Dopamine Transporter Activity Mediates Amphetamine-Induced Inhibition of Akt through a Ca^{2+}/Calmodulin-Dependent Kinase II-Dependent Mechanism

Y. Wei, J. M. Williams, C. Dipace, U. Sung, J. A. Javitch, A. Galli, and C. Saunders

Departments of Pharmacology (U.S., C.S.) and Molecular Physiology and Biophysics (Y.W., J.M.W., C.D., A.G.), Center for Molecular Neuroscience, Vanderbilt University, Nashville, Tennessee; and Departments of Psychiatry and Pharmacology, Center for Molecular Recognition, College of Physicians and Surgeons, Columbia University, New York, New York (J.A.J.)

ABSTRACT

The primary mechanism for clearance of extracellular dopamine (DA) is uptake mediated by the dopamine transporter (DAT), which is governed, in part, by the number of functional DATs on the cell surface. Previous studies have shown that amphetamine (AMPH) decreases DAT cell surface expression, whereas insulin reverses this effect through the action of phosphatidylinositol 3-kinase (PI3K). Therefore, it is possible that AMPH causes DAT cell surface redistribution by inhibiting basal insulin signaling. Here, we show in a heterologous expression system and in murine striatal synaptosomes that AMPH causes a time-dependent decrease in the activity of Akt, a protein kinase immediately downstream of PI3K. This effect was blocked by the DAT inhibitor cocaine, suggesting that AMPH must interact with DAT to inhibit Akt. We also showed that AMPH is able to stimulate Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity, both in the heterologous expression system as well as in murine striatal synaptosomes. The ability of AMPH to decrease Akt activity was blocked by the CaMKII inhibitor 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN93), but not by its inactive analog 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN92). Furthermore, preincubation with KN93 prevented the AMPH-induced decrease in DAT cell surface expression. Thus, AMPH, but not cocaine, decreases Akt activity through a CaMKII-dependent pathway, thereby providing a novel mechanism by which AMPH regulates insulin signaling and DAT trafficking.

Dopaminergic neurotransmission is governed in part by the reuptake of extracellular DA via the dopamine transporter (DAT). Because DA uptake capacity is dependent on the DAT turnover rate, the affinity of the transporter for DA, and the number of functional transporters at the cell surface, regulation of DAT cell surface expression is important for tuning DA neurotransmission (Beckman et al., 1998; Robinson, 2001; Kahlig and Galli, 2003). The psychostimulant amphetamine (AMPH) causes DAT to redistribute away from the cell surface, decreasing DA clearance efficiency and providing an additional mechanism by which this DAT substrate increases extracellular DA (Sulzer et al., 2005).

A variety of receptor signal transduction pathways have been shown to regulate neurotransmitter transporter trafficking as well as activity (Beckman et al., 1998; Blakely and Sung, 2000; Robinson, 2001; Kahlig and Galli, 2003; Blakely et al., 2005). For example, Apparsundaram et al. (1998, 2001) demonstrated that protein kinase C activation leads to a loss in activity and cell surface expression of the norepinephrine transporter. In addition, protein kinase C activation is associated with the rapid redistribution of hDAT away from the cell surface in both native neuronal and heterologous expression systems (Pristupa et al., 1998; Daniels and Amara, 1999; Granas et al., 2003; Loder and Melikian, 2003; Sorkina et al., 2003). It is noteworthy that tyrosine kinases, which are activated by insulin and insulin-like growth factor 1, also regulate DA clearance (Doolen and Zahniser, 2001; Carvelli et al., 2002; Garcia et al., 2005). DAT cell surface expression and activity are reduced by tyrosine kinase inhibitors (Doolen and Zahniser, 2001) and by inhibitors of kinases

ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; AMPH, amphetamine; h, human; PI3K, phosphatidylinositol 3-kinase; GSK, glycogen synthase kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; COC, cocaine; CTR, vehicle; KN93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; KN92, 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; ANOVA, analysis of variance.

activated by insulin signaling, including phosphatidylinositol 3-kinase (PI3K) and Akt (Carvelli et al., 2002; Garcia et al., 2005). These data support the hypothesis that DA clearance is regulated by tonic activity of the insulin pathway (Carvelli et al., 2002; Garcia et al., 2005; Owens et al., 2005). Indeed, in animal models of diabetes, DA uptake is compromised (Owens et al., 2005). Consequently, stimulation of the insulin signaling pathway may afford a way to regulate DAT plasma membrane expression. Moreover, insulin stimulation blocked the AMPH-induced decrease in DAT cell surface expression (Carvelli et al., 2002; Garcia et al., 2005). This "insulin rescue" is mediated by activation of PI3K and Akt (Carvelli et al., 2002; Garcia et al., 2005).

Protein kinase B/Akt functions as an important regulator of cell survival, growth, apoptosis, and glucose metabolism (Hanada et al., 2004). Each of the three Akt isoforms contains a pleckstrin homology domain that interacts with membrane lipid products of PI3K (Wymann and Pirola, 1998; Hanada et al., 2004). This interaction is required for Akt activation (Hanada et al., 2004). Akt is phosphorylated at two sites, Thr-308 in the activation loop of the catalytic domain and Ser-473 in the C-terminal regulatory domain (Hanada et al., 2004). Upon activation, Akt is translocated to the cell membrane, where it is anchored to the membrane via its pleckstrin homology domain. A major target of active Akt is glycogen synthase kinase (GSK)3, the activity of which is regulated by phosphorylation.

