Psychoactive Substrates Stimulate Dopamine Transporter Phosphorylation and Down Regulation by Cocaine Sensitive and Protein Kinase C Dependent Mechanisms

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Running Title: Methamphetamine-Induced Dopamine Transporter Phosphorylation

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Dopamine transporters (DATs) undergo intracellular sequestration and functional down regulation upon exposure to psychostimulant substrates. To investigate the potential mechanism underlying these responses we examined the acute in vitro and in vivo effects of amphetamine (AMPH) and methamphetamine (METH) on phosphorylation and down regulation of rat DAT using wild type and N-terminal truncation mutants. Phosphorylation of DAT assessed by $^{32}$PO$_4$ metabolic labeling was increased up to two-fold by in vitro treatment of rDAT LLC-PK$_1$ cells with AMPH or METH, and was similarly increased in rat striatal tissue by in vitro application or in vivo injection of METH. The dopamine transport blocker (-)-cocaine did not affect DAT phosphorylation but prevented the phosphorylation increase induced by METH. Phosphorylation of DAT induced by METH was also prevented by the protein kinase C blocker bisindoylmaleimide I and was absent in an N-terminally truncated protein that lacks the first twenty-one residues including six serines that also represent the site of phorbol ester induced phosphorylation. Down-regulation of transport induced by METH was also cocaine and protein kinase C dependent, but was retained in the N-terminal truncation mutant. These results demonstrate that transport or binding of METH stimulates DAT phosphorylation and down-regulation by a mechanism that requires protein kinase C, but that METH-induced down-regulation can occur independently of direct transporter phosphorylation. The finding that DAT phosphorylation is stimulated by amphetamines reveals a previously unknown effect of these drugs that is not produced by cocaine and may be related to reinforcement.

The dopamine transporter (DAT$^1$) is a phosphoprotein expressed in dopaminergic neurons that clears extracellular dopamine (DA) from the synapse following neurotransmitter release. This regulates transmitter availability for DA receptors and is crucial for the temporal and spatial shaping of dopaminergic neurotransmission (1). Neurological disorders such as schizophrenia, depression, and attention deficit disorder are associated with abnormal DA levels that may result from functional dysregulation of DAT (2). DAT is also a major site of action for numerous psychostimulant and neurotoxic drugs that modulate DA levels or lead to dopaminergic neurodegeneration (3). Amphetamine (AMPH) and methamphetamine (METH) are DAT substrates that raise extracellular transmitter levels by competing with endogenous DA for transport and inducing DA efflux through transport reversal (4,5). Many DAT substrates including AMPH, METH, and 6-hydroxydopamine (6-OHDA), are toxins that induce production of free radicals and neurodegeneration after transport into dopaminergic cells (2). Other drugs such as cocaine are not transported by DAT but bind to the protein and elevate synaptic DA concentrations by inhibiting substrate translocation.

DAT transport activity, plasma membrane expression, and phosphorylation level are acutely modulated by exogenous kinase and phosphatase treatments (6,7), but the mechanisms underlying these processes are not understood. Protein kinase C (PKC) activators such as phorbol 12 myristate...
acetate (PMA) and protein phosphatase inhibitors such as okadaic acid (OA) induce DAT endocytosis and reduced transport $V_{\text{max}}$ (6,8-11), and stimulate phosphorylation of DAT on a cluster of N-terminal serines (12,13). Removal of N-terminal phosphorylation sites, however, does not prevent PKC-induced DAT down regulation or endocytosis (13,14), indicating that these processes occur through mechanisms involving other PKC-regulated proteins.

DA uptake and DAT surface expression are also reduced by *in vitro* substrate pretreatment or *in vivo* METH injection via mechanisms that are blocked or attenuated by PKC inhibitors and cocaine (15-19). Conversely, DAT surface levels and transport activity have been variously reported to be unaffected (19,20) or upregulated (15,17) by acute cocaine pretreatment. These findings suggest the presence of a rapid feedback process associated with phosphorylation conditions and substrate transport that regulates DA clearance by modulating DAT cell surface expression.

Reverse transport of DAT is also regulated by PKC, as AMPH-induced DA efflux is blocked by PKC inhibitors (21,22), and MPA induces substrate-independent DA efflux (23). Removal or mutation of DAT N-terminal serines suppresses AMPH-induced DA efflux (14), suggesting a relationship between PKC-mediated phosphorylation and an efflux promoting form of the protein.

We have probed the molecular basis and potential relationships between these processes by examining the involvement of psychostimulant substrates and uptake blockers in DAT phosphorylation and down regulation. In this report we demonstrate that AMPH and METH but not cocaine stimulate the level of DAT metabolic phosphorylation in both heterologously expressing cells and rat brain tissue. METH-induced DAT phosphorylation occurs on the same domain identified for PMA-stimulated phosphorylation and requires PKC activity and binding or active transport of drug. However, METH-induced transport down regulation is retained in a phosphorylation-deficient DAT mutant, demonstrating that this process does not require transporter phosphorylation. These findings directly demonstrate the occurrence of PKC-dependent substrate-induced DAT phosphorylation that was previously implicated in mutagenesis studies as a requirement for AMPH-induced efflux (14) and indicate that PKC is part of a common pathway for down regulation of DAT induced by PMA and METH.

