

Regulation of Cyclin-Dependent Kinase 5 and Calcium/Calmodulin-Dependent Protein Kinase II by Phosphatidylinositol-Linked Dopamine Receptor in Rat Brain

Xuechu Zhen, Satindra Goswami, Syed Amir Abdali, Mara Gil, Kalindi Bakshi, and Eitan Friedman.

Department of Physiology/Pharmacology, City University of New York Medical School, New York, New York

Received May 4, 2004; accepted July 29, 2004

ABSTRACT

A brain dopamine receptor that modulates phosphatidylinositol (PI) metabolism via the activation of phospholipase C β (PLC β) has been described previously. The present study aims to define the downstream signaling cascade initiated by the PI-linked dopamine receptor. Incubation of rat brain frontal cortical slices with 6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF83959), a recently identified selective agonist of the PI-linked D₁-like dopamine receptor, elicited transient time- and dose-dependent stimulations of cyclin-dependent kinase 5 (cdk5) and calcium/calmodulin-dependent protein kinase II (CaMK II) activities. The stimulation of these kinases is blocked by 20 μ M *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) or the PLC β antagonist 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122) and is attenuated by the protein kinase inhibitor calphostin C or by the intracellular calcium chelator BAPTA, indicating that SKF83959 stimulates cdk5 and

CaMK II activities via a PI-linked D₁-like dopamine receptor, and PLC β and is dependent on protein kinase C and calcium. Although cdk5 and CaMK II are physically associated in native brain tissue, no change in this association was observed in response to SKF83959 stimulation or to the inhibition of either cdk5 by roscovitine or of CaMK by 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN93), suggesting that SKF83959-mediated stimulation of cdk5 or CaMK II is independent of the other kinase and that the association of the two kinases is not modulated by change of kinase activity. Moreover, we found that cdk5 phosphorylates dopamine and cAMP-regulated phosphoprotein at Thr75, whereas CaMK II is responsible for the activation of cAMP response element-binding protein in response to SKF83959 stimulation. The present data provide the first insight into the signaling mechanism for the PI-linked dopamine receptor. This information, in turn, may help in exploring the functional consequences of stimulation of this brain receptor.

Dopamine, one of the major neurotransmitters in brain, exerts its action via dopamine receptors. So far, at least five dopamine receptors have been cloned. It is known that the

receptors in the D₁ receptor family (D₁, D₅) couple to G_s protein and activate the production of cAMP; the D₂ receptor family (D₂₋₄) couples to G_i/G_o protein and inhibits cAMP production (Missale et al., 1998). Thus, these receptors regulate the levels of cAMP, which in turn regulates the activity of protein kinase A (PKA). In addition to the cAMP/PKA pathway, recent evidence indicates that activation of dopamine receptors also elevates intracellular calcium via the

This work was supported by U.S. Public Health Service grant DA11029 and the City University of New York Incentive Collaborative grant.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.104.002279.

ABBREVIATIONS: PKA, protein kinase A; PLC β , phospholipase C β ; cdk5, cyclin-dependent kinase 5; CaMK II, calcium/calmodulin-dependent protein kinase II; PI, phosphatidylinositol; PKC, protein kinase C; DARPP-32, dopamine and cAMP-regulated phosphoprotein; CREB, cAMP-responsive element-binding protein; IP₃, inositol trisphosphate; FCX, frontal cortex; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; TBS, Tween-20 phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SKF83959, 6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; U-73122, 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; KN93, 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; SKF81297, *R*-(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; PD98059, 2'-amino-3'-methoxyflavone; KN92, 2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; U-73343, 1-[6-((17 β -methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; SKF83566, (\pm)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; SKF38393, 1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride.

activation of phospholipase C β (PLC β), which hydrolyzes phosphatidylinositol (PI) into diacylglycerol and inositol trisphosphate (IP $_3$) (Felder et al., 1989; Undie and Friedman, 1990; Pacheco and Jope, 1997). IP $_3$ is an important second messenger that elicits the release of calcium from intracellular stores. The dopamine receptor that activates the PLC/IP $_3$ pathway has been called the PI-linked dopamine receptor. This receptor seems to pharmacologically resemble the D $_1$ receptor family because it is stimulated selectively by agonists and is inhibited by antagonists of the D $_1$ receptor (Felder et al., 1989; Friedman et al., 1997). SKF83959 is a recently identified selective agonist for the PI-linked dopamine receptor (Panchalingam and Undie, 2001; Jin et al., 2003). However, the functional implications of this novel PI-linked dopamine receptor pathway in brain are completely unknown.

The regulation of intracellular calcium content by IP $_3$ is critical in mediating neuronal responses. Ca $^{2+}$ and calcium-sensing proteins such as calmodulin form functional complexes that interact with various proteins, thus modulating their functions (Soderling, 2000). Ca $^{2+}$ /calmodulin regulates CaMK, a serine/threonine protein kinase. CaMK phosphorylates cAMP response element-binding protein (CREB), a critical transcriptional factor that is involved in synaptic plasticity and in mediating learning and memory processes (Bito et al., 1996). Thus, it is of interest to investigate whether the PI-linked dopamine receptor regulates the CaMK/CREB pathway.

Recent information indicates that cyclin-dependent kinase 5 (cdk5) is an important kinase in the modulation of dopamine receptor signals (Bibb et al., 1999; Nishi et al., 2000). cdk5 was shown to phosphorylate the dopamine and the cAMP-regulated phosphoprotein (DARPP-32) at Thr75. Phospho-DARPP-32 (Thr75) is an inhibitor of protein kinase A. Activation of the D $_1$ dopamine receptor has been shown to decrease the level of phospho-DARPP-32 (Thr75), whereas activation of the D $_2$ dopamine receptor increases its level (Nishi et al., 2000). cdk5 exhibits exclusively high activity in neurons; it has been shown that cdk5 is required for neuronal development and in regulating neuronal migration, process elongation, cortical lamination, and synaptic plasticity (Dhavan and Tsai, 2001; Smith et al., 2001). Abnormal cdk5 activity has been linked to neurodegenerative diseases such as Alzheimer's disease (Dhavan and Tsai, 2001). This kinase was also shown to be associated with calcium regulation (Liu et al., 2001; Dhavan et al., 2002). The interaction between cdk5 and CaMK II was reported recently (Dhavan et al., 2002). However, the potential role of the PI-linked dopamine receptor in the regulation of cdk5 and CaMK II remains unknown.