We have shown that activation of Akt via insulin signaling blocks AMPH-induced DAT cell surface redistribution away from the plasma membrane (Garcia et al., 2005). Furthermore, inhibition of Akt activity mimics the effects of AMPH on DAT surface localization (Garcia et al., 2005). Therefore, under basal insulin signaling, AMPH might inhibit Akt via a transporter-mediated mechanism, and as a consequence, lead to DAT cell surface redistribution. The first preliminary evidence that AMPH inhibits Akt via a receptor-independent mechanism was provided by Garcia et al. (2005). Recently, the Caron group demonstrated a DA D2 receptor-mediated inhibition of Akt for compounds able to increase extracellular DA such as AMPH (Beaulieu et al., 2005). This novel mechanism is mediated by recruitment of β-arrestin and the phosphatase PP2A, which leads to dephosphorylation and inactivation of Akt. In contrast, a mechanism for stimulation of Akt by D2 receptor mediated via extracellular signal-regulated kinase 1/2 has also been proposed (Brami-Cherrier et al., 2002). Here, we provide evidence that in addition to these two mechanisms, AMPH inhibits Akt through a novel DAT-dependent mechanism mediated by stimulation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) function. In addition, this CaMKII stimulation is essential for AMPH-induced DAT cell surface redistribution.

Materials and Methods

Cell Culture

Human embryonic kidney-293 cells were stably transfected with yellow fluorescent protein-tagged hDAT (hDAT cells) as described previously (Ferrer and Javitch, 1998; Saunders et al., 2000; Kahlig et al., 2004). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Preparation of Synaptosomes

Mice were housed in a colony room maintained at constant temperature (37°C) and humidity for 1 week before the experiments. Mice had free access to food and water, and a 12:12-h light/dark cycle was used. Mice were killed by decapitation, and their brains were removed and placed in an ice-cooled dish for dissection. The striatum was dissected and homogenized in ice-cold Krebs-Ringer buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 10 mM glucose, pH 7.4) containing 0.32 M sucrose using a glass homogenizing tube and a Teflon pestle. After centrifugation at 1000g for 10 min at 4°C, the pellet was discarded, and the supernatant was centrifuged at 16,000g for 25 min at 4°C. The resulting P2 pellet was placed on ice and was resuspended immediately before the experiments.

Phospho-Akt Detection and Immunoblotting

hDAT cells were seeded into six-well plates (10^6 cells/well) approximately 24 h before the experiment. Cells were then treated with 10 μM AMPH (d-amphetamine sulfate; Sigma-Aldrich, St. Louis, MO) for the indicated times. The reactions were terminated on ice by removal of drug and addition of lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were separated on SDS-polyacrylamide gel electrophoresis (10%) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Immunoblots were obtained by using antibodies against total Akt, phospho-Akt Ser-473 (1:500), and phospho-Akt Thr-308 (1:200) (Cell Signaling Technology Inc., Danvers, MA). Immune complexes were revealed by using horseradish peroxidase-conjugated goat anti-rat or anti-rabbit antibodies (1:5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with a chemoluminescent reagent for visualization (ECL-Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK) on Hyperfilm ECL film (GE Healthcare). Band densities were quantitated using Scion Image software (Scion Corporation, Frederick, MD) and normalized to the appropriate total extract to control for protein loading. Each immunoblot was performed a minimum of three times.

Assay of Akt Activity

The Akt activity assay kit (BioVision, Mountain View, CA) uses an Akt-specific antibody for immunoprecipitation of Akt from cell lysates, and activity of the immunoprecipitated kinase is measured in vitro using exogenously added recombinant GSK3α as the substrate. The resulting phosphorylated GSK3α is determined by immunoblotting. hDAT cells were seeded at 1.0×10^6 cells in six-well plates for 24 h before treatment. hDAT cells or murine striatum synaptosomes (~400 μg of protein) were starved with Dulbecco's modified Eagle's medium for 1 h without fetal bovine serum and antibiotics. Cells/synaptosomes were then exposed to the indicated treatments in Dulbecco's modified Eagle's medium followed by washing twice with 4°C phosphate-buffered saline-Ca^{2+}-Mg^{2+} before lysis. Cells/synaptosomes were lysed at 4°C for 45 min. Protein assays were performed with cell lysates by using the Bio-Rad DC Protein Assay kit (Bio-Rad). Protein (~400 μg) from cell lysates was immunoprecipitated with anti-Akt monoclonal antibody from the Akt assay kit. The Akt kinase assay was performed as per the manufacturer's protocol. Phospho-specific antibodies to GSK3α (Ser-21) were used to detect phosphorylated protein. Immunoblotting and data analysis were performed as described above.