**MATERIALS AND METHODS**

**Site-directed mutagenesis and cell culture.** The wt rDAT construct was obtained from Dr. David Donovan, National Institute on Aging, and placed in a pcDNA 3.0 plasmid. A mutant construct lacking the first 21 N-terminal amino acids with the addition of an initiation methionine was produced from the wt cDNA using the Stratagene QuikChange® kit. The entire DAT coding region of the resulting V21 rDAT construct was sequenced for accuracy (Northwoods DNA, Solway, MN) and used for transfection. For the production of stable transformants parental LLC-PK1 cells were grown to approximately 50% confluency and transfected using the FuGENE transfection reagent (Roche) and 0.5 µg of the V21 rDAT pcDNA 3.0 plasmid. Transformants were selected 24 hrs later by the addition of 400 µg/ml Geneticin (G418) to the cell culture medium. LLC-PK1 cells stably expressing wt rDAT (24) or pooled V21 rDAT cell lines were maintained in Alpha Minimum Essential Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 µg/ml G418 and 1X penicillin/streptomycin in an incubation chamber gassed with 5% CO₂/95% O₂ at 37 °C. Cells were grown to approximately 70% confluence in 6 or 12 well plates for use. Western blotting of cell lysates with DAT monoclonal antibody (26) revealed comparable expression levels of the mature 100-110 kDa forms of the wt and V21 DAT protein.

**Dopamine uptake.** For [³H]DA uptake assays rDAT LLC-PK1 cells were washed twice with 1 ml of Krebs-Ringer HEPES (KRH) buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 5.6 mM glucose, pH 7.4). Triplicate wells were pretreated at 37 °C for 30 min or other indicated times with 1 ml KRH containing vehicle, test treatments, or phosphorylation modulators prior to assay for transport. Soluble test compounds (METH, AMPH, (-)-cocaine, SCH 23390, sulpiride) were prepared in distilled/deionized water, and OA, PMA, bisindoylmaleimide I (BIM), and H89 were...
prepared at high concentration in dimethyl sulfoxide (DMSO) followed by dilution in the incubation mixture to a final DMSO concentration of 0.1%. At the end of the pretreatment interval cells were placed on ice and rapidly washed three times with ice-cold KRH to remove pretreatment drug prior to assay for DA transport. Uptake was initiated by adding 10 µl of a 100X DA stock solution to bring the final concentration of [3H]DA to 10 nM and total DA to 3 µM. Non-specific uptake was determined with 100 µM (-)-cocaine and was subtracted from total uptake values. Uptake assays were carried out at 37°C for 8 min and terminated by rapidly washing the wells three times with 1 ml ice cold KRH. The cells were solubilized in 500 µl of RIPA buffer consisting of 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 1 µM OA and one Roche Complete Mini protease inhibitor tablet per 10 ml buffer. Lysates were then measured for incorporated radioactivity using a Packard 1900CA liquid scintillation counter at 57% efficiency.

Phosphorylation of DAT in cells. On the day of the experiment cells were incubated in phosphate-free medium for 30 min followed by exchange with fresh medium containing 0.5 mCi/ml of 32PO4. Cells were labeled for 4h at 37°C, followed by application of test compounds to duplicate or triplicate wells for 2-30 min. In all experiments cells in parallel wells were treated with OA or PMA as positive controls for stimulated phosphorylation. At the end of the treatment cells were washed once with 500 µl of ice cold sucrose phosphate buffer (SP), consisting of 0.32 M sucrose and 10 mM sodium phosphate, pH 7.4, and lysed on ice for 15 minutes with 500 µl RIPA buffer. Lysates were centrifuged at 4°C at 20,000 x g for 20 min and the resulting supernatant further centrifuged at 4°C at 100,000 x g for 60 min. The soluble fraction was collected and subjected to immunoprecipitation with DAT antibody 16 followed by SDS-PAGE and autoradiography. In all experiments control and experimental groups were 32PO4 labeled, immunoprecipitated, and subjected to electrophoresis and autoradiography at the same time and exactly in parallel. All autoradiographs shown and analyzed were obtained from equal exposure times to allow for direct comparison of signal intensities.