The present work demonstrates that stimulation of the PI-linked dopamine receptor by SKF83959 induces the transient activation of cdk5 and CaMK II. Activation of cdk5 and CaMK II is mediated via PLC β and requires the activation of PKC and the intracellular release of calcium. Receptor activation increases the phosphorylation of DARPP-32 (Thr75), a known substrate of cdk5, whereas activation of CaMK II results in the activation of CREB. This study therefore provides new insights into the signaling pathways that transduce PI-linked dopamine receptor signals. These findings may facilitate the unraveling of the functional role of the PI-linked dopamine receptor in brain.

Materials and Methods

Materials. SKF83959 was kindly provided by the National Institute of Mental Health synthesis program (Menlo Park, CA). R(+)-SCH23390 hydrochloride, SKF81297, and SKF83959 were purchased from Sigma/RBI (Natick, MA). PD98059, KN92, and KN93 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). Antibodies to cdk5, CaMKs, and horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). U-73122, U-73343, calphostin C, and roscovitine were obtained from Calbiochem (San Diego, CA). CaMK assay kit was obtained from Upstate Biotechnology (Charlottesville, VA). Proteinase inhibitors were from Sigma-Aldrich (St. Louis, MO). Anti-phospho-(Thr75 and Thr34) DARPP-32, were from New England Biolabs (Beverly, MA). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). [γ - 32 P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Other reagents were purchased from standard laboratory suppliers.

Slice Preparation and Treatment. Brain slices of rat frontal cortex (FCX) were prepared as described previously (Zhen et al., 2001a; Dhavan et al., 2002) using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). The prisms were washed in oxygenated Krebs-Ringer buffer. After equilibration at 37°C for 30 min, the slices were subsequently incubated with SKF83959 or other reagents. Reactions were terminated by cooling on ice followed by centrifugation. The tissues were sonicated and lysed in lysis buffer containing 20 mM Tris, pH 7.8, 150 mM NaCl, 50 mM NaF, 2 mM EGTA, 1 mM EDTA, 0.5 mM β -glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethanesulfoxide, and proteinase inhibitor cocktail. The protein content of the supernatant was determined by the Bradford method (Bio-Rad). Aliquots of supernatants (300 μ g) were immunoprecipitated with anti-cdk5, anti-CaMK II or IV, and anti-ERK2 antibody, respectively, using a method published previously, with minor modification (Zhen et al., 2001b). The immunoprecipitates were collected and used for kinase activity assays or in some cases for analyzing the physical association between cdk5 and CaMK II, as described below.

In Vitro Immune Complex Kinase Assays. The immune complex was washed three times with lysis buffer and twice with the corresponding kinase assay buffer (see below). The cdk5 kinase assay was performed in 50 mM HEPES, pH 7.4, containing 10 mM MgCl $_2$, 1 mM dithiothreitol, 100 μ M [γ - 32 P]ATP (5 μ Ci), and substrate peptide (Dhavan et al., 2002) for 20 min at 30°C. ERK kinase activity was assessed for 20 min at 30°C in the presence of 50 μ M [γ - 32 P]ATP (5 μ Ci) and 0.2 mg/ml myelin basic protein, as described previously (Zhen et al., 2001a). The reaction was stopped on ice, and 20 μ l of supernatant was spotted onto P81 paper and washed extensively in 0.425% phosphoric acid. Radioactivity incorporated into peptide was determined by scintillation counting. For CaMK assay, immunocomplexes of CaMK II or IV were washed with kinase assay buffer, and the reaction was conducted in the presence of 10 μ M [γ - 32 P]ATP using peptide- γ as substrate (Kasahara et al., 2001), according to the manufacturer's instruction.

Phosphorylated Proteins and Immunoblot Analysis. The lysate was boiled in preparation buffer, and 30 μ g of protein was loaded onto 12% SDS-PAGE. The proteins were separated electrophoretically and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with 10% (w/v) fat-free dry milk in 0.1% Tween-20 phosphate-buffered saline (TBS) followed by incubation with anti-phospho-CREB (1:2000) for 2 h. The membranes were washed and then incubated for 1 h with species-specific horseradish peroxidase-conjugated secondary IgG antibody (1:5000–1:10,000 dilution) in 0.1% TBS. The membranes were washed once with 0.3% TBS for 20 min followed by four 10-min washes with 0.1% TBS, and the signals were visualized by Supersignal (Pierce, Rockford, IL). For the analysis of DARPP-32, the brain tissues were sonicated and prepared by boiling in 1% SDS-contained sample preparation buffer as described previously (Snyder et al., 1998). Equal

amounts of homogenate protein were loaded onto 12% SDS-PAGE, and the membranes were then probed with anti-phospho-DARPP-32 at Thr34 (1:750) or anti-phospho-DARPP-32 at Thr75 antibody, respectively. For assessing the association of Cdk5 and CaMK II, 300 μ g of lysate protein was incubated with 3 μ g of anti-cdk5 antibody and was incubated with shaking for 2 h before the addition of Protein A/G PLUS (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were then collected and loaded onto 12% SDS-PAGE. The proteins were separated electrophoretically and transferred to nitrocellulose membranes. Membranes were incubated with anti-CaMK II antibody, or vice versa, CaMK II precipitates were separated by SDS-PAGE, and anti-cdk5 antibody was used to detect the association between the kinases.

Measuring Brain PI Hydrolysis in Brain Slices in Response to SKF83959 Challenge. Brain slices were incubated with Krebs' bicarbonate buffer for 45 min with buffer replacement every 15 min. Tissues were then labeled with [*myo*-³H]inositol (10 μ Ci) for 1 h in the presence of 20 mM lithium chloride. Tissues were then incubated with SKF83959 or antagonist SCH23390 for a designated time. The reaction was stopped with 300 μ l of 1 M trichloroacetic acid. The 500- μ l sample of trichloroacetic acid extraction was transferred to 2-ml Eppendorf tubes. With the addition of 10 mM EDTA and freon/tri-*n*-ethylamine (3:1, v/v), samples were thoroughly mixed and centrifuged. A 500- μ l sample of the upper layer was taken in a glass tube containing 0.5 M NaHCO₃ and 1 ml of distilled water. After pass to the AG-1 \times 8 column (0.75 ml), samples were washed extensively. Total immunoprecipitates were then eluted and collected as described previously (Challiss et al., 1988). Results are expressed as counts per minute per milligram of protein.

Cell Culture and Treatment. PC-12 cells that were transfected with D₁ dopamine receptor were prepared as described previously (Zhen et al., 2001b). Cells were seeded in 10-cm dishes. After they reached 80 to 90% confluence, cells were cultured in low serum medium for 4 h before incubating with SKF83959 or other drugs for the indicated times. Cells were then harvested, and lysates were prepared for kinase assay.