CaMKII Activity Assay

CaMKII Activity as Measured by Anti-Phospho-CaMKII Antibody. hDAT cells were seeded into six-well plates approximately 24 h before the experiment (60–80% confluent). After 1 h of serum starvation in Krebs-HEPES-Ringer-Tris buffer containing 120 mM NaCl, 4.7 mM KCl, 10 mM HEPES, 5 mM TRIZMA base, 2.2 mM

CaCl$_2$, and 10 mM dextrose with 100 μM ascorbic acid, the cells were treated with 10 μM AMPH in Krebs-HEPES-Ringer-Tris buffer at 37°C as a function of time. The plates were removed from the incubator and placed on ice, where they were washed with 4°C PBS/Ca^{2+}-Mg^{2+} and then lysed with 500 μl/well of lysis buffer (containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1% SDS, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 μM mycrocystin, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) by rocking the cells on ice for 1 h. The cells were then scraped with a rubber policeman and centrifuged at 16,000g for 30 min at 4°C. The supernatants were collected, assayed for protein, boiled with Laemmli sample buffer for 10 min, and loaded onto 10% gels for SDS-polyacrylamide gel electrophoresis. The gels were subsequently transferred to polyvinylidene difluoride and probed either with anti-phospho-CaMKII antibody (1:2000; catalogue no. ab3908, Abcam Inc., Cambridge, MA) or with anti-CamKII antibody (1:100; catalog no. ab19223-5, Abcam Inc.) followed by donkey anti-rabbit peroxidase-conjugated secondary antibody, and detected with a chemoluminescent reagent. Densitometric analysis was carried out within the linear range using Scion Image software (Scion Corporation) and normalized for protein.

CaMKII Activity as Measured Using a Synthetic Substrate. The ability of CaMKII to phosphorylate autocamtide II, a synthetic and specific substrate, was evaluated with a commercial kit purchased from Upstate Biotechnology (Lake Placid, NY). This assay allows for quantitation of Ca^{2+}-independent phospho-CaMKII activity. The assay kit is designed to measure the phosphotransferase activity of phospho-CaMKII in crude cell lysates, and it is based on phosphorylation of specific substrate peptide (KKALRRQETVDAL) by the transfer of [γ-^{32}P]ATP by phospho-CaMKII. The phosphorylated substrate is then separated from the residual [γ-^{32}P]ATP using P81 phosphocellulose paper and quantitated by using a scintillation counter.

Results

AMPH Reduces Phospho-Akt in hDAT Cells. Akt is activated by phosphorylation at two key regulatory sites: Thr-308 in the activation loop of the catalytic domain and Ser-473 in the COOH-terminal regulatory domain. There-fore, we measured Akt activity by assaying its phosphorylation at Thr-308 as well as Ser-473 in human embryonic kidney-293 cells stably expressing yellow fluorescent protein-tagged hDAT (hDAT cells). The AMPH-induced decrease in Akt activity (Fig. 1) was significant after 30 to 40 min of AMPH exposure as assessed by quantitation of the phosphorylation at either the catalytic site (Fig. 1B) or the regulatory domain (Fig. 1C). This inhibition of Akt persisted for at least 60 min (Fig. 1, B and C). The total level of Akt was unaffected during the 60-min time period studied (Fig. 1A).

We obtained the IC$_{50}$ for AMPH inhibition of Akt by exposing hDAT cells to different concentrations of AMPH and immunoblotting for phospho-Ser-473 (Fig. 2A). The IC$_{50}$ was calculated by nonlinear regression analysis of the data from the quantitation of immunoblots of phospho-Ser-473 (Fig. 2B; IC$_{50}$ = 7.57 $\pm$ 0.86 μM; n = 3). This effective concentration of AMPH is consistent with animal studies demonstrating that administration of AMPH at doses reported to cause hyperactivity and stereotyped behaviors leads to extracellular AMPH concentrations equal to or higher than 10 μM (Clausing et al., 1995). Thus, we chose a concentration of 10 μM AMPH for subsequent studies with pharmacological blockers of DAT.

AMPH Reduces Phospho-Akt in Murine Striatal Synaptosomes. The striatum is highly enriched in DAT (Javitch et al., 1983) and insulin receptors (Hill et al., 1986). In addition, Banks and Kastin demonstrated that the blood-brain barrier is permeable to insulin (Banks and Kastin, 1998). Moreover, insulin signaling has been shown to regulate DA clearance in this brain region (Owens et al., 2005). Using murine striatal synaptosomes, we explored whether the ex vivo application of AMPH inhibits Akt activity. Similar to the results in the heterologous expression system, at 30 min we observed a significant decrease in Akt activity as assessed by reduced phosphorylation of Thr-308 in the catalytic domain (Fig. 3). Thus, both in a heterologous expression
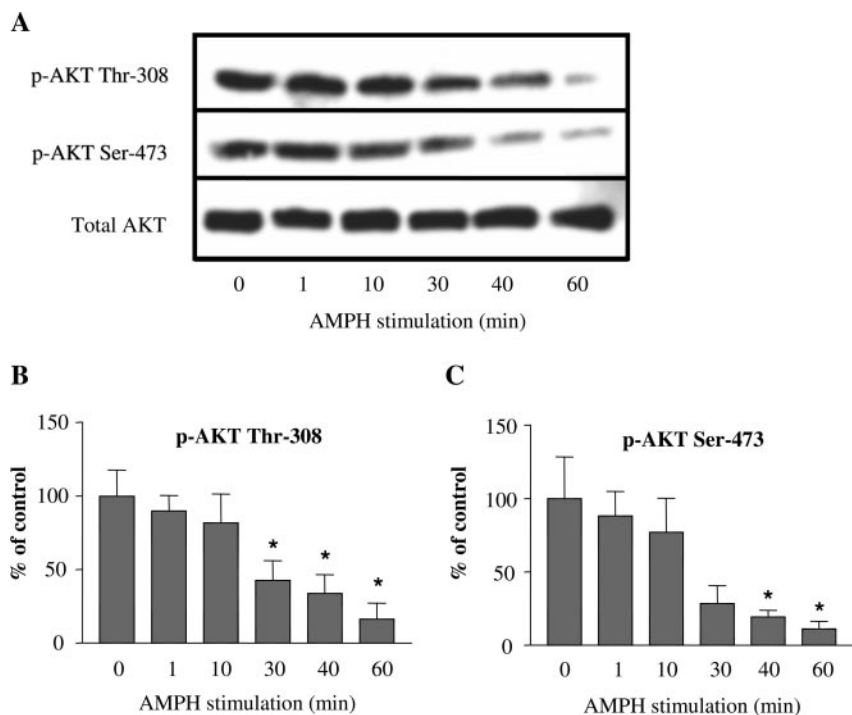