Phosphorylation of DAT in striatal slices. Metabolic phosphorylation of DAT in rat striatal slices was performed as previously described (25). Male Sprague-Dawley rats (175-300 g) were decapitated and the striata were rapidly removed and weighed. The tissue was sliced into 350 µm slices using a McElvain Tissue Chopper and placed into wells of a 12-well culture plate containing oxygenated Krebs-bicarbonate buffer consisting of 25 mM NaHCO3, 125 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 5 mM MgSO4, and 10 mM glucose, pH 7.3. Slices were preincubated for 30 min at 30°C, with shaking at 105 rpm, followed by exchange with fresh buffer containing 1 mCi/ml 32PO4 and continued incubation with shaking at 30°C for 90-120 min. 95% O2/5% CO2 was gently blown across the top of the plate during the incubation and test compounds or vehicle were added for the final 30 minutes or other indicated times. At the end of labeling, tissue slices were transferred to microcentrifuge tubes and centrifuged at 500 x g for 2 min at 4°C. The supernatant fractions were removed and 1 ml ice-cold Krebs-bicarbonate buffer was added to the slices. The tissue was disrupted by 6 passages through a 26 gauge needle, centrifuged at 500 x g for 2 min at 4°C, and the supernatants were removed. The sedimented membranes were solubilized with 0.5% SDS sample buffer (60 mM Tris, pH 6.8, 0.5% SDS, 10% glycerol, 100 mM dithiothreitol) at 50 mg/ml original wet weight and centrifuged at 20,000 x g for 20 minutes to remove SDS insoluble material. The soluble fraction was collected and subjected to immunoprecipitation with DAT antibody 16 followed by SDS-PAGE and autoradiography.

Phosphorylation of DAT in striatal slices following in vivo METH administration. Male Sprague-Dawley rats (175-300 g) were weighed and injected subcutaneously with 1 ml volumes of sterile saline or 15 mg/kg METH. Thirty minutes after injection, the animals were decapitated and the striata were quickly removed, weighed and sliced. Metabolic labeling of the slices was initiated within 10-15 min of tissue harvest and was performed as described above except that the 32PO4 incubation time was reduced to 30 min. During the 32PO4 labeling the striatal slices
obtained from one hemisphere of each animal received no further addition, while the slices from the other hemispheres were treated with 10 µM OA as a positive control for stimulated DAT phosphorylation. The slices were then processed, solubilized and prepared for immunoprecipitation as described above.

**Immunoprecipitation, electrophoresis and autoradiography.**  
$^{32}$PO$_4$-labeled samples from cell lysates or solubilized striatal membranes were immunoprecipitated with rabbit polyclonal antiserum 16 generated against rDAT N-terminal amino acids 42-59 (11). Precipitated samples were electrophoresed on 8% SDS polyacrylamide gels with high range Rainbow molecular mass standards, and gels were dried and subjected to autoradiography using Kodak Biomax film or PhosphorImager analysis. For all experiments samples were labeled, processed, and electrophoresed on the same gel exactly in parallel. For figure preparation, lanes that were not adjacent in the original gel but separated by blank lanes or juxtaposed for purposes of consistent data presentation are separated by black lines.

$^{32}$PO$_4$-labeled DAT migrates as a broad band on these gels (striatal forms 80-90 kDa, expressed forms 100-110 kDa), often as a complex of a doublet or multiple forms. The identity of this complex of bands as DAT was verified in various experiments by co-electrophoresis of samples with photoaffinity labeled DAT, by using preimmune antiserum for immunoprecipitation negative controls, and by western blotting immunoprecipitated $^{32}$PO$_4$-labeled samples with a highly specific monoclonal DAT antibody (26) to demonstrate correspondence of $^{32}$PO$_4$-labeled and immunoreactive bands. Similar and additional peptide blocking controls for the identity of the phosphorylated DAT bands have been documented in previous studies (10,11). For quantification the 80-90 kDa region of the autoradiographs shown in Figures 2-8 were scanned and quantified with Molecular Analyst (BioRad) or ImageQuant (Molecular Dynamics) software. The phosphorylation intensities of treated samples were expressed as percent of the basal phosphorylation level, which was defined as 100%. Normalized phosphorylation values from multiple experiments were averaged for statistical analysis by students t-test or ANOVA using Prism 3 software.

**Materials.** Carrier free $^{32}$PO$_4$ was from ICN; [$^3$H]DA (41 Ci/mmol) and high range Rainbow molecular mass standards were from Amersham; OA and PMA were from Calbiochem; sulpiride and SCH 23390 were from RBI; DA, AMPH, METH, (-)-cocaine, BIM, H89, and other reagents were from Sigma. Rats were purchased from Charles River Laboratories and were housed and treated in accordance with regulations established by the University of North Dakota Institutional Animal Care and Use Committee.

**RESULTS**

Transports substrates down-regulate DA transport activity. [$^3$H]DA transport and regulation of DAT by PMA have been well characterized in rDAT LLC-PK1 cells (10,24). To examine the responsiveness of this system to DAT substrates, cells were pretreated with METH for 30 min, washed extensively to remove extracellular drug, and assayed for [$^3$H]DA transport. METH pretreatment resulted in a 28 ± 4% reduction of transport activity compared to control values (Fig. 1A), comparable to a 30 ± 3% decrease induced by PMA (both p<0.001). Time course studies showed that down-regulation induced by 10 µM METH occurred with a half time of approximately 2 minutes (15 ± 2%, p<0.01) and reached a plateau of 20–28% down-regulation (p<0.001) by 5-10 min (Fig. 1B). The finding that there was no loss of activity seen with 1 min of pretreatment demonstrates that the washout process effectively removed the METH and that the reduced [$^3$H]DA uptake observed in other samples is not caused by transport competition from residual drug. Down-regulation was dose-dependent beginning at 0.1 µM METH and showing strongest responses (25 ± 3% and 46 ± 6%, p<0.001) at 10 and 100 µM METH (Fig. 1C). Transport activity down regulates to similar levels in these cells with DA pretreatment (27), demonstrating a response to endogenous as well as psychostimulant substrates. These characteristics are qualitatively comparable to those previously reported for DA-, AMPH-, and METH-induced reduction of DA transport in rat striatal tissue and hDAT expressing cells and oocytes (15-17,19).

