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Negative regulation of cyclin-dependent kinase 5 targets by protein kinase C

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Abstract

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed protein serine/threonine kinase essential for brain development and implicated in synaptic plasticity, dopaminergic neurotransmission, drug addiction, and neurodegenerative disorders. Relatively little is known about the molecular mechanisms that regulate the activity of Cdk5 *in vivo*. In order to determine whether protein kinase C (PKC) regulates Cdk5 activity in the central nervous system, the phosphorylation levels of two Cdk5 substrates were evaluated under conditions of altered PKC activity *in vivo*. Treatment of acute striatal slices with a PKC-activating phorbol ester caused a time- and dose-dependent decrease in the levels of phospho-Ser6 inhibitor-1, phospho-Ser67 inhibitor-1, and phospho-Thr75 dopamine- and cAMP-regulated phosphoprotein, M_r 32,000 (DARPP-32). This effect was reversed by the PKC inhibitor, Ro-32-0432. Moreover, phospho-Ser6 inhibitor-1, phospho-Ser67 inhibitor-1, and phospho-Thr75 DARPP-32 levels were elevated in brain tissue from mice lacking the gene for PKC- α . PKC did not phosphorylate Cdk5 or its cofactor, p25, *in vitro*. Striatal levels of the Cdk5 cofactor, p35, did not change in response to phorbol ester treatment. Furthermore, Cdk5 immunoprecipitated from striatal slices treated with phorbol ester had unaltered activity toward a control substrate *in vitro*. These results suggest that PKC exerts its effects on the phosphorylation state of Cdk5 substrates through an indirect mechanism that may involve the regulatory binding partners of Cdk5 other than its neuronal cofactors.

Keywords

Cdk5; PKC; inhibitor-1; DARPP-32; phorbol ester

1. Introduction

A unique member of the cyclin-dependent kinase family with no apparent role in cell cycle progression, cyclin-dependent kinase 5 (Cdk5) is a proline-directed protein serine/threonine kinase that is highly expressed in post-mitotic, terminally differentiated neurons (Dhavan and Tsai, 2001). To become active, Cdk5 requires association with one of its membrane-bound

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neuronal activators: p35 (Tsai et al., 1994) or its homolog, p39 (Tang et al., 1995). Mice lacking Cdk5 (Gilmore et al., 1998; Ohshima et al., 1996) or p35 and p39 (Ko et al., 2001) display abnormal corticogenesis and perinatal lethality, underscoring the critical role of Cdk5 activity in the developing brain. While p35-deficient mice are viable, with less severe signs of disrupted cortical development (Tsai et al., 1994), and mice lacking p39 appear normal (Ko et al., 2001), the genetic deletion of both cofactors mimics loss of Cdk5, suggesting that p35 and p39 are the only relevant cofactors of Cdk5 in the central nervous system (Ko et al., 2001). The Ca^{2+} -dependent protease, calpain, cleaves the first ~100 N-terminal residues of p35 or p39, resulting in the soluble proteolytic fragment, p25 or p29, respectively (Kusakawa et al., 2000; Patzke and Tsai, 2002). Cdk5 bound to this cleaved cofactor shows prolonged activation, mislocalization, and altered substrate specificity—mechanisms believed to be responsible for aberrant Cdk5-dependent protein phosphorylation in a number of neurodegenerative disorders (Hashiguchi et al., 2002; Patrick et al., 1999).

Cdk5 levels remain high throughout adulthood in rodents (Connor et al., 1998). Ischemia (Green et al., 1997; Hayashi et al., 1999), excitotoxicity (Henchcliffe and Burke, 1997), neurotoxicity (Neystat et al., 2001), and chronic cocaine exposure (Bibb et al., 1999) have been reported to elevate these levels. While earlier studies focused on the role of Cdk5 in brain development, more recent work has implicated Cdk5 in synaptic transmission in mature neurons. Presynaptically, Cdk5 has been suggested to regulate neurotransmitter release and synaptic vesicle endocytosis through the phosphorylation of synapsin I (Matsubara et al., 1996), the α -subunit of the P/Q-type voltage-dependent Ca^{2+} channel (Tomizawa et al., 2002), amphiphysin I (Floyd et al., 2001), dynamin I (Tan et al., 2003; Tomizawa et al., 2003), and synaptojanin I (Lee et al., 2004). In the postsynaptic compartment, Cdk5 modulates synaptic plasticity (Hawasli et al., 2007) and phosphorylates the NR2A subunit of the N-methyl-D-aspartate-type glutamate receptor (Li et al., 2001; Wang et al., 2003), the postsynaptic density protein, PSD-95 (Morabito et al., 2004), and the protein phosphatase-1 inhibitors, inhibitor-1 (Bibb et al., 2001) and its striatal homolog, the dopamine- and cAMP-regulated phosphoprotein, M_r 32,000 (DARPP-32) (Bibb et al., 1999).