A

p-AKT Thr-308

p-AKT Ser-473

Total AKT

0   1   10   30   40   60

AMPH stimulation (min)

B   **C**

p-AKT Thr-308   p-AKT Ser-473

Fig. 1. AMPH induces a time-dependent decrease in phospho-Akt. A, immunoblots of phospho-Akt Thr-308, phospho-Akt Ser-473, and total Akt from extracts obtained from hDAT cells treated either with vehicle or with 10 μM AMPH for the indicated time. B and C, evaluation of band densities of the immunoblots in A by using Scion Image software. Band densities of the phospho-Akt Thr-308 (n = 4) and phospho-Akt Ser-473 (n = 3) were normalized to their respective total protein extract (Total Akt) to correct for differences in cell seeding and then expressed as a percentage of vehicle-treated control. Data are expressed as means $\pm$ S.E.M. (one-way ANOVA followed by Dunnett's post hoc test; *, $p < 0.05$ relative to vehicle treatment).

system and in a neuronal preparation, AMPH inhibits Akt activity.

Cocaine Blocks the AMPH-Induced Decrease of Akt Activity in hDAT Cells and in Murine Striatal Synaptosomes. We wanted to determine whether the ability of the DAT substrate AMPH to decrease Akt activity is mediated by its action at DAT. To assess Akt activity in these studies, we measured its ability to phosphorylate GSK3α (see *Materials and Methods*). With this methodology, a reduction in Akt activity results in decreased phosphorylation of GSK3α. As seen in Fig. 4A, 10-min pretreatment of hDAT cells with 10 μM cocaine before addition of 10 μM AMPH in the continued presence of cocaine (COC + AMPH) blocked the ability of AMPH to inhibit Akt activity. Cocaine by itself had no effect on Akt activity (COC).

Likewise, in murine striatal synaptosomes, a 10-min pretreatment with 10 μM COC before addition of 10 μM AMPH in the continued presence of cocaine (COC + AMPH) blocked the ability of AMPH to decrease Akt activity (Fig. 4B). Again, cocaine by itself was without effect. In contrast, as seen in Fig. 4C, when cocaine was added after treatment with AMPH, cocaine was unable to block the AMPH-induced decrease in Akt activity (AMPH + COC). Thus, the ability of cocaine to block AMPH-induced inhibition of Akt activity results from its blockade of AMPH transport by DAT and not on a DAT-independent rescue of Akt signaling.

Acute AMPH Activates CaMKII. Repetitive administration of AMPH has been shown to activate kinases such as CaMKII in cell lines as well as in vivo (Iwata et al., 1997a;

Kantor et al., 1999; Tan, 2002). Acute administration of AMPH has been shown to increase intracellular calcium (Gnegy, 2003; Kantor et al., 2004). Elevations in intracellular calcium could activate CaMKII activity (Hudmon and Schulman, 2002). In an attempt to identify signaling pathways triggered by the actions of AMPH at DAT that might lead to inhibition of Akt activity, we explored the ability of AMPH to activate CaMKII. Using a phospho-specific antibody raised against a synthetic phospho-peptide corresponding to amino acid residues surrounding the phosphorylated Thr-286 (the autophosphorylation site associated with CaMKII activation), we determined that AMPH activates CaMKII in hDAT cells and in murine striatal synaptosomes. Figure 5A shows an immunoblot of Thr286 phospho-CaMKII (top lane) and total CaMKII (bottom lane) after treatment of hDAT cells with 10 μM AMPH for different durations. The AMPH-induced increase in phospho-CaMKII was significant at 10 min and further increased at 30 min (Fig. 5B). In contrast, 10 μM DA failed to increase CaMKII autophosphorylation in hDAT cells (data not shown). To further confirm that AMPH stimulates CaMKII activity, we measured the Ca^{2+}-independent phosphotransferase activity of phospho-CaMKII in crude cell lysates obtained from hDAT cells treated for 30 min with AMPH (see *Materials and Methods*). In AMPH-treated cells CaMKII activity was increased 1.65 $\pm$ 0.14-fold [390,706 $\pm$ 95,014 and 662,823 $\pm$ 155,012 cpm phosphate incorporated for vehicle treatment and AMPH treatment, respectively;
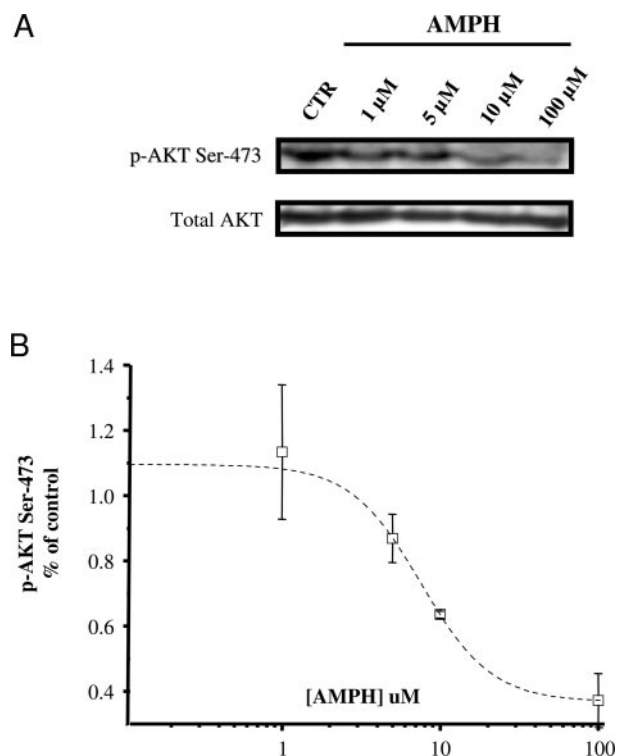