AMPH and METH increase DAT phosphorylation in cells. To assay for DAT
phosphorylation responses to substrates, cells were metabolically labeled with $^{32}$PO$_4$ and treated with or without AMPH or METH for 10-30 min followed by immunoprecipitation and autoradiography of DAT (Fig. 2). In all experiments parallel sets of cells were treated with OA or PMA as positive controls for stimulated phosphorylation. DATs from untreated cells showed a basal level of constitutive $^{32}$PO$_4$ incorporation as previously described (10,11), which is defined as 100% for purposes of comparison with phosphorylation levels obtained in experimental conditions. After treatment of cells with 2 µM AMPH, DAT phosphorylation was increased to an average of $139 \pm 8\%$ of basal, compared to an OA-stimulated level of $225 \pm 21\%$ (Fig. 2, left three lanes). In separate experiments, DATs from cells treated with 10 µM METH or 10 µM PMA showed stimulation of phosphorylation to $154 \pm 9\%$ and $255 \pm 22\%$ of basal, respectively (Fig. 2, right three lanes). The level of phosphorylation induced by these psychostimulant substrates was statistically greater than basal (AMPH, p<0.01; METH, p<0.001) and ranged between 31-35% of the levels stimulated by OA or PMA.

METH induces DAT phosphorylation in striatal slices. We then examined DAT phosphorylation in a more native context using rat striatal slices and focusing on the effects of METH (Fig. 3). DATs from $^{32}$PO$_4$ labeled striatal slices treated with 10 µM METH showed an increase in DAT phosphorylation to $191 \pm 27\%$ of basal (p<0.01) compared to a $450 \pm 67\%$ level (p<0.001) induced by OA (Fig. 3A). Dose response studies (Fig. 3B) showed a trend toward increased phosphorylation at 1 µM METH ($120 \pm 12\%$) with statistically significant increases seen at 10 µM METH ($191 \pm 27\%$, p<0.01), which was the highest concentration tested. Time course analysis showed that the effect is rapid, with increased phosphorylation apparent in some experiments after 2-5 min of treatment and statistically significant peak levels ($188 \pm 26\%$ to $182 \pm 33\%$, p<0.01) reached at 10-15 minutes (Fig. 3C). Phosphorylation levels did not remain statistically elevated with 20 min treatment times ($125 \pm 18\%$), suggesting that in striatal tissue the response is rapidly transient.

DAT phosphorylation is increased by in vivo METH. To further examine the potential for METH-induced phosphorylation of DAT to be a physiologically relevant response we analyzed $^{32}$PO$_4$ labeling of DAT after injection of rats with 15 mg/kg METH, a dose of drug that produces clearly detectable locomotor activation and induces significant down-regulation of in vitro synaptosomal DA uptake (18). For this experiment rats were injected subcutaneously with METH or saline, sacrificed after 30 min, and sliced striatal tissue was labeled with $^{32}$PO$_4$ for 30 min. The slices from one striatum of each animal received no further treatment during the $^{32}$PO$_4$ labeling while slices from the corresponding striata received in vitro OA. DATs from the saline-treated animals showed detectable $^{32}$PO$_4$ incorporation indicative of rapid constitutive phosphate turnover (Fig. 4). In five independent experiments, DAT phosphorylation in the animals that received METH was increased to an average of $183 \pm 13\%$ of basal (p<0.001), showing that the presence of the drug in vivo induced a process leading to stimulation of DAT phosphorylation that was detectable by ex vivo $^{32}$PO$_4$ labeling. Application of in vitro OA to the tissue from untreated and treated animals strongly stimulated DAT phosphorylation to an average of $437 \pm 89\%$ and $396 \pm 89\%$ relative to basal (p<0.05), verifying the responsiveness of the tissue to phosphorylation activators. When tissue harvested after 30 min of in vivo METH was labeled with $^{32}$PO$_4$ for longer times (60-120 min), DAT phosphorylation was not stimulated above basal (not shown), consistent with a transient DAT phosphorylation response to in vivo METH.

