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Regulation of cdk5 and CaMK II by PI-linked dopamine receptor in rat brain

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Running title: PI-linked dopamine receptor and cdk5 and CaMK II pathways

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Abbreviation: cdk5, cyclin-dependent kinase 5; CaMK II, calcium/calmodulin-dependent protein kinase II; MAPK, Mitogen-activated protein kinase; PI, phosphatidylinositol; DARPP-32, dopamine and cAMP-regulated-phosphoprotein; CREB, cyclic AMP-responsive element-binding protein; IP_3, inositol trisphosphate.

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Abstract

A brain dopamine receptor that modulates phosphatidylinositol metabolism via the activation of PLCβ has been previously described. The present study aims to define the down stream signaling cascade initiated by the PI-linked dopamine receptor. Incubation of rat brain frontal cortical slices with SKF83959, a recently identified selective agonist of the PI-linked D_1-like dopamine receptor, elicited transient time-and dose-dependent stimulations of cdk5 and CaMK II activities. The stimulation of these kinases is blocked by 20 μM SCH23390 or the PLCβ antagonist, 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 D_1 –like dopamine receptor and PLCβ and is dependent on PKC and calcium. Although cdk5 and CaMK II is physically associated in native brain tissue, no change in this association was observed in response to SKF 83959 stimulation, or to the inhibition of either cdk5 by roscovitine or of CaMK, by 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 DARPP-32 (Thr-75) while CaMK II is responsible for the activation of CREB in response to SKF83959 stimulation. The present data provides 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.

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Introduction

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 D_1 receptor family (D_1, D_5) couple to Gs protein and activate the production of cAMP; and the D_2 receptor family ($D_{2\text{-}4}$) couples to Gi/G_0 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 indicate that activation of dopamine receptors also elevates intracellular calcium via the activation of phospholipase C_β (PLC $_\beta$), which hydrolyzes phosphatidylinositol (PI) into diacylglycerol and inositol trisphosphate (IP_3) (Felder et al., 1989; Undie and Friedman, 1990; Liu et al., 1992; Frail et al, 1993 et al., Pacheco and Jope, 1997; Lezano and Bergson, 2001). 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 appears to pharmacologically resemble the D_1 receptor family since it is selectively stimulated by agonists and inhibits by antagonists of the D_1 receptor (Felder et al., 1989; Friedman et al., 1997). SKF 83959 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^{++} and calcium sensing proteins such as calmodulin form functional

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complexes that interact with various proteins thus modulating their functions (Soderling, 2001). CaMKs, a serine/threonine protein kinase, is regulated by Ca^{++}/calmodulin. CaMKs 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 if 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 threonine 75 (Thr-75). Phospho-Thr-75 DARPP-32 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 (Thr-75), while 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 (Smith, Greer and Tsai, 2001; Dhavan and Tsai, 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 (Dhavan et al, 2002; Liu et al, 2001). The interaction between cdk5 and CaMK II was recently reported (Dhvan et al, 2002). However, the potential role of the PI-linked dopamine receptor in the regulation of cdk5 and CaMK II remains unknown.

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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 (Thr-75), a known substrate of cdk5, whereas activation of CaMK II results in the activation of CREB. The present study, therefore, provides new insights into the signaling pathways that transduce PI-linked dopamine receptor signals. These findings may, therefore, facilitate the unraveling of the functional role of the PI-linked dopamine receptor in brain.

Materials and Methods

Materials. SKF 83959 was kindly provided by the NIMH synthesis program (Menlo Park, CA 94025). R(+)-SCH-23390 hydrochloride, SKF 81297, SKF83599 were purchased from RBI (Natick, MA). PD98059, KN92, KN93 were from BioMol (Plymouth Meeting, PA). Antibodies to cdk5, CaMKs and horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotech. Inc (Santa Cruz, CA). U-73122, U-73343, Calphostin C, roscovitine, were obtained from Calbiochem (La Jolla, CA). CaMK assay kit was obtained from Upstate (Charlottesville, VA). Proteinase inhibitors were from Sigma (St. Louis, MO). Anti-phospho(Thr-75 and -34) DARPP-32, were from New England Biolab. Electrophoresis reagents were obtained from Bio Rad (Richmond, CA). [γ-^{32}P]-ATP (3000 Ci/mmol) was purchased from NEN (Boston, MA, USA). Other reagents were purchased from standard laboratory suppliers.

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Slice preparation and treatment. Brain slices of rat frontal cortex (FCX) were prepared as described (Zhen et al, 2001; Dhavan et al, 2002) using a McIllwain tissue chopper (Brinkmann Instruments, Westbury, NY). The prisms were washed in oxygenated Krebs-Ringer buffer (KRB). After equilibration at 37°C for 30 min, the slices were subsequently incubated with SKF 83959 or other reagents. Reactions were terminated by cooling on ice and followed by centrifugation. The tissues were sonicated and lysed in lysis buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 50 M NaF, 2 mM EGTA, 1 mM EDTA, 0.5 mM β-glycerophosphotate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethysulfoxide and proteinase inhibitor cocktail. The protein content of the supernatant was determined by the method of Broadford (BioRad). Aliquots of supernatants (300 μg) were immunoprecipitated with anti-cdk5, anti-CaMKII or IV anti-ERK2 antibody respectively, using a previously published method with minor modification (Zhen et al, 2002). 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 DTT, 100 μM [γ^{32}P]ATP (5 μCi), and substrate peptide (Dhvan 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 μM [γ^{32}P]ATP (5 μCi), and 0.2 mg/ml MBP as described previously (Zhen et al., 2001). The reaction was stopped on ice and 20 μl supernatant was spotted onto P81 paper and washed extensively in 0.425% phosphoric acid. Radioactivity incorporated into peptide