In spite of a growing list of physiological substrates, surprisingly little is known about the regulation of Cdk5 activity in neurons, other than the role of cofactor metabolism (Bibb et al., 2001). When phosphorylated by Cdk5, p35 is rapidly turned over by the proteasome (Bibb et al., 2001; Patrick et al., 1998), a process promoted by physiological levels of glutamatergic stimulation (Wei et al., 2005). Unphosphorylated, p35 is susceptible to calpain-dependent conversion to its more stable and soluble form, p25 (Bibb et al., 2001; Kerokoski et al., 2002). Cleavage of p35 by calpain is stimulated by high intracellular Ca^{2+} levels (Kusakawa et al., 2000), as may occur in acute neuronal injury (Snyder et al., 1992) and glutamate excitotoxicity (Kerokoski et al., 2004; Lee et al., 2000). The following studies investigate a possible role for protein kinase C (PKC) in the regulation of Cdk5 substrates and Cdk5 activity in the brain.

2. Materials and methods

2.1. Chemical, enzymes, and animals

All chemicals were from Sigma, except where indicated. PKC (a mixture of the Ca^{2+} -dependent conventional isoforms, α , β , and γ) was purified from rat brain (Woodgett and Hunter, 1987). Cdk5 and p25-His₆ were co-expressed in insect Sf9 cultures using baculovirus vectors and affinity-purified (Bibb et al., 2001). ATP was from Roche. [γ - ^{32}P] ATP was from PerkinElmer Life Sciences. Phorbol-12,13-dibutyrate (PDBu) and Ro-32-0432 were from Calbiochem. Recombinant inhibitor-1 was generated as previously described (Bibb et al., 2001). Histone H1 was from Upstate. Mice were housed 4-5 per cage in a colony maintained as previously

described (Hawasli et al., 2007). All procedures were approved by the UT Southwestern Institutional Animal Care and Use Committees.

2.2. Preparation and incubation of acute striatal slices

Male C57BL/6 mice (8-10 weeks old) were killed by decapitation. The brains were rapidly removed and placed in cold, oxygenated Krebs buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 1.5 mM MgSO₄, and 10 mM D-glucose, pH 7.4). 350- μ m coronal sections were prepared using a vibrating blade microtome. Striatal slices were dissected from these sections in cold, oxygenated Krebs buffer using a dissecting light microscope. Each slice was transferred to a polypropylene incubation tube containing 2 ml of Krebs buffer and allowed to recover at 30°C under constant oxygenation with 95% O₂/5% CO₂ for 60 min, with a change of buffer following the first 30 min of recovery. Slices were subsequently treated with drugs as specified for each experiment, transferred to microfuge tubes, snap-frozen on dry ice, and stored at -80°C until further analysis. Prior to use, PDBu and Ro-32-0432 were dissolved in dimethyl sulfoxide (DMSO) to generate 1000X stock solutions corresponding to each dose tested in these experiments. Control slices were treated with 0.1% DMSO, the final DMSO concentration achieved in striatal slices treated with PDBu and Ro-32-0432.