Data Analysis. Data are expressed as mean \pm S.E.M. and analyzed by analysis of variance followed by Newman-Keuls test unless otherwise indicated. Statistical significance was considered at $p < 0.05$.

Results

SKF83959 Stimulates cdk5 Activity via D₁-Like Dopamine Receptor in Rat Brain Slices. FCX slices were incubated with 20 μ M SKF83959 for 2, 5, 10, 20, and 30 min, and cdk5 activity was then measured in tissue lysates. SKF83959 induced a time-dependent stimulation of kinase activity (Fig. 1A). SKF83959-induced activation of cdk5 reached a peak within 2 min (2.37 ± 0.21 -fold, $n = 3$) and returned to basal levels at 10 min, indicating that SKF83959 activates cdk5 transiently. A dose-response analysis indicates that SKF83959 activated cdk5 activity at 10 μ M, and peak activation occurred at 20 μ M (Fig. 1B). Because SKF83959 has been found to be a selective agonist of the PI-linked D₁ dopamine receptor, we therefore checked whether activation of cdk5 is also mediated by the receptor. FCX slices were preincubated with 20 μ M of the selective D₁ receptor antagonist SCH23390 for 20 min before the stimulation of SKF83959. As shown in Fig. 1C, SCH23390 almost completely blocked SKF83959-stimulated cdk5, suggesting that the stimulation of cdk5 by SKF83959 is mediated via a D₁-like dopamine receptor. Moreover, we found that another D₁ receptor antagonist, SKF83566 (100 nM), also blocked SKF83959- and SKF81297-stimulated cdk5 activity (Fig. 1D), whereas prazosin (1 μ M), spiperone (10 μ M), and mesulergine (10 μ M) did not (data not shown).

Activation of cdk5 by SKF83959 Is Mediated by PLC β and Is Dependent on PKC and Intracellular Calcium. Because SKF83959 is a selective agonist for the PI-linked D₁-like dopamine receptor and this receptor is known to couple to PLC β /PKC/IP₃ pathway, we tested whether this signaling pathway is involved in cdk5 activation. Brain slices were preincubated with U-73122 (10 μ M), U-73343 (10 μ M), calphostin C (1 μ M), or BAPTA (10 μ M) for 20 min before the addition of SKF83959, and cdk5 activity was determined. As shown in Fig. 2A, the putative phospholipase C inhibitor U-73122 blocked SKF83959-induced cdk5 activation. In contrast, its inactive analog, U-73343, did not interfere with

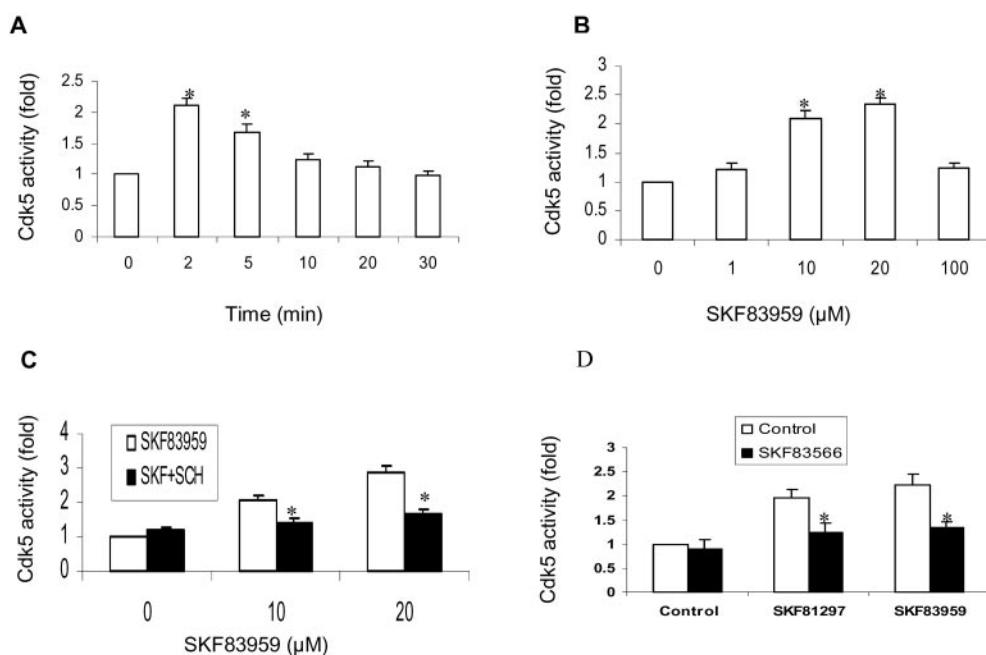


Fig. 1. Stimulation of the PI-linked D₁ dopamine receptor by SKF83959 induces a transient activation of cdk5 in rat brain slices. FCX slices were incubated with either 20 μ M SKF83959 for 2 to 30 min (A) or 1, 10, 20, or 100 μ M SKF83959 for 2 min (B). cdk5 activity was assayed in cdk5 immunoprecipitates of tissue lysates. *, $p < 0.01$ compared with control. C, FCX slices were preincubated for 20 min with vehicle or with 20 μ M SCH23390 before the addition of 10 or 20 μ M SKF83959, and the cdk5 activity was measured. D, slices were preincubated with either SKF83566 (100 nM) or vehicle for 20 min before the addition of SKF81297 or SKF83959. *, $p < 0.01$, Student's *t* test, compared with respective SKF83959-treated group. The results are expressed as fold change from control and are presented as means \pm S.D. obtained from at least three independent experiments. SKF, SKF83959; SCH, SCH23390.

activation of this kinase. Moreover, inhibition of PKC by calphostin C or intracellular calcium release by BAPTA also attenuated cdk5 activation, whereas PKA inhibitor had little effect (Fig. 2B). These results suggest that SKF83959-mediated activation of cdk5 is mediated via PLC β and is dependent on PKC and Ca²⁺ but not on PKA. Phosphorylation of PLC β by PKC has been shown to inhibit PLC β activity (Ryu et al., 1990; Litosch, 1996). We therefore tested whether PKC activation influences SKF83959-induced cdk5 activation by preincubated FCX slices with 1 μ M PMA before stimulating with SKF83959, carbachol, or NaF. Carbachol and NaF are known to stimulate the PLC β pathway, therefore serving as positive controls. As expected, both carbachol and NaF stimulated cdk5 activity in FCX slices, whereas activation of PKC by PMA significantly reduced carbachol- or NaF-stimulated cdk5 activation (Fig. 3). Likewise, the SKF83959-elicited response was also inhibited by prior PKC stimulation (Fig. 3). Taken together, the present data demonstrate that stimulation of the PI-linked D₁ dopamine receptor by SKF83959 activates cdk5 through PLC β -mediated PKC activation and elevation in intracellular calcium.