Fig. 2. AMPH-induced decrease in phospho-Akt is concentration dependent. A, immunoblots of phospho-Akt Ser-473 and total Akt from extracts obtained from hDAT cells treated for 30 min either with vehicle or with the indicated concentrations of AMPH. B, data obtained from the quantitation of the immunoblots as in A were plotted against the different concentrations of AMPH, and the data were fit to a sigmoidal inhibition curve (OriginLab Corp., Northampton, MA) to derive the IC$_{50}$ for AMPH.
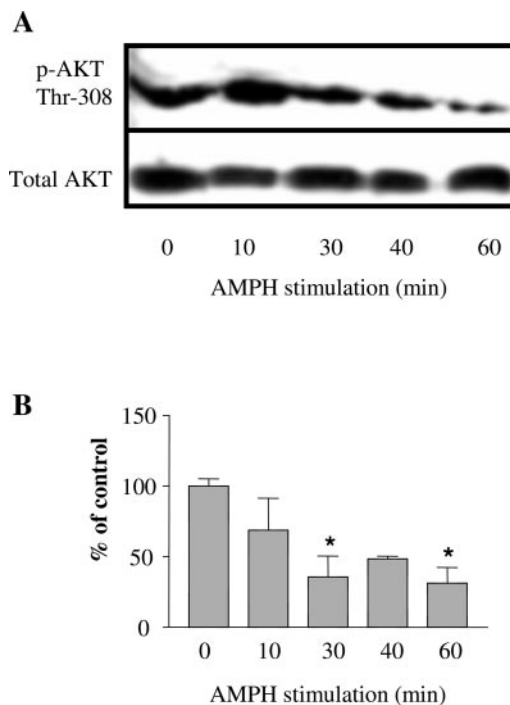

Fig. 3. AMPH induces a decrease in phospho-Akt Thr-308 in murine striatal synaptosomes. A, immunoblots of phospho-Akt Thr-308 and total Akt from extracts of murine striatal synaptosomes treated either with vehicle or with 10 μM AMPH for the indicated times. B, densitometric analysis of the immunoblots in A using Scion Image software. The densities of the phospho-Akt Thr-308 samples were normalized to their respective total protein extracts (Total Akt) and then expressed as a percentage of vehicle-treated control. Data obtained from three independent experiments are expressed as means $\pm$ S.E.M. [one-way ANOVA followed by Dunnett's post hoc test ($n = 3$); $*, p < 0.05$ relative to vehicle treatment].

mean ± S.E.M. ($n = 6$); the level of significance was $p < 0.004$ as measured by paired Student's t test].

AMPH significantly stimulated CaMKII activity in murine striatal synaptosomes as well. Figure 5C shows an immunoblot of Thr-286 phospho-CaMKII (top lane) and total CaMKII (bottom lane) after treatment of striatal synaptosomes with 10 μM AMPH (Fig. 5C). After 40 min of AMPH exposure, phospho-CaMKII was significantly increased with respect to control conditions (Fig. 5D).
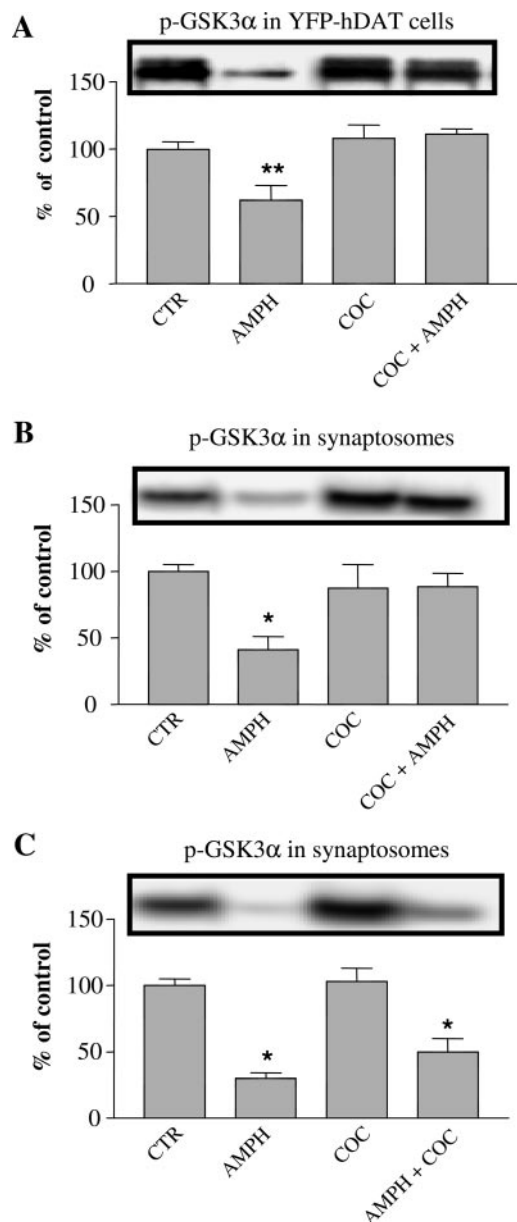