DAT phosphorylation is not affected by cocaine. The effect of cocaine on DAT phosphorylation was examined in metabolically labeled rat striatal slices. In four independent experiments, DATs from striatal slices treated in vitro with 30 µM (-)-cocaine for 15 min displayed no average change in phosphorylation compared to untreated controls ($106 \pm 28\%$, p>0.05) (Fig. 5A) while OA induced substantial phosphorylation increases ($285 \pm 63\%$, p<0.05). The slight variability between the autoradiographic intensities of the control and cocaine samples in this figure is an indication of scatter that does not reflect a consistent alteration in DAT phosphorylation in the presence of cocaine (Fig 5A histogram, Fig. 5B, Fig 6A, reference 27). We also found that no cocaine effects were
conclusively produced with treatment times as short as two minutes (Fig. 5B), suggesting that a transient phosphorylation change did not occur unless it was completed within this time frame. In vitro cocaine applied to rDAT LLC-PK1 cells for up to 30 min at higher concentrations (100 µM) also failed to produce a significant effect on DAT phosphorylation (Fig. 6A, 27).

METH-induced DAT phosphorylation and down-regulation are blocked by cocaine. AMPH and METH are actively transported into cells by DAT but can also cross the plasma membrane by passive diffusion. To determine if METH-induced phosphorylation of DAT is due to diffusion we incubated rDAT LLC-PK1 cells with 100 µM (-)-cocaine prior to and during METH pretreatment to block METH binding and/or transport (Fig. 6A). DAT phosphorylation was not statistically different from basal in cells treated with cocaine alone (128 ± 5%, p>0.05) but was increased to 158 ± 15% (p<0.001) in the cells that received METH. However, DAT phosphorylation levels were not different from basal (107 ± 9%, p>0.05) in cells that were co-treated with cocaine and METH.

Down-regulation of DA transport induced by METH (20 ± 5%, p<0.001) was also blocked (2 ± 4% increase, p>0.05) by co-application of 10 µM (-)-cocaine (Fig. 6B). Pretreatment of cells with cocaine alone did not cause a change in subsequent transport activity (12 ± 5% increase, p>0.05). The blockade of METH-induced DAT phosphorylation and down-regulation by cocaine indicates that these processes require either active transport or binding of METH to DAT and do not occur in response to passive diffusion of drug through the cell membrane.

METH-induced DAT phosphorylation and down-regulation are PKC dependent. To date PKC is the only kinase that has been well characterized as contributing to DAT phosphorylation. We used the PKC inhibitor bisindoylmaleimide I (BIM), which blocks PMA-stimulated DAT phosphorylation (11), to investigate the potential role of PKC in substrate-induced DAT phosphorylation (Fig. 7A). In rDAT LLC-PK1 cells, DAT phosphorylation was not affected by treatment with BIM alone (92 ± 6% of control, p>0.05) while DATs from cells treated with METH alone showed a phosphorylation level increased to 124 ± 4% of control (p<0.05). However, DAT phosphorylation was not different from basal (87 ± 7%, p>0.05) when cells were treated with METH in the presence of BIM, indicating that PKC activity is required for the METH-induced response. Similar experiments performed for transport (Fig. 7B), showed that down-regulation induced by METH (13 ± 2%, p<0.001) was effectively blocked by BIM (3 ± 4%, p>0.05) in a manner comparable to blockade of PMA-induced transport down regulation (PMA 19 ± 3%, p<0.001; PMA plus BIM 10 ± 3%, p>0.05).

Although the concentration of BIM used in these experiments (10 µM) can also inhibit cAMP dependent protein kinase (PKA), this dose was used because lower BIM concentrations (1 µM) do not block PMA-induced DAT phosphorylation or down-regulation (11, data not shown). To investigate a potential role for PKA in these processes, we treated cells with H89, a specific PKA inhibitor. H89 had no effect on METH- or PMA- induced down regulation (METH plus H89, 19 ± 3%; PMA plus H89, 24 ± 5%, both p<0.001) (Fig. 7B). Neither BIM nor H89 used alone had any effect on DA transport activity (97 ± 4% and 96 ± 3% of control, respectively, both p > 0.05). In two independent preliminary experiments a trend towards increased METH-induced DAT phosphorylation (126 ± 9% of control) that was suppressed by BIM (82 ± 12% of control), showed no obvious inhibition by H89 (147 ± 33% of control), although statistical significance was not obtained in any of the conditions. These results support the interpretation that BIM effects on phosphorylation and down regulation occur via inhibition of PKC.

N-terminal serines are required for METH-induced DAT phosphorylation but not for METH-induced down-regulation. It has been demonstrated that removal of the first 22 residues of hDAT containing the N-terminal serine cluster prevents PKC-induced hDAT phosphorylation but not down regulation or endocytosis (13,14). To further examine the relationship between METH- and PMA-induced phosphorylation we generated a comparable rDAT N-terminal truncation mutant, D21 rDAT, that lacks the first six serines at residues 2, 4, 7, 12, 13, and 21. The wt and D21 rDAT proteins display similar levels of immunoblot staining (Fig. 8A), and of [3H]DA transport and [3H]CFT binding activity (data not
Furthermore, METH inhibited \[^{3}H\]DA transport in wt and \(\Delta 21\) proteins with statistically equivalent IC\(_{50}\)s (929 ± 129 nM and 726 ± 96 nM, respectively, \(p>0.05\)), indicating that METH was either transported by or bound to the truncated rDAT protein with an affinity comparable to the wt protein.