SKF83959 Activates CaMK II Activity in a Time- and Dose-Dependent Manner. Calcium has been shown to play an important role in regulating CaMK activity. To study the effect of SKF83959 on CaMK activity, FCX slices were treated with SKF83959, and CaMK II and IV activities were measured in anti-CaMK II or anti-CaMK IV immunoprecipitates. As shown in Fig. 4, A and B, SKF83959 induced a

transient activation of CaMK II in FCX slices. Similar to the result with cdk5 shown in Fig. 1, the maximal stimulation was achieved at 2 min of agonist stimulation; a return to basal level occurred within 10 min. The dose-response curve indicates that stimulation of CaMK II is observed at 10 μ M of SKF83959, and it reaches maximum stimulation at 20 μ M. However, significant changes in CaMK IV activity were not detected by this treatment (data not shown), indicating that SKF83959 selectively stimulates CaMK II activity. Similar, we did not detect significant stimulation of ERK activity in the present experimental condition (data not shown). As expected, SCH23390 blunted the drug-induced activation of CaMK II. Another D₁ receptor antagonist SKF83566 also blocked the SKF83959-induced CaMK II activation, whereas D₂, α_1 , or 5-hydroxytryptamine_{2A/2C} receptor antagonist did not (Fig. 4C). It is interesting to note that stimulation of CaMK II was also mediated through PLC β and was dependent on PKC and intracellular calcium because U-73122, calphostin C, or BAPTA blocked the activation of CaMK II (Fig. 4D). Thus, it seems that SKF83959-mediated CaMK II activation shares upstream mechanisms with cdk5 activation in FCX slices.

SKF83959 Did Not Alter cdk5 and CaMK II Activity in D₁-Expressing PC-12 Cells. One may argue that classic D₁ dopamine receptor contributes to the activation of cdk5 and CaMK II because SKF83959 was originally shown to have a high affinity for the D₁ dopamine receptor, and the FCX is enriched with this receptor. We, therefore, tested whether cdk5 and CaMK II activation in brain slices is mediated by the D₁ dopamine receptor. PC-12 cells that stably express the D₁ dopamine receptor were incubated with either 1 μ M SKF83959 or with the selective D₁ receptor agonist SKF81297, and kinase activities were assessed. As shown in Fig. 5A, activation of D₁ receptors by SKF81297 did not affect cdk5 or CaMK II activity although this treatment resulted in a dramatic stimulation on cAMP (data not shown; Jin et al., 2003). As expected, SKF83959 also did not activate these kinases in the cells (Fig. 5B). However, carbachol induced time-dependent stimulation on the CaMK II activity in the cells (Fig. 5C). Our previous study demonstrated that both SKF83959 and SKF81297 do not stimulate PI hydrolysis in these cells (Jin et al., 2003) but do so in brain slices (Fig. 5D) (Jin et al., 2003), indicating that classic D₁ dopamine recep-

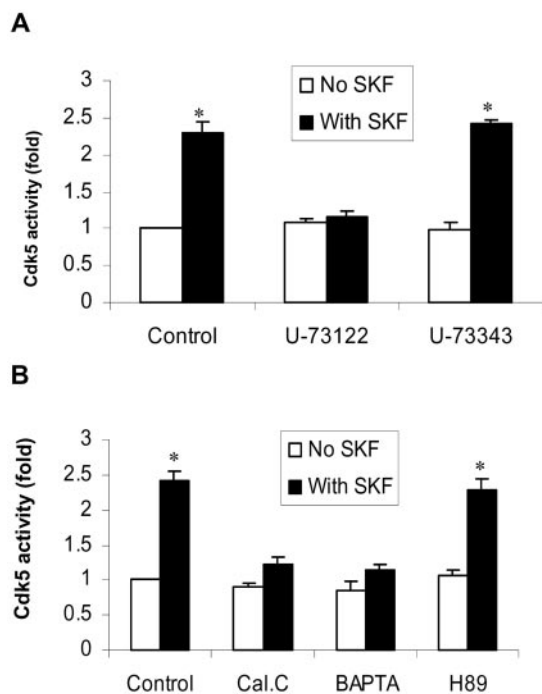


Fig. 2. Activation of cdk5 by SKF83959 is dependent on PKC and intracellular calcium and is mediated via PLC β but not PKA. A, FCX slices were incubated with 10 μ M U-73122 and 10 μ M U-73343 (dissolved in dimethyl sulfoxide) before the addition of 20 μ M SKF83959. cdk5 activity was assayed in cdk5 immunoprecipitates. B, FCX slices were incubated with 1 μ M PKC inhibitor calphostin C, 10 μ M BAPTA, or 5 μ M PKA inhibitor H89 before the addition of 20 μ M SKF83959. cdk5 activity was assayed as described above. The results are expressed as fold change from control and presented as means \pm S.D. obtained from at least three independent experiments. SKF, SKF83959; Cal. C, calphostin C; *, $p < 0.01$, Student's t test, compared with respective control.

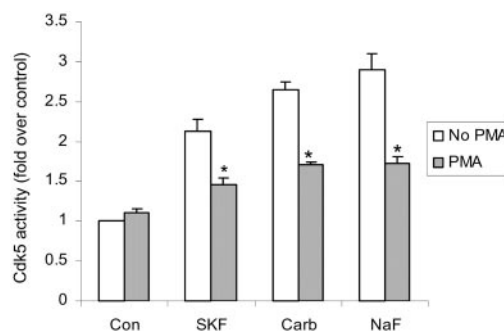


Fig. 3. PKC stimulation attenuates SKF83959-stimulated cdk5 activity. Brain slices were incubated with 1 μ M PMA for 20 min before the addition of 20 μ M SKF83959, 20 mM NaF, or 100 μ M carbachol. Tissue was homogenized, and Cdk5 activity was assayed. Data are expressed as fold change over control and summarized as means \pm S.D. of four independent experiments. Con., control (0.1% dimethyl sulfoxide); SKF, SKF83959; Carb, carbachol; NaF, NaF; *, $p < 0.01$ compared with respective non-PMA control.

tors are not involved in SKF83959-mediated activation of cdk5 and CaMK II in brain slices.