2.3. Immunoblot analysis of tissue homogenates

Frozen striatal tissue samples were sonicated in boiling 1% sodium dodecyl sulfate (SDS) containing 50 mM NaF and boiled for an additional 5 min. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard. An equal amount of total protein (80-100 μ g) from each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer to nitrocellulose membranes (0.2 μ m) (Whatman). The membranes were immunoblotted using antibodies for phospho-Ser6 inhibitor-1 (1:750) (Nguyen et al., 2007), phospho-Ser67 inhibitor-1 (1:5000) (Bibb et al., 2001), total inhibitor-1 (1:4000) (Gustafson et al., 1991), phospho-Thr75 DARPP-32 (1:5000) (Bibb et al., 1999), total DARPP-32 (1:8000) (Ouimet et al., 1984), or p35 (1:1000) (Santa Cruz). This was followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:8000) (Chemicon). Antibody binding was detected by autoradiography using the enhanced chemiluminescence immunoblotting detection system (Amersham Biosciences) and quantified by densitometry using NIH Image software. In all experiments, apparent phosphoprotein levels were adjusted to reflect relative levels of total protein loaded per lane. Where specified, these corrected values were further normalized to the means of indicated controls. The pharmacological manipulations used in this study did not alter the total amount of inhibitor-1, DARPP-32, or p35 in striatal slices. In some experiments, phosphoprotein levels were assessed in frozen tissue samples from the brains of *Prkca*^{-/-} mice and wild-type littermates (Braz et al., 2003).

2.4. *In vitro* protein phosphorylation reactions

All protein phosphorylation reactions were performed at 30°C in a final volume of at least 30 μ l containing 10 μ M substrate, 100 μ M ATP, and 0.2 mCi/ml [γ -³²P] ATP, except where indicated. The PKC reaction solution included 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.2, 25 mM β -glycerol phosphate, 1 mM dithiothreitol, 1 mM CaCl₂, 10 mM MgCl₂, 0.1 mg/ml phosphotidylserine, and 0.01 mg/ml diacylglycerol, where the final two components were added to the reaction mixture following sonication on ice for 1 min. Cdk5 reactions were conducted in 30 mM MOPS, pH 7.2, and 5 mM MgCl₂. Time course reactions were performed by removing 10- μ l aliquots from the reaction solution at various time points and adding an equal volume of 5X SDS protein sample buffer to stop the reaction. [³²P]-phosphate incorporation was assessed by SDS-PAGE and PhosphorImager analysis. To

calculate reaction stoichiometries, radiolabeled reaction products and radioactive standards were quantified by densitometry using ImageQuant software (Amersham Biosciences). Standards consisted of spotted aliquots from serial dilutions of each reaction mixture (1:100, 1:500, 1:1000), with the moles of phosphate per unit volume of each dilution defined using the ATP concentration of the original reaction. Division of the signal per mole of substrate by the signal per mole of phosphate yielded the stoichiometry of phosphate incorporation (mol phosphate/mol substrate).

2.5. Immunoprecipitation and assay of Cdk5

For Cdk5 immunoprecipitation, acutely prepared mouse striatal slices incubated in the absence or presence of 5 μ M PDBu for 30 min were lysed with a Dounce homogenizer in 1 ml of lysis buffer containing 150 mM NaCl, 20 mM Trizma-HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid, 0.5% Igepal CA-630, 10 mM NaF, 5 mM sodium orthovanadate, and protease inhibitors. Lysates were centrifuged at $10,000 \times g$ for 10 min at 4°C. Supernatants were pre-cleared by incubation with rabbit IgG conjugated to agarose (Santa Cruz) for 30 min at 4°C. Following centrifugation at $1,500 \times g$ for 5 min at 4°C, pellets were washed three times with 1 ml of lysis buffer and once with kinase assay buffer containing 50 mM HEPES, pH 7.0, 10 mM MgCl₂, and 1 mM dithiothreitol, and saved as controls. Pre-cleared supernatants were incubated with 25 μ l of anti-Cdk5 antibody conjugated to agarose (Santa Cruz) for 60 min at 4°C and centrifuged at $1,500 \times g$ for 4 min at 4°C. Pellets were washed three times with 1 ml of lysis buffer and once with kinase assay buffer. Cdk5 assays were performed in a volume of 30 μ l containing 2 μ g of histone H1, 500 mM ATP, and 0.2 mCi/ml [γ -³²P] ATP. Following incubation at 30°C for 60 min, reactions were stopped by the addition of an equal volume of 5X SDS protein sample buffer. Samples were boiled for 5 min, separated by SDS-PAGE, and analyzed by autoradiography, as described above.