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was determined by scintillation counting. For CaMK assay, immunocomplexes of CaMK II or IV were washed with kinase assay buffer and reaction was conducted in presence of 10 μM [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 lyate was boiled in preparation buffer and 30μg 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 PBS (TBS), followed by incubation with anti-phospho-CREB (1:2000) for 2 hr. The membranes were washed and then incubated for 1 hr with species-specific HRP-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 4 X 10 min washes with 0.1 % TBS and the signals 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 (Snyder et al., 1998). Equal amounts of homogenate protein were loaded onto 12 % SDS-PAGE, the membrane were then probed with anti-phospho-DARPP-32 at Thr-34 (1:750) or anti-phospho-DARPP-32 at Thr-75 antibody respectively. For assessing the association of Cdk5 and CaMK II, three hundred μg of lysate protein was incubated with 3μg of anti-cdk5 antibody and incubated with shaking for 2 hours before addition of protein A/G PLUS. 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

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vice versa, CaMK II precipitates were separated by SDS-PAGE and anti-cdk5 antibody was used for detect the association between the kinases.

Measuring brain PI hydrolysis in brain slices in response to SKF83959 challenge. Brain slices were incubated with Kreb's bicarbonate buffer for 45 min with buffer replacement every 15 min. Tissues were then labeled with [^{3}H] myo-inositol (10 μci) for 1hour in presence of 20 mM Lithium chloride. Tissues were then incubated with SKF 83959 or antagonist SCH23390 for designated time. The reaction was stopped with 300 μl of 1M TCA. The 500 μl of TCA extraction was transferred to 2 ml eppendorff tubes. With addition of 10 MM EDTA and freon:tri-n-ethyllamine (3:1, v/v), samples were thoroughly mixed and centrifuged. Five hundred μl of the upper layer was taken in a glass tube containing 0.5 M NaHCO3 and 1 ml distilled water. After pass to the AG-1 x 8 column (0.75 ml), samples were washed extensively. Total IPs was then eluted and collected as described (Challiss et al., 1988). Results are expressed as cpm/mg protein.

Cell culture and treatment. PC12 cells that were transfected with D_1 dopamine receptor prepared as previously described (Zhen et al, 2002). Cells were seeded in 10-cm dishes. After reached 80-90% confluence, cells were cultured in low serum medium for 4 hours before incubating with SKF83959 or other drugs for indicated times. Cells were then harvested and lysates were prepared for kinase assay.

Data analysis. Data are expressed as mean ± SEM and analyzed by ANOVA followed by Newman-Keuls test unless otherwise indicated. Statistical significance was considered at $p<0.05$.

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RESULTS

SKF83959 stimulates cdk5 activity via D_1-like dopamine receptor in rat brain slices. Frontal cortical (FCX) slices were incubated with 20 μM 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 SKF 83959 activates cdk5 transiently. A dose-response analysis indicates that SKF83959 activated cdk5 activity at 10 μM, and peak activation occurred at 20 μM (Fig.1B). Since SKF83959 has been found to be a selective agonist of the PI-linked D_1 dopamine receptor, we therefore checked if activation of cdk5 is also mediated by the receptor. FCX slices were preincubated with 20 μM of the selective D_1 receptor antagonist, SCH23390 for 20 min before the stimulation of SKF83959. As shown in Figure 1C, SCH23390 almost completely blocked SKF 83959-stimulated cdk5, suggesting that the stimulation of cdk5 by SKF83959 is mediated via a D_1-like dopamine receptor. Moreover, we found that another D_1 receptor antagonist SKF83566 (100 nM) also blocked SKF83959- and SKF81297- , another D_1 selective agonist, stimulated cdk5 activity (Fig.1 D), whereas prazosin (1 μM), spiperone (10 μM) and mesulergine (10 μM) did not (data not shown).

Activation of cdk5 by SKF83959 is mediated by PLC_β and dependent on PKC and intracellular calcium. Since SKF 83959 is a selective agonist for the PI-linked D_1-like dopamine receptor and this receptor is known to couple to PLC_β/PKC/IP3 pathway, we tested if this signaling pathway is involved in cdk5 activation. Brain slices were pre-incubated with U-73122 (10 μM), U-73343 (10 μM), Calphostin C (1 μM), or BAPTA (10

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μM) for 20 min prior to the addition of SKF 83959 and cdk5 activity was determined. As shown in Figure 2A, the putative phospholipase C inhibitor, U-73122 blocked SKF 83959-induced cdk5 activation. In contrast, its inactive analogue, U-73343, did not interfere with activation of this kinase. Moreover, inhibition of PKC by Calphostin C or intracellular calcium release by BAPTA also attenuated cdk5 activation whereas PKA inhibitor has little effect (Fig. 2B). These results suggest that SKF83959-mediated activation of cdk5 is mediated via PLC_β and is dependent on PKC and Ca^{+2} but not on PKA. Posphorylation 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 μM PMA prior to 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, while activation of PKC by PMA significantly reduced carbachol- or NaF–stimulated cdk5 activation (Fig 3). Similarly, 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 D_1 dopamine receptor by SKF 83959 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 SKF 83959 and CaMK II and IV activities were measured in anti-CaMK II, or anti-CaMK IV immunoprecipitates. As shown in Fig. 4 (A&B), SKF83959 induced a transient activation

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of CaMK II in FCX slices. Similar to the result with cdk5 in Figure 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 μM of SKF83959, it reaches maximum at 20 μM. 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 experiment condition (data no shown). As expected, SCH23390 blunted the drug-induced activation of CaMK II. Another D_1 receptor antagonist SKF 83566 also blocked the SKF83959-induced CaMKII activation whereas D_2, α_1, or 5-$HT_{2A/2C}$ receptor antagonist did not (Fig 4C), Interestingly, stimulation of CaMK II was also mediated through PLCβ and dependent on PKC and intracellular calcium since U-73122, Calphostin C or BAPTA blocked the activation of CaMK II (Fig.4 D). Thus, it appears 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 D_1 –expressing PC12 cells. One may argue that classical D_1 dopamine receptor contributes to the activation of cdk5 and CaMK II since SKF83959 was originally shown to have a high affinity for the D_1 dopamine receptor and the FCX is enriched with this receptor. We, therefore, tested if cdk5 and CaMK II activation in brain slices is mediated by the D_1 dopamine receptor. PC12 cells that stably express the D_1 dopamine receptor were incubated with either 1 μM SKF 83959 or with the selective D_1 receptor agonist, SKF 81297 and kinase activities were assessed. As shown in Fig. 5A**,** activation of D_1 receptors by SKF81297 did not affect cdk5 or CaMKII activity although this treatment resulted in a dramatic stimulation on cAMP (data