In \(^{32}\text{PO}_4\) labeling studies of the \(\Delta 21\) mutant performed in parallel with wt rDAT we found that the wt protein displayed the characteristic pattern of basal \(^{32}\text{PO}_4\) incorporation that was increased by both METH and PMA (Fig 8A upper panel). In contrast, although immunoblotting showed that equal amounts of DAT protein were present in all wt and \(\Delta 21\) samples (Fig. 8A lower panel), the \(^{32}\text{PO}_4\) labeled \(\Delta 21\) rDAT samples showed only diffuse background staining throughout the lanes without any obvious indication of the presence of a phosphoprotein band (Fig. 8A upper panel). Although in this experiment the background staining present in the METH sample was lighter than in the other samples this was not found in three other independent experiments that showed no basal or METH-induced phosphorylation of the \(\Delta 21\) rDAT protein.

Similar to what was found for the hDAT N-terminal truncation mutant, the \(\Delta 21\) rDAT protein retained transport down regulation in response to PMA (79 ± 5% of control, \(p<0.001\)) (Fig. 8B). We also found that \(\Delta 21\) rDAT displayed a level of down-regulation in response to METH (75 ± 4% of control, \(p<0.001\)) (Fig. 8B), that was similar to that of the wt protein. These results demonstrate that METH- and PMA-induced phosphorylation of rDAT occur on the same N-terminal tail serine cluster but that phosphorylation of this domain is not required for PMA- or METH-induced phosphorylation of the \(\Delta 21\) rDAT protein.

METH-induced DAT phosphorylation in LLC-PK\(_1\) cells is independent of DA receptor activation. LLC-PK\(_1\) cells have been reported to express dopamine D\(_1\) receptors and to synthesize DA when supplied with L-DOPA (28). L-DOPA was not added to the cells in these experiments, making it unlikely that the METH- and AMPH-stimulated increases in DAT phosphorylation could be due to drug-induced efflux of endogenous DA leading to activation of DA receptors. However, we investigated this possibility by examining the effects of METH on \(^{32}\text{PO}_4\) labeling of DAT in cells pretreated with the dopamine D\(_1\) and D\(_2\) receptor antagonists sulpiride and SCH 23390 to block activation of potential DA receptors. In two independent experiments performed in duplicate, DAT phosphorylation stimulated by METH was similar in the absence (120 ± 3%) or presence (121 ± 6%) of sulpiride plus SCH 23390 (both \(p<0.05\) relative to basal), compared to a 146 ± 9% stimulation by PMA (\(p<0.01\)). Thus in LLC-PK\(_1\) cells the effects of METH on DAT phosphorylation occur independently of DA receptor activation.

**DISCUSSION**

This study demonstrates that metabolic phosphorylation of DAT is increased after in vitro treatment of heterologously expressing cells with AMPH or METH, and after in vitro and in vivo exposure of rat striatal tissue to METH. These results show for the first time that substrate treatments can directly affect the phosphorylation state of DAT in the absence of exogenous kinase or phosphatase modulators and extend the significance of these and related processes from model cell culture systems to the brain and conditions of in vivo drug administration. These findings reveal a previously unknown metabolic effect of amphetamines on DAT that is distinct from direct pharmacological inhibition of uptake and may function in processes that could persist after systemic drug clearance. In contrast to the effects of AMPH and METH, acute exposure of striatal tissue or cells to cocaine did not affect \(^{32}\text{PO}_4\) labeling or transport activity of DAT, demonstrating that DAT phosphorylation and regulation display differential responses to these pharmacologically distinct classes of psychostimulant drugs.

The ability of METH to induce DAT phosphorylation does not appear to be mechanistically linked to its induction of down-regulation, as the removal of the N-terminal phosphorylation sites abolishes METH-induced phosphorylation without preventing METH-induced transport down-regulation. This is also consistent with the lack of similarity between the time courses of METH-induced phosphorylation and down-regulation. It is more likely that AMPH/METH-induced DAT phosphorylation is required for or regulates transporter reversal and substrate efflux. It has previously been shown that
AMPH-induced DA efflux requires PKC activity (22) and is impaired by removal of DAT N-terminal serines and restored by replacement of the sites with aspartic acid residues (14), consistent with the presence of negative charges in this domain promoting an efflux permissive conformation of the protein. Thus the PKC-dependent METH-induced metabolic phosphorylation of DAT demonstrated here possesses characteristics compatible with efflux properties and processes implicated by mutagenesis approaches. Substrate-induced phosphorylation of DAT may therefore play a role in the in vivo reversal of DA transport related to dendrodendritic-inhibition of dopaminergic neurons and dopaminergic neural sensitivity (29).