2.6. Statistical analysis

Differences between data groups were evaluated for significance using a Student's *t* test of unpaired data $\pm$ S.E.M.

3. Results

3.1. Effects of PDBu and Ro-32-0432 on the level of phosphorylation of Cdk5 substrates in the striatum

Treatment of striatal slices with the PKC-activating phorbol ester, PDBu, resulted in a time- and dose-dependent decrease in the level of phosphorylation of three Cdk5 targets in this tissue, Ser67 of inhibitor-1, Thr75 of DARPP-32, and Ser6 of inhibitor-1 (Fig. 1A-C). Relative to baseline, PDBu treatment maximally reduced phospho-Ser67 inhibitor-1 levels by $60 \pm 8\%$ and phospho-Thr75 DARPP-32 levels by $84 \pm 5\%$ ($n = 4$).

To evaluate the possibility that PDBu exerts a non-specific, PKC-independent inhibitory effect on Cdk5 activity, *in vitro* phosphorylation Cdk5 reactions were conducted in the presence and absence of PDBu. PDBu had no effect on the ability of Cdk5/p25 to phosphorylate recombinant inhibitor-1 *in vitro* (Fig. 1D), arguing against the direct inhibition of Cdk5 by PDBu.

To determine whether the effect of PDBu on the phosphorylation state of Cdk5 substrates in the striatum is PKC-dependent, levels of phospho-Ser67 inhibitor-1 were assessed in striatal slices treated with PDBu following incubation in the absence or presence of the PKC inhibitor, Ro-32-0432 (Fig. 2A). Pretreatment with Ro-32-0432 reversed the effect of PDBu on phospho-Ser67 inhibitor-1 levels, indicating that PDBu mediates its effect through the activation of PKC. Similar results were obtained with phospho-Thr75 DARPP-32 (data not shown).

3.2. Effect of *Prkca* deletion on the level of phosphorylation of Cdk5 substrates in the brain

The phosphorylation state of these Cdk5 substrates was next assessed in untreated brain tissue from wild-type and *Prkca*^{-/-} mice (Fig. 2B-D). Deletion of the gene encoding PKC- α had no effect on total inhibitor-1 or DARPP-32 levels in the brain (data not shown). However, relative to controls, basal levels of phospho-Ser6 inhibitor-1 were elevated 1.6 ± 0.1 -fold and 6.1 ± 0.2 -fold in *Prkca*^{-/-} cortex and striatum, respectively (Fig. 2B). No phospho-Ser6 inhibitor-1 was detected in cerebellar tissue from *Prkca*^{-/-} and wild-type animals. Relative to controls, basal levels of phospho-Ser67 inhibitor-1 were elevated 2.6 ± 0.3 -fold and 2.6 ± 0.2 -fold in *Prkca*^{-/-} cortex and striatum, respectively (Fig. 2C). On the other hand, the level of phosphorylation of this Cdk5 site was unaltered in cerebellar tissue from *Prkca*^{-/-} animals, suggesting that phospho-Ser67 inhibitor-1 may not be subject to regulation by PKC- α in the cerebellum. Differences between phospho-Ser6 and phospho-Ser67 inhibitor-1 levels also suggest that PKC- α differentially regulates Cdk5 sites in a tissue-specific manner.

As expected, DARPP-32 phosphorylation at Thr75 was detectable in the striatum, but not in the cortex or cerebellum of wild-type and *Prkca*^{-/-} mice (Fig. 2D). Compared to phospho-Ser67 inhibitor-1, phospho-Thr75 DARPP-32 levels in *Prkca*^{-/-} striatum showed a more modest, but significant, increase of 1.7 ± 0.2 -fold relative to wild-type controls.

Taken together, these results indicate that PKC negatively regulates the phosphorylation state of at least three Cdk5 substrates in the striatum, raising the possibility that Cdk5 activity may be under the control of PKC-mediated signaling pathways in this tissue.