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not shown; also see 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 CaMKII activity in the cells (Fig.5C). Our previous study demonstrated that both SKF 83959 and SKF 81297 do not stimulate PI hydrolysis in these cells (see Jin, et al 2003), but do so in brain slices (Fig.5 D and Jin et al, 2003). Indicating that classical D_1 dopamine receptors are not involved in SKF83959-mediated activation of cdk5 and CaMK II in brain slices.

Activation of cdk5 and CaMK II by SKF 83959 is independent to each other. As shown above, activation of cdk5 and CaMK II by SKF 83959 share a similar time course. A previous study also indicated that the cdk5 activators, p35 and p39, interact with the alpha-subunit of Ca^{2+}/CaMKII. We therefore tested if activation of the two kinases by SKF83959 is interrelated. Brain slices were pretreated with 50 μM KN 93, a selective inhibitor of CaMK II or its negative control, KN92 prior to the addition of SKF 83959. Inhibition of CaMK II did not alter SKF 83959-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.6 C), suggesting that cdk5 or CaMK II activity may not be required for their association. It is therefore clear that while SKF 83959-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 SKF 83959. CaMKs are known to phosphorylate CREB at ser-

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133 and this activates CREB. We first check if CREB phosphorylation is altered by SKF83959 in FCX slices. As shown in Fig. 7A, SKF83959 activated CREB transiently; phosphorylation of CREB (ser-133) 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 pre-incubated with SCH23390, U-73122, U-73343 for 20 min prior to the addition of SKF 83959. Activation of CREB was blunted by SCH23390 or U-73122, but not by U-73343 (Fig. 7B), indicating that CREB activation by SKF 83959 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 SKF-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 SKF 83959, whereas cdk5 is not.

We next examined the potential target of cdk5 activation by SKF83959. DARPP-32 phosphorylation in response to SKF 83959 stimulation in FCX slices was analyzed using specific anti-phospho-DARPP-32 antibodies (Thr-34, and Thr-75). As shown in Fig. 7D, SKF 83959 induced a significant increase in the level of phospho-DARPP-32 (Thr-75) whereas no significant change in phospho- DARPP-32 (Thr-34) was observed. Moreover, Roscovitine, a selective inhibitor for cdk5, largely attenuated SKF83959-induced phosphorylation of DARPP-32 at Thr-75, indicating that cdk5 activation contributes to SKF83959-mediated Thr-75 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.

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Discussion

The present studies demonstrate that SKF 83959 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 appears 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 PLCβ and is dependent on PKC and intracellular calcium release, since 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 (Th-75) and of CREB that are associated with cdk5 and CaMKII activation. To our knowledge, this is the first report that describes the detailed signaling cascades that are activated by the PI-linked D_1 dopamine receptor.

Although previously a number of reports described that Gαq couples the dopamine receptor to PLC (Felder et al., 1989; Undie and Friedman, 1990; Liu et al., 1992; Frail et al, 1993 et al., Pacheco and Jope, 1997; Lezano And Bergson, 2001), the recent identification of a selective agonist, SKF 83959, (Panchalingam & 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 D_1 dopamine receptor differentially regulates the cdk5 and CaMK II pathways. The conclusion

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is based on the following observations: 1) the selective D_1 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) SKF 83959 did not stimulate PI hydrolysis (Jin et al, 2003) nor did its activate cdk5 or CaMK II (Fig. 5) in D_{1A} expressing PC12 cells indicating that the –stimulation of cdk5 or CaMK II by SKF 83959 in brain slices is not mediated by the classical Gαs -coupled D_{1A} dopamine receptor, 4) the D_1 dopamine receptor agonist, SKF81297 did not stimulate cdk5 and CaMK II activities in D_{1A}/ PC12 cells although it induced a dramatic stimulation of cAMP/PKA, further confirming that activation of the classical Gαs -coupled D_1 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 (Tsai et al., 1994) and the association is essential for its activation (Lew et al., 1994; Tang et al., 1995). Cdk5 has been demonstrated to play an important role in neurite outgrowth and migration (Nikolic et al., 1996; Zheng et al., 1998; Chae et al, 1997; Ohshima et al, 1996; Dhavan and Tsai, 2001), and in neuroplasticity (Fischer et al., 2003). While 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 recently identified. 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 D_1-and D_2- like dopamine receptors was shown to regulate DARPP-32

phosphorylation. Phosphorylation of DARPP-32 at Thr-75 converts DARPP-32 into a potent PKA inhibitor and reduces phsphorylation of DARPP-32 at Thr-34 (Hemmings et al., 1989; Bibb et al, 1999). The latter is an inhibitor of protein phosphatase 1 (PP1). Dopamine D_1 receptor stimulation decreases the striatal phospho- DARPP-32 Thr-75 whereas D_2 receptor stimulation increases phosphorylated at this site probably via alteration in protein phosphatase-2A (Nishi A 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-indued change in levels of phospho-Thr-75 DARPP-32 in this brain region. Thus, our results reveal additional mechanism for dopamine receptor-regulated DARPP-32/PP1 pathway in brain. It is interesting to note that stimulation of metabotropic glutamate receptors (mGluRs) also results in a transient activation of cdk5 in brain slices. This activation of cdk5 is associated with mGluRs receptor agonist-mediated stimulation of phospho- DARPP-32 at Thr-75 (Liu et al, 2001), thus, implying that activation of Gq-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 MAP kinases, PKA and CaMKs (Kornhauser and Greenberg, 1997; Frey et al., 1990; Zhen et al., 1998; 2001; Jay, 2003). These protein kinases are able to phosphorylate the cAMP regulatory element binding protein (CREB) at ser-133 and CREB is considered a central transcription factor in the formation of memory. The present data demonstrate that the PI-linked dopamine receptor is

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involved in regulating activation of CREB via CaMK II indicates the potential role of this receptor in neuroplasticity.