Activation of cdk5 and CaMK II by SKF83959 Is Independent of Each Other. As shown above, the activations of cdk5 and CaMK II by SKF83959 share a similar time course. A previous study also indicated that the cdk5 activators p35 and p39 interact with the α -subunit of Ca^{2+} /CaMK II. We therefore tested whether the activation of the two

kinases by SKF83959 is interrelated. Brain slices were pretreated with 50 μM KN93, a selective inhibitor of CaMK II, or its negative control, KN92, before the addition of SKF83959. Inhibition of CaMK II did not alter SKF83959-induced cdk5 stimulation (Fig. 6A). On the other hand, inhibition of cdk5 by roscovitine also did not affect the activation of CaMK II by SKF83959 (Fig. 6B). Moreover, we have observed a physical association between cdk5 and CaMK II; however, this association was not altered significantly by change of either cdk5 or CaMK II activity (Fig. 6C), suggesting that cdk5 or CaMK II activity may not be required for their association. It is therefore clear that although SKF83959-induced activation of cdk5 and CaMK II share similar upstream pathways, the activations of the two kinases are regulated independently.

Activation of CaMK II or cdk5 Differentially Regulates Phosphorylation of Distinct Substrates in Response to SKF83959. CaMKs are known to phosphorylate CREB at Ser133, and this activates CREB. We first determined whether CREB phosphorylation is altered by SKF83959 in FCX slices. As shown in Fig. 7A, SKF83959 activated CREB transiently; phosphorylation of CREB (Ser133) increased at 2 min, reached maximal at 5 min, and returned to control level within 10 min. To detect the upstream mechanism for CREB activation, brain slices were preincubated with SCH23390, U-73122, and U-73343 for 20 min before the addition of SKF83959. Activation of CREB was blunted by SCH23390 or U-73122 but not by U-73343 (Fig. 7B), indicating that CREB activation by SKF83959 is mediated by the PI-linked dopamine receptor via activation of PLC β . We further found that KN93, a selective inhibitor of CaMKs, also blocked SKF83959-stimulated CREB phosphorylation, whereas KN92 and roscovitine did not alter the drug-induced activation of CREB (Fig. 7C), indicating that CaMK II is required for CREB activation by SKF83959, whereas cdk5 is not.

We next examined the potential target of cdk5 activation by SKF83959. DARPP-32 phosphorylation in response to

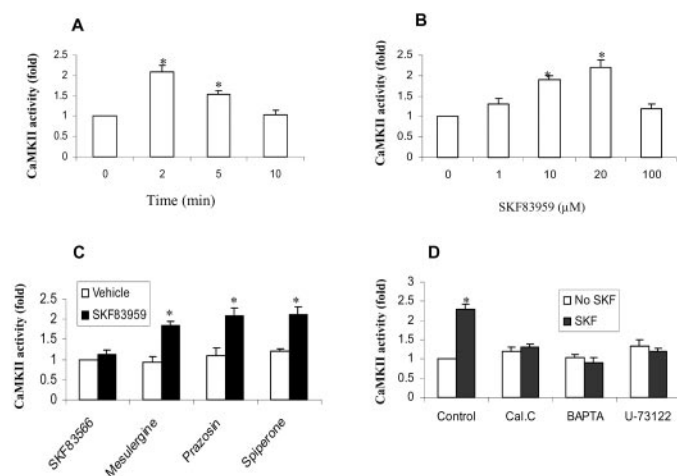


Fig. 4. SKF83959 induced a transient activation of CaMK II in rat brain slices. FCX slices were incubated with either 20 μM SKF83959 for the indicated periods of time (A) or with 1, 10, 20, or 100 μM SKF83959 for 2 min (B) before the tissue was collected and homogenized. CaMK II activity was assayed in CaMK II immunoprecipitates using peptide- γ as substrate as described under *Materials and Methods*. C, FCX slices were preincubated with vehicle or 20 μM SCH23390, 10 μM U-73122, 1 μM calphostin C, or 20 μM BAPTA for 20 min before the addition of SKF83959. Two minutes later, the incubation was terminated, tissue was homogenized, and enzyme activity was conducted as described under *Materials and Methods*. D, FCX slices were preincubated with vehicle or 100 nM SKF83566, 1 μM prazosin, 10 μM mesulergine, or spiperone for 20 min before the addition of 20 μM SKF83959 for 2 min. Data are expressed as fold change over control and are summarized as means \pm S.D. of three or four independent experiments. SCH, SCH23390; Cal. C, calphostin C; SKF, SKF83959. *, $p < 0.01$ compared with control.