Fig. 4. Cocaine blocks the AMPH-induced decrease of Akt activity. Immunoblots and band density analysis of phospho-GSK3α obtained from extracts from hDAT cells (A) or murine striatal synaptosomes (B) stimulated with 10 μM AMPH for 30 min, 10 μM COC for 40 min, or 10 μM COC for 10 min followed by 10 μM AMPH (in the continued presence of 10 μM COC) for 30 min, or vehicle treatment for 40 min (CTR). C shows data obtained from murine striatal synaptosomes treated with 10 μM AMPH for 30 min, 10 μM COC for 40 min, or 10 μM AMPH for 10 min followed by 10 μM COC (in the continued presence of 10 μM AMPH) for 30 min, or vehicle treatment for 40 min (CTR). Data were normalized to vehicle-treated control and are expressed as means ± S.E.M. Normalized values were compared using one-way ANOVA followed by Tukey's post hoc test ($n = 4$; ∗∗, $p < 0.01$ and ∗, $p < 0.05$).

CaMKII Mediates the AMPH-Induced Decrease of Akt Activity. Extracellular DA has been shown to inhibit Akt activity via a D2 receptor-mediated pathway (Beaulieu et al., 2005). However, our results suggest that AMPH may also act through a novel signaling mechanism sustained by DAT function but independent of DA release and its action at D2 receptors. Because AMPH activates CaMKII, we wanted to explore its potential role in mediating the effect of AMPH on Akt activity. Therefore, we conducted these studies in hDAT cells in which DA has no effect on CaMKII.

To determine whether CaMKII activity is involved in the AMPH-induced inhibition of Akt activity, we used KN93, a pharmacological inhibitor of CaMKII. Pretreatment with 10 μM KN93 for 30 min blocked the ability of subsequently applied AMPH to inhibit Akt activity, as assessed by the phosphorylation of GSK3α (Fig. 6A, KN93 + AMPH), suggesting an important role of CaMKII in regulating Akt. However, KN93 by itself had no effect on Akt activity (Fig. 6A, KN93). Figure 6B shows an immunoblot obtained from hDAT cells treated either with vehicle (CTR), 10 μM AMPH, 10 μM KN92, an inactive analog of KN93, or 30-min pretreatment with KN92 followed by treatment with AMPH. KN92 by itself did not alter Akt activity but, unlike KN93, it also failed to inhibit the action of AMPH.

CaMKII Regulates AMPH-Induced DAT Cell Surface Redistribution. Activation of Akt via insulin signaling has been shown to block AMPH-induced reduction in DAT cell surface expression (Garcia et al., 2005). Furthermore, inhibition of Akt activity mimics the effects of AMPH on DAT plasma membrane expression (Garcia et al., 2005). Therefore, under basal insulin signaling, AMPH activation of CaMKII might inhibit Akt, and, consequently, lead to DAT cell surface redistribution. Figure 7A shows immunoblots obtained from biotinylated (top lane) and total (bottom lane) extracts of hDAT cells treated either with CTR for 30 min, with 10 μM AMPH for 30 min, with 10 μM KN93 for 30 min followed by 10 μM AMPH for an additional 30 min (in the continued presence of KN93) (KN93 + AMPH), with 10 μM KN92 for 60 min, or with 10 μM KN92 for 30 min followed by 10 μM AMPH for an additional 30 min (in the continued presence of KN93) (KN92 + AMPH). Incubation of hDAT cells with AMPH significantly decreased surface biotinylated hDAT (Fig. 7A, top lane), whereas preincubation with KN93 followed by AMPH resulted in hDAT cell surface expression similar to that of CTR. It is noteworthy that preincubation with KN92, the inactive analog of KN93, did not block AMPH-induced hDAT cell surface redistribution (Fig. 7A, top lane). Quantification of the biotinylated bands obtained from three different experiments is shown in Fig. 7B.

Discussion

Hormonal regulation of DAT, and subsequently of extracellular DA, is emerging as an important mechanism linking neurotransmitter transporter function to psychostimulant abuse. Profound alterations in the neuronal dopaminergic system occur in experimentally induced diabetic mice (Saitoh et al., 1998). DA clearance in hypoinsulinemic rats is markedly reduced compared with control rats (Patterson et al., 1998; Owens et al., 2005). In addition, diabetic animals are resistant to both the behavioral and locomotor effects of

AMPH (Marshall, 1978; Saitoh et al., 1998). In experimentally induced diabetic rats (i.e., alloxan-treated), acute AMPH administration had an attenuated ability to induce anorexia, stereotyped behavior, and increased locomotor activity; subsequent insulin administration reversed this attenuation (Marshall, 1978). More recently, Galici et al. (2003) demonstrated that there is a selective decrease in AMPH self-administration in diabetic rats that is associated with a decrease in DA uptake in the striatum. Considering that the striatum is highly enriched (Banks and Kastin, 1998) in insulin receptors (Hill et al., 1986) as well as in DAT, these studies strongly support a role for the neuronal PI3K pathway in regulating DAT activity and extracellular DA levels as well as in the actions of AMPH.