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Footnotes

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Figure legends

Figure 1. Stimulation of the PI-linked D_1 dopamine receptor by SKF83959 induces a transient activation of cdk5 in rat brain slices. Frontal cortex (FCX) slices were incubated either with 20 μM SKF 83959 for 2-30 min (**A**), or with 1, 10, 20 or 100 μM SKF 83959 for 2 min (**B**). Cdk5 activity was assayed in cdk5 immnoprecipitates of tissue lysates. * $p<0.01$, compared to control. **C**: FCX slices were preincubated for 20 min with vehicle or with 20 μM of SCH 23390 prior to the addition of 10 or 20 μM of SKF 83959 and the cdk5 activity was measured. **D.** Slices were preincubated with either SKF83566 (100nM) or vehicle for 20 min prior to the addition of SKF81297 or SKF83959. *$p<0.01$, student *t*–test, compared to respective SKF83959-treated group. The results are expressed as fold change from control and presented as Mean± SD obtained from at least three independent experiments. SKF: SKF83959; SCH: SCH23390.

Figure 2. Activation of cdk5 by SKF 83959 is dependent on PKC and intracellular calcium and is mediated via PLCβ but not PKA. A: Frontal cortex (FCX) slices were incubated with 10 μM U-73122, 10 μM U-73343 (dissolved in DMSO) prior to the additional 20 μM SKF 83959. Cdk5 activity was assayed in cdk5 immnoprecipitates. **B**.FCX slices were incubated with 1 μM PKC inhibitor, calphostin C, 10 μM BAPTA, or 5 μM PKA inhibitor, H89 prior to the addition of 20 μM SKF 83959. Cdk5 activity was assayed as described. The results are expressed as fold change from control and presensted as Mean± SD obtained from at least three independent experiments. SKF: SKF 83959; Cal.C: calphostin C. *$p<0.01$, student *t*-test, compared to respective control.

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Figure 3. PKC stimulation attenuates SKF 83959-stimulated cdk5 activity. Brain slices were incubated with 1μM PMA for 20 min prior to the addition of 20μM SKF 83959, 20 mM NaF or 100 μM carbachol. Tissue was homogenized and Cdk5 activity was assayed. Data are expressed as fold change over control and summarized as Mean± SD of four independent experiments. Con. Control (0.1% DMSO); SKF: SKF83959; Carb: carbachol; NaF: sodium fluoride. * $p< 0.01$ compared to respective non-PMA control.

Figure 4. SKF83959 induced a transient activation of CaMK II in rat brain slices. Frontal cortex (FCX) slices were incubated with either 20 μM SKF 83959 for the indicated periods of times (**A**) or with 1, 10, 20 or 100 μM SKF 83959 for 2 min (**B**) before the tissue was collected and homogenized. CaMK II activity was assayed in CaMK II immnoprecipitates using peptide-γ as substrate as described in Methods. **C**. FCX slices were preincubated with vehicle or with 20 μM SCH 23390, 10 μM U-73122, 1 μM calphostin C (1 μM) or 20 μM BAPTA for 20 min before the addition of SKF 83959. Two min later the incubation was terminated, tissue was homogenized and enzyme activity was conducted as described in Methods. **D**. FCX slices were preincubated with vehicle or with 100nM 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 summarized as Mean± SD of 3 or 4 independent experiments. SCH: SCH23390; Cal.C: calphostin C; SKF: SKF 83959; BAPTA. * $p<0.01$, compared to control.

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Figure 5. Stimulation of D_{1A} dopamine receptor did not alter cdk5 or CaMK II activity in D_{1A}/PC12 cells. PC12 cells that stably express the D_{1A} dopamine receptor (D_{1A}/PC12) were incubated with either 10μM of the D_1 dopamine receptor agonists, SKF 38393 (**A**) or SKF 83959 (**B**) for the indicated times. Cells were then collected, lysed and kinase activity assayed in cdk5 or CaMK II immunoprecipitates. **C**. Cells were treated with 100 μM Carbachol for indicated times. **D**. FCX slices were labeled with [^{3}H] myo-inositol (10 μCi) for 1hour in presence of 20 mM Lithium chloride. Tissues were then incubated with 20 μM SCH23390 for 20 min before 30 min SKF 83959 stimulation. PI hydrolysis was measured as described in Methods and expressed as fold change from control. Data are presented as Mean± SD of three or four independent experiments. *p<0.01, compared to control; # P<0.01, compared to SKF 83959 stimulation. SCH: SCH23390; SKF: SKF83959.

Figure 6. Cdk5 and CaMK II are independently activated by SKF 83959. Frontal cortex **(**FCX) slices were incubated either with 50 μM KN 93 or KN92 or with various concentrations of roscovitine for 20 min prior to a 2 min incubation with 20 μM SKF 83959. The slices were lysed and kinase activities were assayed. **A**: cdk5 activity; **B**: CaMK II activity. Data are expressed as fold change over control (mean± SD) from 3- 4 independent experiments. * P< 0.01, compared to that of the respective control group by the student *t* test. **C**: Aliquots of supernatants (300 μg) were immunoprecipitated with anti-cdk5 antibody and Protein A/G PLUS was added and incubated for 2h. The precipitates were collected and subjected to SDS-PAGE electrophoresis. Following transferring, the membrane was probed with anti-CaMK IIα 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.