The ability of amphetamines to promote DAT phosphorylation appears to be dependent on drug binding or transport, as METH-induced phosphorylation is blocked by cocaine and does not require DA receptor activation. Thus in cells, drug binding or active transport is sufficient as well as necessary for METH-stimulated DAT phosphorylation. The same may occur in the brain, although we cannot exclude the possibility that DA efflux stimulated by in vivo METH may activate neuronal receptors or neural circuits that could modulate DAT phosphorylation. However, the physiological pathways controlling DAT phosphorylation in vivo are not known and we did not attempt to address this question with respect to METH in striatal tissue.

In contrast to the ability of substrates to induce transport down regulation, we found that DA uptake was not significantly altered by acute cocaine pretreatment. This differs from the finding of cocaine-induced surface recruitment and upregulation of DAT reported in hDAT EM4 cells (20) but matches results obtained in rat striatal synaptosomes and hDAT HEK cells (15,17). While cocaine may function to prevent or counteract substrate-induced down-regulation, our findings indicate that these processes occur independently of increases or decreases in DAT phosphorylation. Additional studies are warranted, however, as it cannot be ruled out that specific subcellular pools of DAT or subsets of DAT phosphorylation sites may respond to cocaine but be masked by the overall phosphorylation signal, that a transient response to cocaine occurred that was concluded within our treatment intervals, or that DAT phosphorylation might be affected by cocaine in cell lines that support cocaine-induced upregulation.

The mechanism leading to increased phosphorylation of DAT by amphetamines is unknown. One possibility is that in the brain amphetamines regulate neuronal circuits or neurotransmitter levels linked to PKC or other signals that alter DAT phosphorylation. Since such processes are unlikely to occur in isolated cultured cells, a second possibility is that translocation of amphetamines into the cell interior affects physiological processes, for example by altering intracellular H⁺, Na⁺ or Ca²⁺ levels that lead to changes in the activities of DAT kinases or phosphatases or by affecting PKC subcellular localization (5,30-33). This could be compatible with the necessity for high drug doses to elicit responses if lower doses of drug entering the cells are insufficient to induce these physiological changes. A mechanism of this type could also explain down-regulation or internalization processes independent of direct DAT phosphorylation, as PKC activation or translocation could lead to phosphorylation of accessory proteins required for these processes. A third possibility is that changes in DAT conformation or protein-protein interactions occurring during the substrate binding or transport cycle alter its ability to be phosphorylated or dephosphorylated. However, our lab has found that DAT phosphorylation is not significantly changed by doses of DA equal to those used in this study for METH (27). This potentially indicates that conformational changes produced by substrate transport and/or binding are by themselves insufficient to lead to phosphorylation, unless the mechanisms of endogenous and psychostimulant substrate transport vary in a way that can allow such a difference.

It is not known if substrate- or PKC-induced regulation of DA transport or efflux occurs through effects on the intrinsic activity of surface transporters or by regulation of plasma membrane expression, and some evidence has been obtained in support of both processes (34,35). Evidence consistent with surface regulation of related γ-aminobutyric acid (GAT1) and norepinephrine transporters comes from studies showing PKC- and substrate-dependent regulation of transport capacity via interactions of transporter N-terminal
tail domains with the plasma membrane SNARE protein syntaxin 1A (36-38). We have recently found that cleavage of syntaxin 1A with botulinum toxin C leads to increased DA transport and reduced phosphorylation of rat striatal DAT (M. Cervinski and R. Vaughan, unpublished data), suggesting a potentially similar regulatory mechanism for DAT that involves the transporter phosphorylation state.

Modulation of transporter phosphorylation and function by substrates may be a common theme for neurotransmitter uptake carriers. Recently serotonin transporter (SERT) phosphorylation has been demonstrated to be increased by AMPH through mitogen-activated protein kinase dependent mechanism (39). Furthermore, amphetamine-stimulated reverse transport of substrates through SERT and SERT-GAT1 concatamers is attenuated by PKC inhibitors (40). However, PKC-induced phosphorylation, endocytosis, and down regulation of SERT are also attenuated during transport of serotonin and AMPH, correlating with retention of SERT at the plasma membrane under conditions of high transport demand (41). Similarly, substrate-induced upregulation of GAT1 activity and surface expression is counteracted by PKC-dependent phosphorylation (36,42). Thus for GAT1 and SERT, substrates induce homeostatic mechanisms that counteract PKC-dependent phosphorylation and down-regulation and lead to promotion of transporter surface expression and substrate clearance, whereas for DAT PKC is required for both substrate-induced down regulation and efflux, which together lead to reduced transmitter clearance.