PI3K and its downstream target Akt have been shown to be essential for insulin modulation of hDAT function in striatal synaptosomes and hDAT cells (Carvelli et al., 2002; Garcia et al., 2005). Indeed, insulin signaling increases DA uptake capacity and cell surface expression (Carvelli et al., 2002; Garcia et al., 2005). In contrast, in vitro inhibition of

either PI3K or Akt causes a decrease in DA uptake capacity and a redistribution of DAT away from the plasma membrane (Garcia et al., 2005).

Likewise, AMPH decreases DAT cell surface expression, which leads to a decrease in DA uptake and possibly contributes to its ability to increase extracellular DA in vivo (Saunders et al., 2000; Gulley et al., 2002; Little et al., 2002; Chi and Reith, 2003; Sorkina et al., 2003). Insulin application blocks the ability of AMPH to decrease hDAT plasma membrane expression as well as DA uptake, and this "insulin rescue" is mediated by activation of PI3K and Akt (Carvelli et al., 2002; Garcia et al., 2005). Therefore, inhibition of Akt might be a primary mechanism through which AMPH decreases cell surface redistribution of DAT. Indeed, preliminary evidence that AMPH inhibits Akt via a receptor-independent mechanism was provided by Garcia et al. (2005). Here, we have demonstrated that AMPH-induced stimulation of CaMKII is a novel mechanism by which AMPH inhibits Akt activity. This mechanism does not require increased extracellular DA or D2 receptor-activation, in contrast to
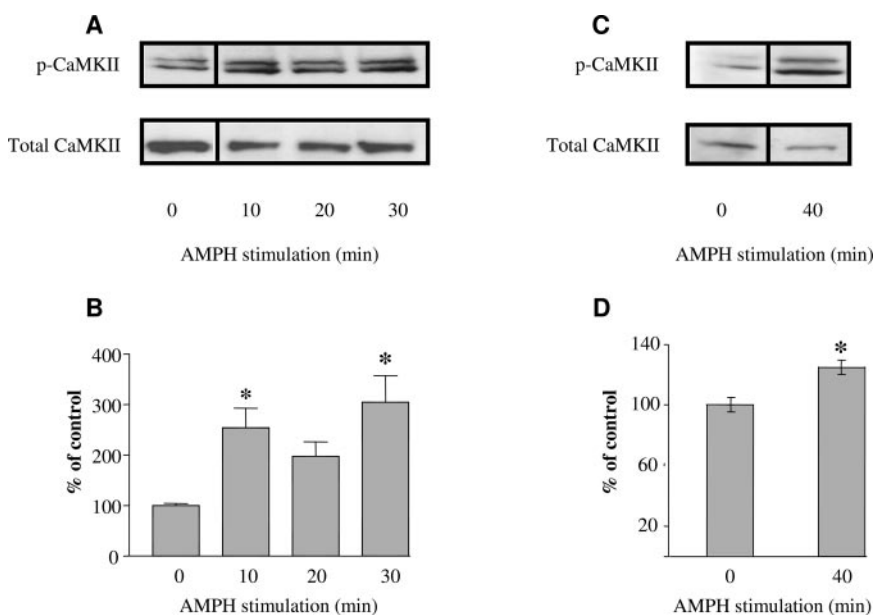

Fig. 5. AMPH enhances CaMKII activity. A, immunoblot of phospho-CaMKII and total CaMKII obtained from extracts of hDAT cells treated with 10 μM AMPH for the indicated times. B, analysis of band density of immunoblots as in A. C, immunoblot of phospho-CaMKII and total CaMKII obtained from extracts of murine striatal synaptosomes treated with 10 μM AMPH for 40 min. D, analysis of band density of immunoblots as in C. Data were normalized to total protein concentration to correct for differences in cell seeding and then normalized to vehicle-treated control. Data are expressed as the means $\pm$ S.E.M. Normalized values were compared using one-way ANOVA followed by Tukey's post hoc test ($n = 3$; *, $p < 0.05$) (B) or Student's t test (D).

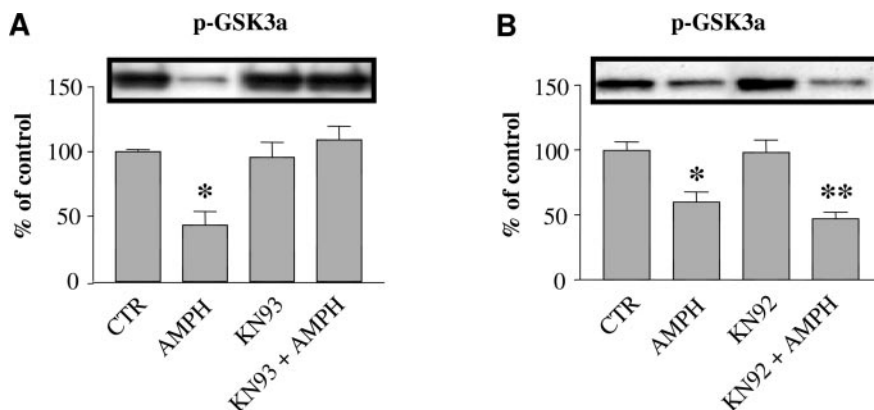Fig. 6. KN93 blocks the AMPH-induced decrease of Akt activity. Immunoblots and densitometric analysis of phospho-GSK3α from extracts obtained from hDAT cells stimulated with 10 μM AMPH for 30 min, 10 μM KN93 for 60 min, 10 μM KN93 for 30 min followed by 10 μM AMPH (in the continued presence of KN93) for 30 min, or vehicle treatment (CTR) (A); and with 10 μM AMPH for 30 min, 10 μM KN92 for 60 min, 10 μM KN92 for 30 min followed by 10 μM AMPH (in the continued presence of KN92) for 30 min, or vehicle treatment (CTR) (B). The densities of the phospho-GSK3α bands were normalized to their respective total protein concentrations and then expressed as a fraction of vehicle-treated control. Normalized values were compared using one-way ANOVA followed by Tukey's post hoc test ($n = 3$; *, $p < 0.05$ and **, $p < 0.01$).