Figure 7. CaMK II and Cdk5 are responsible for SKF 83959-induced phosphorylation of CREB and DARPP-32 (Thr-75). A: Frontal cortex (FCX) slices were incubated with 20μM SKF83959 for the indicated times. The tissues were prepared for western blot analyses and probed with anti-phospho-CREB Ser 133 antibody. **B**. FCX slices were incubated either with 10 μM U-73122 or U-73343 or with 20 μM SCH23390 for 20 min prior to a 5 min additional incubation with 20 μM SKF 83959. Phospho-CREB ser-133 was detected in the slice lysates. **C**. FCX slices were incubated either with 50 μM KN 92, KN93, or with10 μM roscovitine for 20 min prior to the addition of 20 μM SKF 83959 and an additional incubation for 5 min. Phospho-CREB ser-133 was probed in the lysate. **D**. FCX slices were incubated either with 50 μM KN93, or with10 μM roscovitine for 20 min prior to 20 μM SKF 83959 stimulation for an additional 5 min Phospho-DARPP-32 was detected in slice lysates using anti-phospho-DARPP-32 (Thr-75) or anti-phospho-DARPP-32 (Thr-34) antibodies. Each experiment was repeated at least three times. Representative blots are shown.

Figure 1

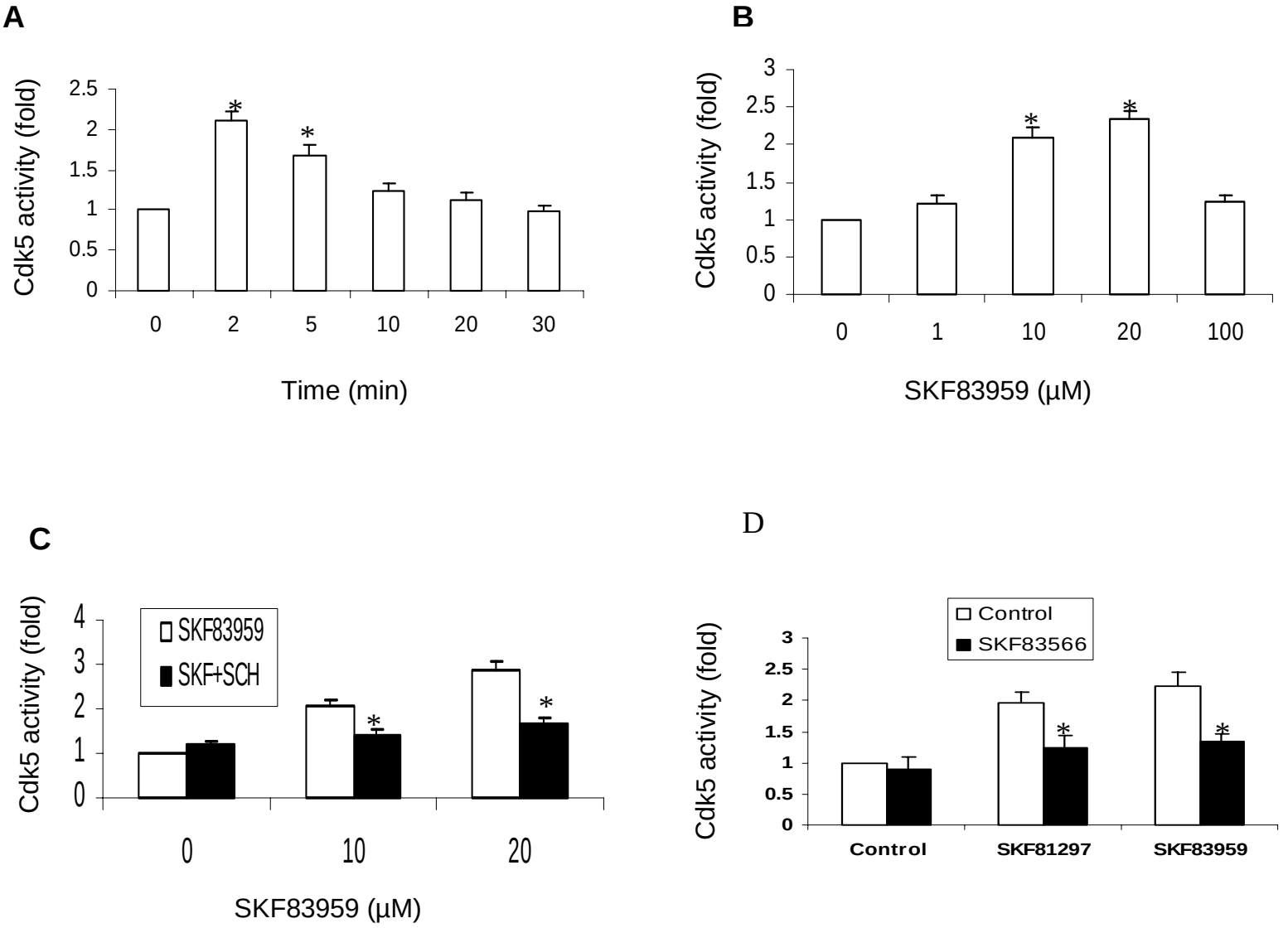

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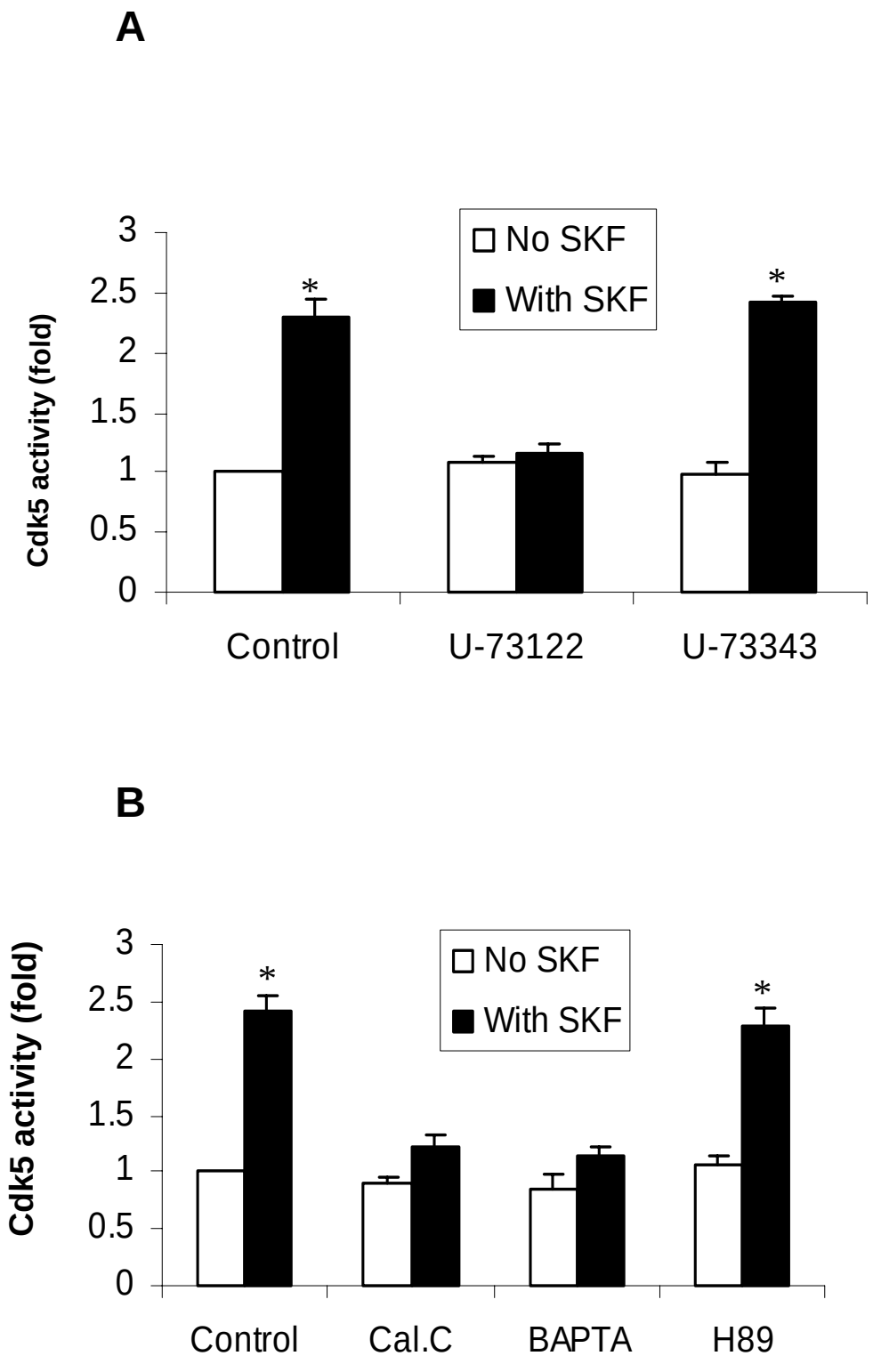
Figure 2



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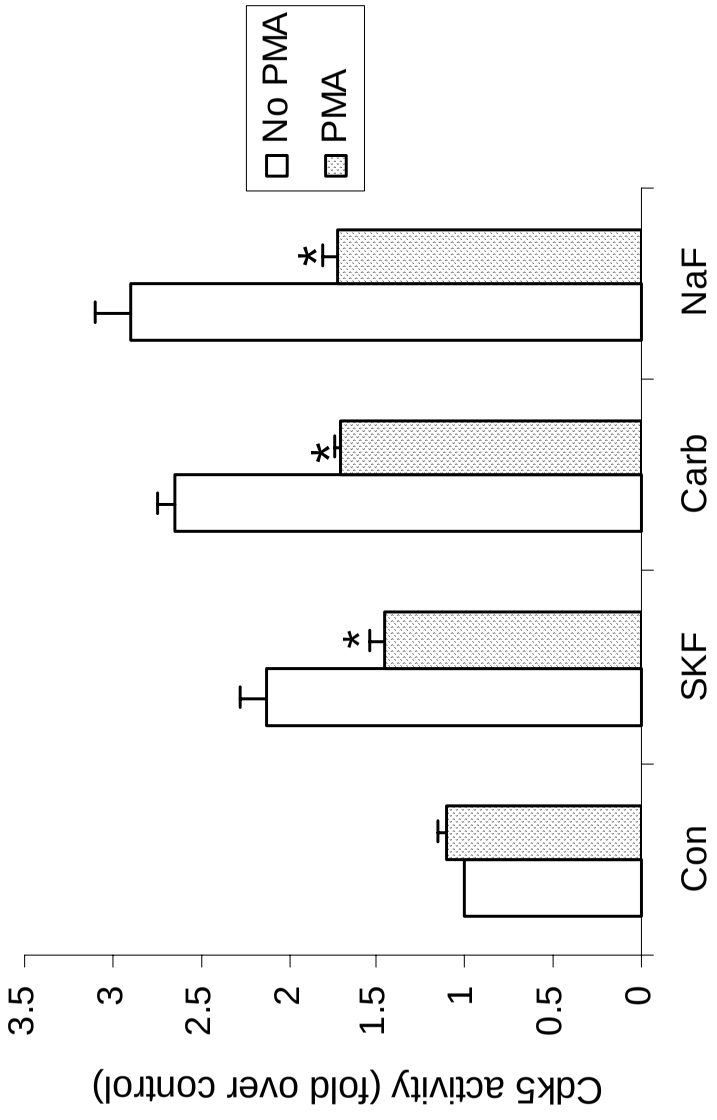