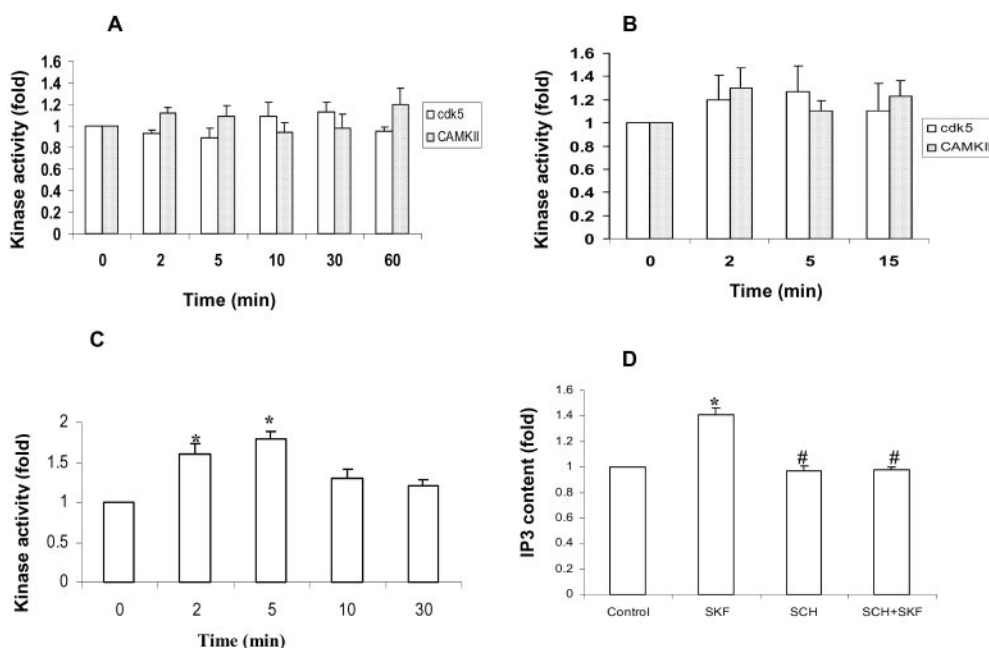


Fig. 5. Stimulation of D_{1A} dopamine receptor did not alter cdk5 or CaMK II activity in $\text{D}_{1A}/\text{PC-12}$ cells. PC-12 cells that stably express the D_{1A} dopamine receptor ($\text{D}_{1A}/\text{PC-12}$) were incubated with either 10 μM of the D_1 dopamine receptor agonists SKF38393 (A) or SKF83959 (B) for the indicated times. Cells were then collected and lysed, and kinase activity was assayed in cdk5 or CaMK II immunoprecipitates. C, cells were treated with 100 μM carbachol for indicated times. D, FCX slices were labeled with [^3H]inositol (10 μCi) for 1 h in the presence of 20 mM lithium chloride. Tissues were then incubated with 20 μM SCH23390 for 20 min before 30-min SKF83959 stimulation. PI hydrolysis was measured as described under *Materials and Methods* and was expressed as fold change from control. Data are presented as means \pm S.D. of three or four independent experiments. *, $p < 0.01$ compared with control; #, $p < 0.01$ compared with SKF83959 stimulation. SCH, SCH23390; SKF, SKF83959.

SKF83959 stimulation in FCX slices was analyzed using specific anti-phospho-DARPP-32 antibodies (Thr34 and Thr75). As shown in Fig. 7D, SKF83959 induced a significant increase in the level of phospho-DARPP-32 (Thr75), whereas no significant change in phospho-DARPP-32 (Thr34) was observed. Moreover, roscovitine, a selective inhibitor for cdk5, largely attenuated SKF83959-induced phosphorylation of DARPP-32 at Thr75, indicating that cdk5 activation contributes to SKF83959-mediated Thr75 phosphorylation of DARPP-32. The results demonstrate that activation of the PI-linked dopamine receptor which stimulates the PLC/PKC/ IP_3 pathway results in activation of cdk5 and CaMK II, and

these regulate the phosphorylation of DARPP-32 and CREB, respectively.

Discussion

The present studies demonstrate that SKF83959 activates cdk5 and CaMK II in a time- and dose-dependent manner. The stimulations are transient and return to basal levels within 10 min. Activation of cdk5 and CaMK II seems to be mediated by the PI-linked dopamine receptor in brain. We further demonstrated that both cdk5 and CaMK II activation by SKF83959 is mediated by PLC β and is dependent on PKC