3.3. Evaluation of Cdk5 inhibition by PKC as a mechanism for the PKC-dependent down-regulation of Cdk5 substrate phosphorylation in the striatum

It is possible that PKC decreases the phosphorylation level of Cdk5 substrates *in vivo* by inhibiting Cdk5 activity, perhaps through the direct phosphorylation of Cdk5 or one of its cofactors. However, the incubation of Cdk5/p25 with PKC resulted in no detectable change in the phosphorylation of Cdk5 or p25 *in vitro* whereas inhibitor-1 was efficiently phosphorylated by PKC (Fig. 3A).

Cdk5 activity is regulated *in vivo* by the availability of its neuronal cofactors (Bibb et al., 2001). To assess whether PKC activation alters Cdk5 cofactor stability in the striatum, p35 levels were assessed in striatal slices treated with PDBu in time course and dose-response experiments (Fig. 3B). However, PDBu had no apparent effect on the levels of striatal p35. Cdk5 levels were likewise unaffected by this treatment (data not shown).

These findings argue against a PKC-mediated effect on the phosphorylation state or stability of Cdk5 or its cofactors. However, the possibility remains that PKC may alter Cdk5 activity through a less direct mechanism, perhaps by impinging on upstream regulators of the Cdk5 pathway. To evaluate this possibility, Cdk5 immunoprecipitated from striatal slices treated with or without PDBu was assessed for its ability to phosphorylate histone H1 *in vitro* (Fig. 3C). Treatment with PDBu had no apparent effect on Cdk5 activity in this assay.

4. Discussion

The results indicate a role for PKC in the regulation of Cdk5 targets in the brain, raising the possibility that PKC may indirectly control Cdk5 activity. Pharmacological activation of PKC caused a time- and dose-dependent decrease in the level of phosphorylation of three Cdk5 substrates in the striatum, an effect that was reversed by a specific PKC inhibitor. Conversely, genetic deletion of PKC- α activity resulted in elevated basal levels of phosphorylation of the same Cdk5 substrates in this tissue. Thus, PKC negatively regulates the level of phosphorylation of at least three Cdk5 targets in the striatum.

PKC may mediate this effect through the inhibition of Cdk5 activity or the up-regulation of protein phosphatase activity. However, the effect of PKC on these Cdk5-dependent phosphorylation sites is unlikely to be fully explained by the PKC-dependent activation of the protein phosphatases responsible for dephosphorylating them. Phospho-Ser67 inhibitor-1 and phospho-Thr75 DARPP-32 are dephosphorylated by the type 2 protein serine/threonine phosphatases, PP-2A and/or PP-2B (Bibb et al., 2001; Nishi et al., 2000). Phospho-Ser6 inhibitor-1 is dephosphorylated by PP-2A and/or PP-1 (Nguyen et al., 2007). PP-2A and PP-2B have been reported to down-regulate PKC activity in some systems (Hansra et al., 1996; Klumpp et al., 1998; Ricciarelli and Azzi, 1998). However, there is no evidence in the literature to suggest the regulation of these type 2 protein phosphatases by phorbol esters or PKC. Furthermore, treatment of striatal slices with PDBu has no effect on the phosphorylation state of PP-1, PP-2A, and PP-2B substrates phosphorylated by protein kinases other than Cdk5 (personal observation), strongly suggesting that PKC mediates its effects on the level of phosphorylation of Cdk5 targets through a Cdk5-dependent mechanism.

There is some evidence to suggest that Cdk5 is subject to regulation by phosphorylation (Liu et al., 2001; Sharma et al., 1999; Zukerberg et al., 2000). However, PKC did not phosphorylate recombinant Cdk5 or p25 *in vitro*. Moreover, PKC activation *in vivo* did not result in altered p35 stability, the most commonly accepted determinant of Cdk5 activity and substrate specificity in neurons. Perhaps least expectedly, PDBu had no effect on the activity of Cdk5 immunoprecipitated from acute striatal slices, although it caused a reduction of up to 84% in the phosphorylation level of three Cdk5 substrates in this tissue. It should be noted, however, that Cdk5 generally immunoprecipitates as a stable heterodimer with its cofactor, making immunoprecipitation an especially useful tool for monitoring changes in Cdk5 activity due to altered cofactor metabolism (Bibb et al., 2001; Wei et al., 2005). There is a growing body of evidence that the Cdk5/p35 heterodimer often occurs within larger protein assemblies inside the cell and these protein-protein interactions are important in the regulation of Cdk5 activity and subcellular localization (Lim et al., 2003). Thus, it remains possible that PKC controls Cdk5 activity at this higher level of quaternary regulation, through a mechanism that may be susceptible to disruption by the challenges of tissue lysis and immunoprecipitation.