other proposed mechanisms (Brami-Cherrier et al., 2002; Beaulieu et al., 2005).

The mechanism by which CaMKII inhibits Akt is not known. One possibility is that CaMKII might regulate a phosphatase, which would in turn lead to dephosphorylation of Akt. Indeed, it has been shown that in vitro treatment with PP2A leads to inactivation of Akt (Andjelkovic et al., 1996) and that CaMKII targets and regulates PP2A complexes/activity (Takahashi and Suzuki, 2006). Other kinases and/or phosphatases might also play a role in this regulation.

Concentrations of AMPH that we have previously shown to cause DAT cell surface redistribution (Saunders et al., 2000; Gulley et al., 2002; Little et al., 2002; Chi and Reith, 2003; Sorkina et al., 2003) caused a significant decrease in Akt activity, both in hDAT cells (Fig. 1) and in striatal synaptosomes (Fig. 3). The IC_{50} for AMPH-induced inhibition of Akt activity was ~8 μM. The time dependence and extent to which AMPH reduced Akt activity in hDAT cells were similar to those in the synaptosomal preparation. This validates the use of the hDAT cells as a reliable experimental tool to examine the action of AMPH on insulin signaling. Because DA did not inhibit Akt in hDAT cells, these data also suggest that D2 receptor activation does not play a significant role in this novel effect of AMPH.

Cocaine pretreatment blocked the effect of AMPH on Akt activity in hDAT cells as well as striatal synaptosomes (Fig. 4), suggesting that AMPH mediates its effect on Akt through its interaction with DAT. Cocaine by itself had no effect on Akt activity in either system. Thus, although AMPH and cocaine are both psychostimulants and drugs of abuse, they differ mechanistically in their actions on Akt.
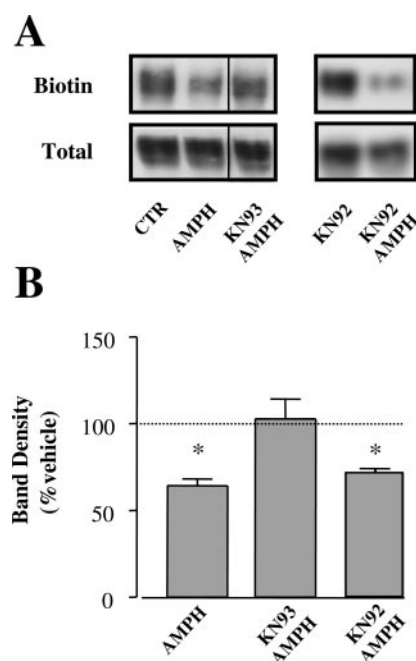

Fig. 7. CaMKII modulates AMPH-induced hDAT cell surface redistribution. A, representative immunoblot of hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells treated as indicated. B, quantification of the immunoblots using the Scion Image system. The density of the biotinylated samples was normalized to the density of the parallel total extract and expressed as a percentage of vehicle-treated controls. (For the K92/AMPH the vehicle was KN92.) The normalized data are expressed as mean ± S.E.M. and compared with respective controls by one-way ANOVA followed by the Bonferroni's test; *, $p < 0.05$.

Because AMPH results in release of intracellular calcium (Gnegy et al., 2004; Kantor et al., 2004), we asked whether CaMKII is involved in the AMPH-mediated Akt effect. We have shown that AMPH activates CaMKII in hDAT cells as well as in synaptosomes (Fig. 5). Our results are consistent those of Iwata et al. (1997b), who reported an increase in synaptosomal CaMKII activity after repeated AMPH administration in rats (Iwata et al., 1997b) as well as with those of Choe and Wang (2002), who observed a 2-fold increase in phospho-CaMKII in the rat striatum after acute AMPH administration.

The CaMKII-specific inhibitor KN93 blocked the ability of AMPH to decrease Akt activity (Fig. 6A), whereas the inactive analog KN92 had no effect (Fig. 6B). Neither of the compounds had an effect on Akt activity by themselves. In addition, KN93 blocked the ability of AMPH to cause DAT cell surface redistribution (Fig. 7). These data suggest that decreased activity of the downstream insulin-signaling component Akt mediates the ability of AMPH to decrease DAT cell surface expression through a mechanism requiring stimulation of CaMKII.

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Address correspondence to: Dr. Aurelio Galli, Department of Molecular Physiology and Biophysics, Center for Molecular Neuroscience, Vanderbilt University, 465 21st Ave. South, Nashville, TN 37232-8548. E-mail: aurelio.galli@vanderbilt.edu