Figure 3

Figure 4

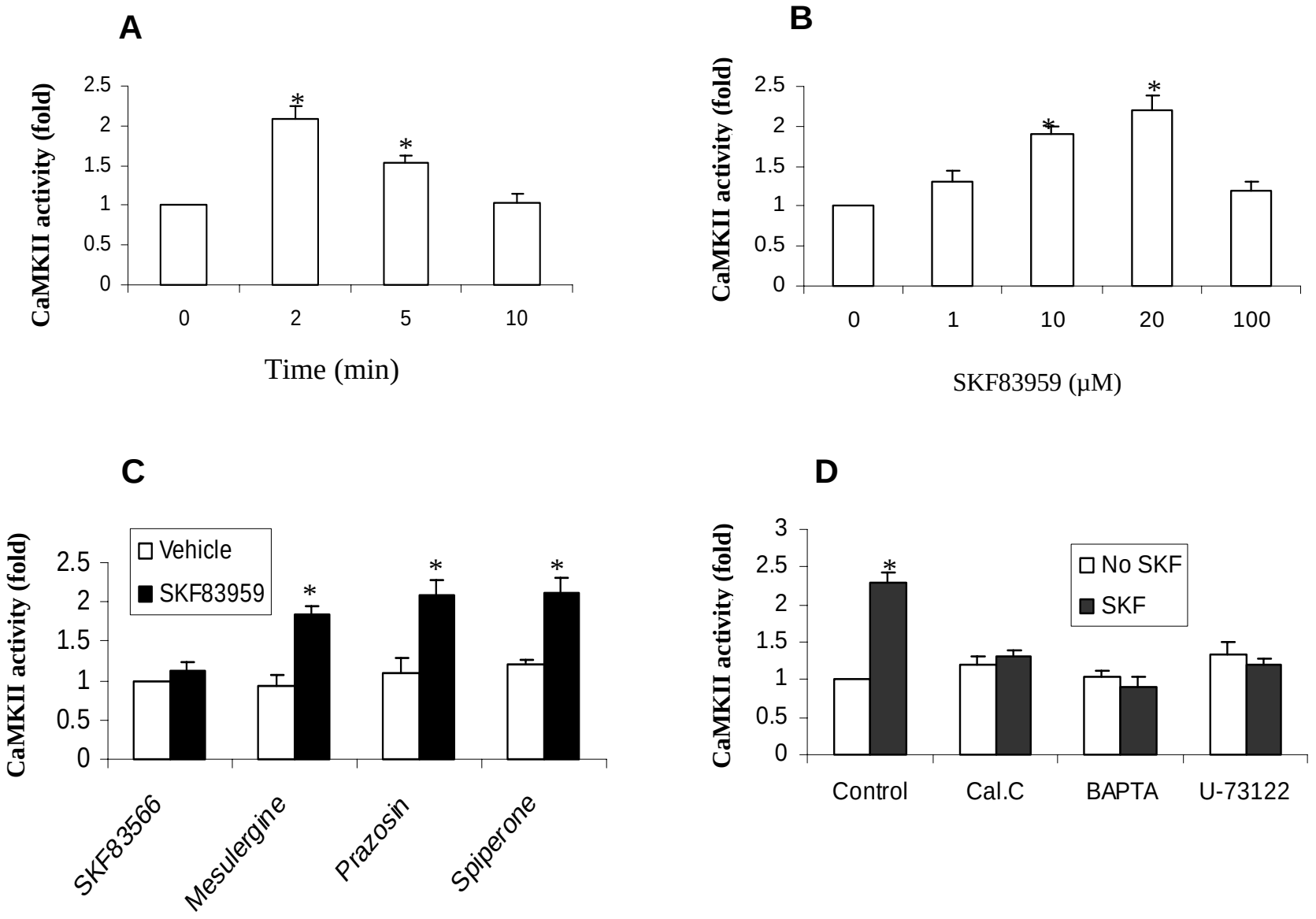

Figure 5

