consistent with the demonstrated role of the enzyme in signal transduction and suggests that spines evolved as specialized organelles to facilitate interactions among various signal transduction pathways—i.e., to integrate the wide variety of information being presented to the neuron by multiple afferent nerve terminals. By confining second messengers, protein kinases, protein phosphatases, and their substrates in a small subcompartment, the dendritic spine would contain in close proximity the numerous elements needed to

react to a variety of first messengers with appropriate integrative physiological responses.

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