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Fig. 1. The effect of PDBu on the phosphorylation of Cdk5 substrates in the striatum and *in vitro*
A, time course and PDBu dose-response curves of phospho-Ser67 inhibitor-1 (*P-S67 I-1*) levels determined by quantitative immunoblot analysis of striatal slices treated with 5 μ M PDBu for 0-30 min (*left*), or with 0-25 μ M PDBu for 15 min (*right*). Data represent means $\pm$ S.E.M. for 4 time course and 4 dose-response experiments. **B**, time course and PDBu dose-response curves of phospho-Thr75 DARPP-32 (*P-T75 D32*) levels determined by quantitative immunoblot analysis of striatal slices treated as described in **A**. Data represent means $\pm$ S.E.M. for 4 time course and 4 dose-response experiments. **C**, Immunoblot analysis of phosphor-Ser6 inhibitor-1 levels in striatal slices treated with 5 μ M PDBu for 0-30 min. **D**, Cdk5-dependent

phosphorylation of inhibitor-1 (*I-1*) in the absence or presence of 5 μ M PDBu *in vitro*. The panel shows SDS-PAGE analysis of [32 P]-labeled inhibitor-1.

Fig. 2. The effect of pharmacological inhibition or genetic deletion of PKC- α on the phosphorylation of Cdk5 substrates in the brain

A, quantitative immunoblot analysis of phospho-Ser67 inhibitor-1 levels in striatal slices treated with PDBu (5 μ M, 15 min) following incubation in the absence or presence of the PKC inhibitor, Ro-32-0432 (Ro, 5 μ M, 60 min). *, $p < 0.001$, Student's unpaired t test, $n = 4$ slices per condition. **B**, quantitative immunoblot analysis of phospho-Ser6 inhibitor-1 (P-S6 I-1) levels in 100 μ g of total protein from wild-type (+) and *Prkca*^{-/-} (-) cerebral cortex (CTX), cerebellum (CRB), and striatum (STR). Phospho-Ser6 inhibitor-1 levels are normalized to the mean value obtained from the tissue in which phospho-Ser6 inhibitor-1 was least abundant. *, $p < 0.02$ compared to wild-type cortex, Student's unpaired t test; **, $p < 0.001$ compared to wild-type striatum, Student's unpaired t test, $n = 4$ slices per condition. **C**, the membranes in **B** immunoblotted and quantified for phospho-Ser67 inhibitor-1 (P-S67 I-1). *, $p < 0.01$ compared to wild-type striatum, Student's unpaired t test; **, $p < 0.01$ compared to wild-type striatum, Student's unpaired t test, $n = 4$ slices per condition. **D**, the membranes in **B** immunoblotted and quantified for phospho-Thr75 DARPP-32 (P-T75 D32). *, $p < 0.05$ compared to wild-type striatum, Student's unpaired t test, $n = 4$ slices per condition.

Fig. 3. Investigation of a possible mechanism of PKC-mediated Cdk5 regulation

A, PKC-dependent phosphorylation of Cdk5/p25 and inhibitor-1 (*I-1*) *in vitro*. The panel shows SDS-PAGE analysis of Cdk5/p25 that was [32 P]-labeled in the absence and presence of PKC. Inhibitor-1 phosphorylated by PKC was included as a control. **B**, immunoblot analysis of p35 levels in striatal slices treated with 5 μ M PDBu for 0-30 min (*top*), or 0-25 μ M PDBu for 15 min (*bottom*). **C**, phosphorylation of histone H1 (*H1*) by Cdk5 immunoprecipitated from the lysates of striatal slices treated with or without 5 μ M PDBu for 30 min (α -Cdk5). In control experiments, rabbit IgG-conjugated agarose (*IgG*) pellets from the pre-clearing step were used as a source of Cdk5 activity. The panels show SDS-PAGE analysis of [32 P]-labeled (*top*) and Coomassie Brilliant Blue (CBB)-stained (*bottom*) histone H1.