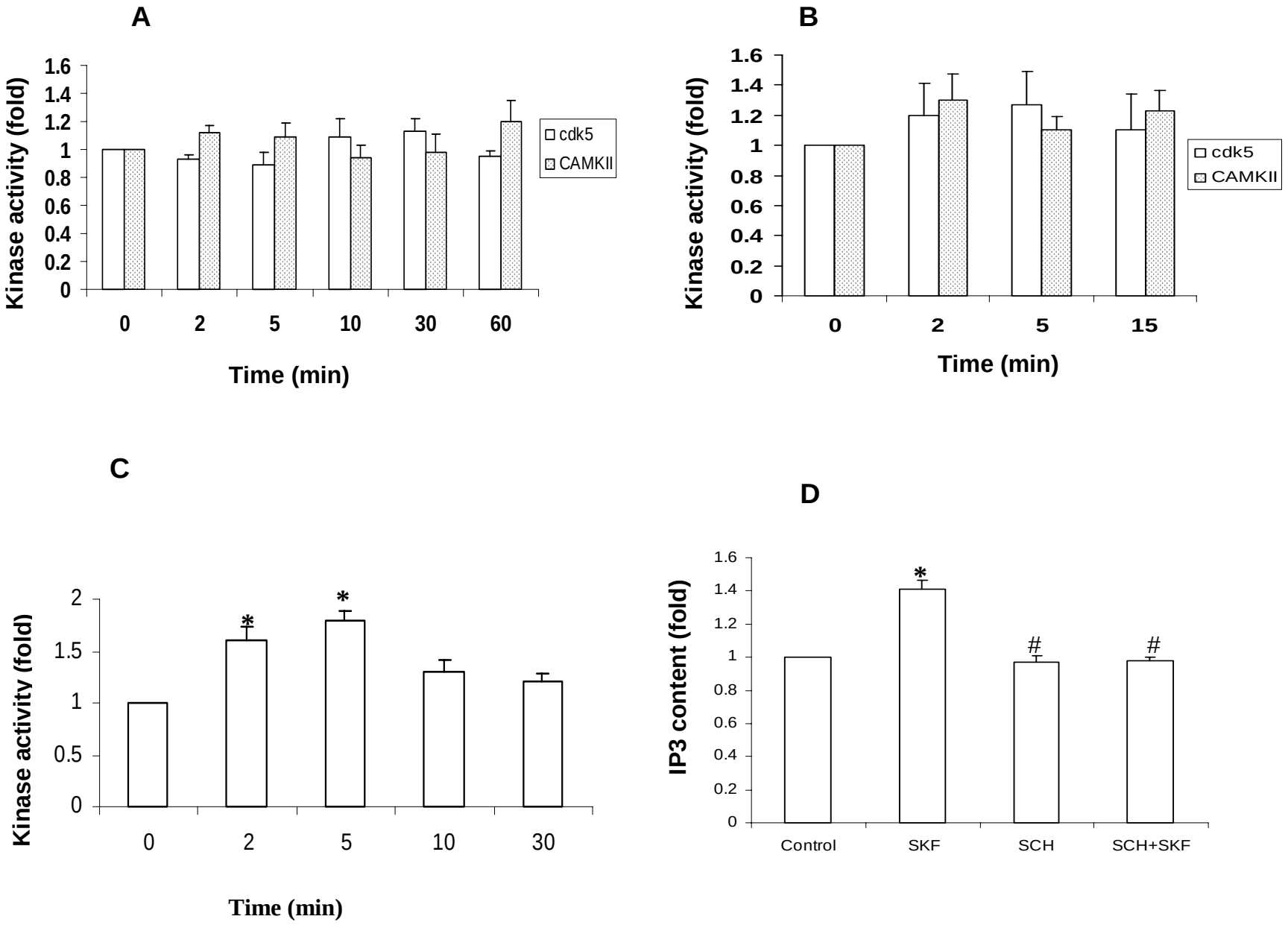

Figure 6

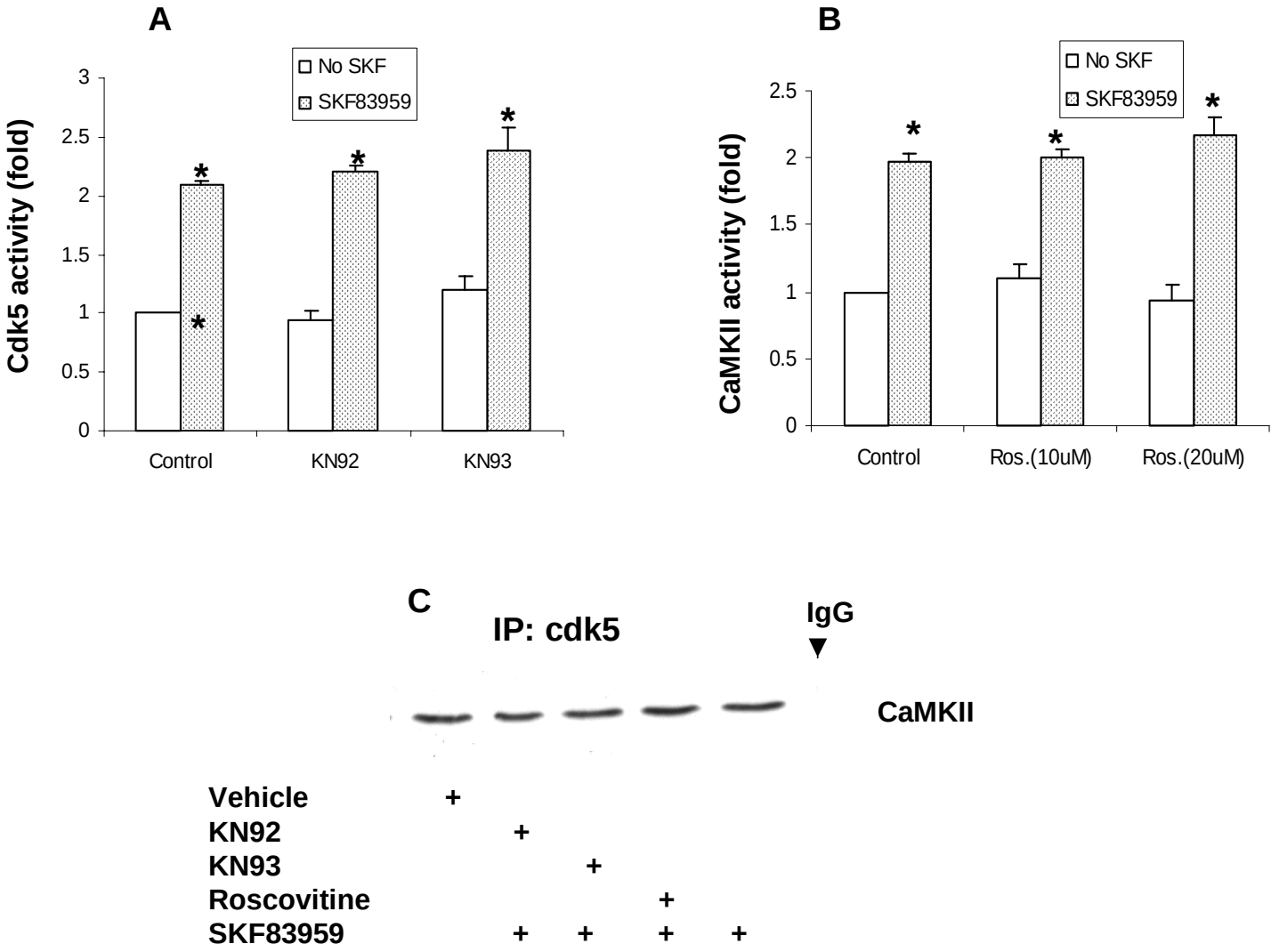

Figure 7