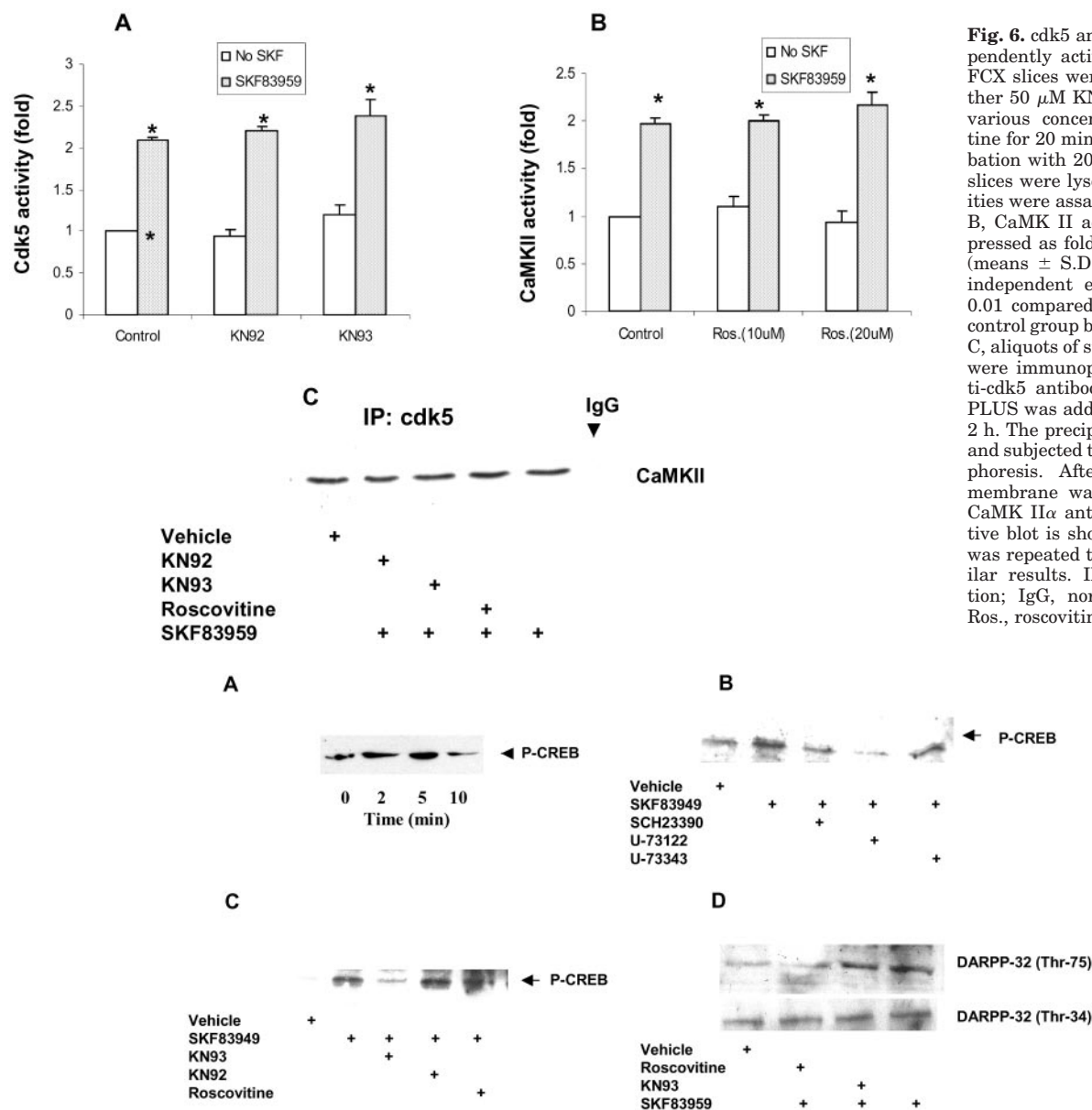


Fig. 7. CaMK II and cdk5 are responsible for SKF83959-induced phosphorylation of CREB and DARPP-32 (Thr75). A, FCX slices were incubated with 20 μ M SKF83959 for the indicated times. The tissues were prepared for Western blot analyses and probed with anti-phospho-CREB Ser133 antibody. B, FCX slices were incubated either with 10 μ M U-73122 or U-73343 or with 20 μ M SCH23390 for 20 min before a 5-min additional incubation with 20 μ M SKF83959. Phospho-CREB Ser133 was detected in the slice lysates. C, FCX slices were incubated either with 50 μ M KN92 or KN93 or with 10 μ M roscovitine for 20 min before the addition of 20 μ M SKF83959 and an additional incubation for 5 min. Phospho-CREB Ser133 was probed in the lysate. D, FCX slices were incubated with either 50 μ M KN93 or 10 μ M roscovitine for 20 min before 20 μ M SKF83959 stimulation for an additional 5 min. Phospho-DARPP-32 was detected in slice lysates using anti-phospho-DARPP-32 (Thr75) or anti-phospho-DARPP-32 (Thr34) antibodies. Each experiment was repeated at least three times. Representative blots are shown.

Fig. 6. cdk5 and CaMK II are independently activated by SKF83959. FCX slices were incubated with either 50 μ M KN93 or KN92 or with various concentrations of roscovitine for 20 min before a 2-min incubation with 20 μ M SKF83959. The slices were lysed, and kinase activities were assayed. A, cdk5 activity; B, CaMK II activity. Data are expressed as fold change over control (means \pm S.D.) from three to four independent experiments. *, $p < 0.01$ compared with the respective control group by the Student's t test. C, aliquots of supernatants (300 μ g) were immunoprecipitated with anti-cdk5 antibody, and Protein A/G PLUS was added and incubated for 2 h. The precipitates were collected and subjected to SDS-PAGE electrophoresis. After transferring, the membrane was probed with anti-CaMK II α antibody. A representative blot is shown. The experiment was repeated three times with similar results. IP, immunoprecipitation; IgG, normal serum control; Ros., roscovitine; SKF, SKF83959.

and intracellular calcium release, because inhibition of PKC or chelation of intracellular released calcium abolished drug-induced kinase activations. Although the activation of cdk5 and CaMK II share the same upstream pathway and are physically associated, inhibition of cdk5 did not interrupt SKF83959-induced activation of CaMK II, and cdk5 activation was not affected by CaMK inhibition, indicating that the activations of the kinases are independent of each other. We also found that SKF83959 induces the phosphorylation of DARPP-32 (Thr75) and of CREB that are associated with cdk5 and CaMK II activation. To our knowledge, this is the first report that describes the detailed signaling cascades that are activated by the PI-linked D₁ dopamine receptor.

Although a number of studies have found that G_{αq} couples the dopamine receptor to PLC (Felder et al., 1989; Undie and Friedman, 1990; Pacheco and Joep, 1997), the recent identification of a selective agonist, SKF83959 (Panchalingam and Undie, 2001; Jin et al., 2003), for this pathway has allowed the detailed investigation of signal transduction and function of this dopamine receptor. The present data demonstrate that activation of the PI-linked D₁ dopamine receptor differentially regulates the cdk5 and CaMK II pathways. The conclusion is reached from the following observations: 1) the selective D₁ dopamine receptor antagonist SCH23390 blocked SKF83959-stimulated cdk5 and CaMK II activities; 2) inhibition of PLCβ attenuated SKF83959-stimulated cdk5 and CaMK II activation; 3) SKF83959 did not stimulate PI hydrolysis (Jin et al., 2003), nor did it activate cdk5 or CaMK II (Fig. 5) in D_{1A}-expressing PC-12 cells, indicating that the stimulation of cdk5 or CaMK II by SKF83959 in brain slices is not mediated by the classic G_{αs}-coupled D_{1A} dopamine receptor; and 4) the D₁ dopamine receptor agonist SKF81297 did not stimulate cdk5 and CaMK II activities in D_{1A}/PC-12 cells, although it induced a dramatic stimulation of cAMP/PKA, further confirming that activation of the classic G_{αs}-coupled D₁ receptor is not responsible for SKF83959-stimulated cdk5 and CaMK II activation in brain slices.

cdk5, the catalytic component of the kinase, and its neuron-specific activator p35 have received great deal of attention in physiology and pathology. cdk5 is a proline-directed serine/threonine kinase and is enriched in neurons. It forms a complex with p35 in neurons, and the association is essential for its activation. cdk5 has been demonstrated to play an important role in neurite outgrowth and migration (Dhavan and Tsai, 2001) and in neuroplasticity (Fischer et al., 2003). Although neuronal cytoskeletal proteins such as Tau were originally identified as substrates for cdk5, suggesting that the kinase may be involved in the pathology of neurodegenerative diseases (Smith et al., 2001), there are other intracellular substrates that were identified recently. Among them, DARPP-32 is of particular interest because it is considered to be a central signaling molecule in the dopamine signaling system of the brain (Greengard et al., 1999). Stimulation of both D₁- and D₂-like dopamine receptors was shown to regulate DARPP-32 phosphorylation. Phosphorylation of DARPP-32 at Thr75 converts DARPP-32 into a potent PKA inhibitor and reduces phosphorylation of DARPP-32 at Thr34 (Bibb et al., 1999). The latter is an inhibitor of protein phosphatase 1. Dopamine D₁ receptor stimulation decreases the striatal phospho-DARPP-32 Thr75, whereas D₂ receptor stimulation increases phosphorylation at this site probably via alteration in protein phosphatase-2A (Nishi et al., 2000).

The present data demonstrate that activation of the PI-linked dopamine receptor stimulates cdk5 activity in frontal cortex, and this activation is responsible for SKF83959-induced change in levels of phospho-DARPP-32 Thr75 in this brain region. Thus, our results reveal additional mechanisms for dopamine receptor-regulated DARPP-32/protein phosphatase 1 pathway in brain. It is interesting to note that stimulation of metabotropic glutamate receptors also results in a transient activation of cdk5 in brain slices. This activation of cdk5 is associated with metabotropic glutamate receptor agonist-mediated stimulation of phospho-DARPP-32 at Thr75 (Liu et al., 2001), thus implying that activation of G_q-linked G-protein coupled receptors may share a common cdk5 pathway that regulates DARPP-32.

Brain dopamine systems have been considered to play a critical role in synaptic plasticity associated with learning and memory (Jay, 2003). Recent evidence indicates that many signaling molecules may be involved in transducing dopamine signals that are involved in neuroplasticity and in cognitive processes. These include mitogen-activated protein kinases, PKA, and CaMKs (Frey et al., 1993; Kornhauser and Greenberg, 1997; Zhen et al., 1998, 2001a; Jay, 2003). These protein kinases are able to phosphorylate CREB at Ser133, and CREB is considered to be a central transcription factor in the formation of memory. The present data demonstrate that the PI-linked dopamine receptor is involved in regulating the activation of CREB via CaMK II, indicating the potential role of this receptor in neuroplasticity.

Acknowledgments

We thank Regine Brioché and Rochelle Ledgister for excellent technical assistance.

References

- Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, and Czernik A (1999) Phosphorylation of DARPP-32 by cdk5 modulates dopamine signaling in neurons. *Nature (Lond)* **402**:669–671.
- Bito H, Deisseroth K, and Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca²⁺- and stimulus duration-dependent switch for hippocampal gene expression. *Cell* **87**:1203–1214.
- Challiss RA, Batty IH, and Nahorski SR (1988) Mass measurements of inositol(1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem Biophys Res Commun* **157**:684–691.
- Dhavan R, Greer PL, Morabito MA, Orlando LR, and Tsai LH (2002) The cyclin-dependent kinase 5 activators p35 and p39 interact with the α subunit of Ca²⁺/Calmodulin-dependent protein kinase II and α-actin-1 in a calcium-dependent manner. *J Neurosci* **22**:7879–7891.
- Dhavan R and Tsai LH (2001) A decade of cdk5. *Nat Rev Mol Cell Biol* **2**:749–759.
- Felder CC, Jose PA, and Axelrod J (1989) The dopamine-1 agonist, SKF82526, stimulates phospholipase-C activity independent of adenylate cyclase. *J Pharmacol Exp Ther* **248**:171–175.
- Fischer A, Sananbenesi F, Spiess J, and Radulovic J (2003) Cdk5: a novel role in learning and memory. *Neurosignals* **12**:200–208.
- Frey U, Huang YY, and Kandel ER (1993) effect of cAMP stimulate a late stage of LTP in hippocampal CA1 neurons. *Science (Wash DC)* **260**:1661–1664.
- Friedman E, Jin LQ, Cai GP, Hollon TR, Drago J, Sibley DR, and Wang HY (1997) D₁-like dopaminergic activation of phosphoinositide hydrolysis is independent of D_{1A} dopamine receptors: evidence from D_{1A} knockout mice. *Mol Pharmacol* **51**:6–11.
- Greengard P, Allen PB, and Nairn AC (1999) Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. *Neuron* **23**:435–447.
- Jay TM (2003) Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. *Prog Neurobiol* **69**:375–390.
- Jin LQ, Goswami S, Cai G, Zhen X, and Friedman E (2003) SKF83959 selectively regulates phosphatidylinositol-linked D₁ DAR receptors in rat brain. *J Neurochem* **85**:378–386.
- Kasahara J, Fukunaga K, and Miyamoto E (2001) Activation of calcium/calmodulin-dependent protein kinase IV in long term potentiation in the rat hippocampal CA1 regions. *J Biol Chem* **276**:24044–24050.
- Kornhauser JM and Greenberg ME (1997) A kinase to remember: dual roles for MAPK kinase in long-term memory. *Neuron* **18**:839–842.
- Missale C, Nash SR, Robinson SW, Jaber M, and Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* **78**:189–225.

- Litosch I (1996) Protein kinase C inhibits the Ca^{2+} -dependent stimulation of phospholipase C-beta1 in vitro. *Recept Signal Transduct* **6**:87–98.
- Liu F, Ma XH, Ule J, Bibb JA, Nishi A, Demaggio AJ, Yan Z, Nairn AC, and Greengard (2001) Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. *Proc Natl Acad Sci USA* **98**:11062–11086.
- Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC, and Greengard P (2000) Amplification of dopaminergic signaling by a positive feedback loop. *Proc Natl Acad Sci USA* **97**:12840–12845.
- Pacheco MA and Jope RS (1997) Comparison of [^3H]phosphatidylinositol and [^3H]phosphatidylinositol 4, 5-bisphosphate hydrolysis in postmortem human brain membranes and characterization of stimulation by dopamine D_1 receptors. *J Neurochem* **69**:639–644.
- Panchalingam S and Undie AS (2001) SKF83959 exhibits biochemical agonism by stimulation [^{35}S] GTP γ S binding and phosphoinositide hydrolysis in rat and monkey brain. *Neuropharmacology* **40**:826–837.
- Ryu SH, Kim U-H, Wahl MI, Brown AB, Carpenter G, Huang K-P, and Rhee SG (1990) Feedback regulation of phospholipase C- β by protein kinase C. *J Biol Chem* **265**:17941–17945.
- Smith DS, Hreer PL, and Tsai LH (2001) cdk5 on the brain. *Cell Growth Diff* **12**:277–283.
- Snyder GL, Fienberg AA, Haganir RL, and Greengard P (1998) A dopamine/ D_1 receptor/protein kinase A/dopamine-and cAMP-regulated phosphoprotein (Mr 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor. *J Neurosci* **18**:10297–10303.
- Soderling TS (2000) CaM-kinases: modulators of synaptic plasticity. *Curr Opin Neurobiol* **10**:375–380.
- Undie AS and Friedman E (1990) Stimulation of a dopamine D_1 receptor enhances inositol phosphates formation in rat brain. *J Pharmacol Exp Ther* **253**:987–992.
- Zhen X, Du W, Romano AG, Friedman E, and Harve JA (2001a) P38 Mitogen-activated protein kinase is involved in associative learning in rabbits. *J Neurosci* **21**:5513–5519.
- Zhen X, Zhang J, Johnson J, and Friedman E (2001b) D_4 dopamine receptor differentially regulates Akt/NF- κB pathways in D4MN9D cells. *Mol Pharmacol* **60**:857–864.
- Zhen XC, Uryu K, Wang HY, and Friedman E (1998) D_1 dopamine receptor agonists mediate activation of p38 mitogen-activated protein kinase and c-Jun amino-terminal kinase by a protein kinase A-dependent mechanism in SK-N-MC human neuroblastoma cells. *Mol Pharmacol* **54**:453–458.

Address correspondence to: Dr. Xuechu Zhen, Department of Physiology/ Pharmacology, City University of New York Medical School, 138th Street at Convent Avenue, New York, NY 10031. E-mail: xuechu@med.cuny.edu