The finding that DA clearance is reduced by substrates seems counterintuitive, but in the case of DAT such a process may be compatible with a protective response that limits the intracellular accumulation of neurotoxic substrates such as AMPH, METH, and 6-OHDA. The high METH doses required to induce DAT phosphorylation and down regulation (10–100 μM) are consistent with this idea, as these concentrations are substantially above the K_i for inhibition of DA transport (290 nM) (43) and compatible with responses occurring primarily at high transport velocities in which significant amounts of drug may be imported into the neuron. These responses might therefore only occur to significant levels under conditions of extreme transport challenge such as high dosage drug administration known to cause neuronal damage. If substrate-induced DAT phosphorylation, trafficking, or down-regulation are related to a neuroprotective mechanism, then individual variability in these processes may contribute to vulnerability to drug abuse or toxic insults that lead to neurodegenerative pathologies. Clarification of the mechanisms involved in these processes may reveal molecular opportunities for therapeutic interventions for regulation of DAT activity and fine-tuning of DA levels in psychiatric disorders.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. METH induces down-regulation of DA transport in rDAT LLC- PK1 cells. LLC-PK1 cells expressing rDAT were treated with (A) vehicle (control), 200 nM PMA or 10 μM METH for 30 min, (B) vehicle 10 μM METH for 0, 1, 2, 5, 10, 20, or 30 min, or (C) the indicated doses of METH for 30 min. Cells were washed to remove pretreatment drug followed by assay for [3H]DA transport. Values shown are means ± S.E. of 3 or indicated number of independent experiments, performed in triplicate. *, p<0.05; **, p<0.01; ***, p<0.001 relative to control by ANOVA.

Figure 2. AMPH and METH induce DAT phosphorylation in rDAT LLC-PK1 cells. LLC-PK1 cells expressing rDAT were metabolically labeled with ³²PO₄ and treated with or without 2 μM AMPH or 10 μM OA for 30 minutes (left three lanes), or 10 μM METH for 10 minutes or 1 μM PMA for 20 minutes (right three lanes), followed by immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panel: Autoradiographs of representative experiments, lanes correspond to treatments indicated directly below on histogram. The order of some lanes was altered for consistent data presentation. Lower panel: Summary of DAT phosphorylation levels (means ± S.E. of 8 or 11 independent experiments, performed in duplicate for AMPH or METH respectively). **, p<0.01 relative to basal; ***, p<0.001 relative to basal; [p<0.001 relative to AMPH; [p<0.001 relative to METH by ANOVA.

Figure 3. METH induces DAT phosphorylation in rat striatal tissue. Rat striatal slices were metabolically labeled with ³²PO₄ and treated with (A) vehicle or 10 μM METH for 10 minutes or 10 μM OA for 20 minutes, (B) the indicated doses of METH for 10 minutes, or (C) 10 μM METH for the indicated times, followed by immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panels: Autoradiographs of representative experiments, lanes correspond to treatments indicated directly below on histogram. Lower panel: Summary of DAT phosphorylation levels (means ± S.E. of 10 or indicated number of independent experiments performed in duplicate). **, p<0.01; ***, p<0.001 relative to basal by t-test.

Figure 4. In vivo METH increases DAT phosphorylation. Rats were injected with saline or 15 mg/kg METH 30 minutes prior to decapitation. Striata from both hemispheres of each animal were isolated, sliced, and labeled with ³²PO₄ for 30 minutes. Tissue from one hemisphere of each animal received no
additional treatment while 1 µM OA was applied to the matching hemispheres. Samples were subjected to immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panel: Autoradiograph of representative experiment, lanes correspond to treatments indicated directly below on histogram. Lower panel: Summary of DAT phosphorylation levels (means ± S.E. of 5 independent experiments). *, p<0.05; ***, p<0.001, relative to basal by t-test

Figure 5. DAT phosphorylation is not affected by cocaine. Rat striatal slices metabolically labeled with $^{32}$PO$_4$ were treated with (A) 30 µM (-)-cocaine for 15 minutes or 10 µM OA for 20 minutes or (B) 30 µM (-)-cocaine for the times indicated, followed by immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panel: Autoradiograph of representative experiment, lanes correspond to treatments indicated directly below on histogram. Lower panels: Summary of DAT phosphorylation levels (means ± S.E. of 4 or indicated number of independent experiments performed in duplicate). *, p<0.05 relative to basal by ANOVA.

Figure 6. METH-induced phosphorylation and down-regulation of DAT are blocked by cocaine. (A) rDAT LLC-PK$_1$ cells were metabolically labeled with $^{32}$PO$_4$ and pre-incubated for 5 minutes with or without 100 µM (-)-cocaine followed by the addition of 10 µM METH for an additional 10 minutes. Samples were subjected to immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panel: Autoradiograph of representative experiment, lanes correspond to treatments indicated directly below on histogram. The order of some lanes was altered for consistent data presentation. Lower panel: Summary of DAT phosphorylation levels (means ± S.E. of 4 independent experiments performed in triplicate). ***, p<0.001 relative to basal by ANOVA. (B) rDAT LLC-PK$_1$ cells were pre-treated for 5 minutes with or without 10 µM (-)-cocaine followed by the addition of 10 µM METH for an additional 10 minutes. Cells were washed to remove drug and assayed for $[^3]$H]DA uptake. Results shown are means ± S.E. of 3 independent experiments, performed in triplicate. ***, p<0.001 relative to control by ANOVA.

Figure 7. METH-induced DAT phosphorylation and down-regulation are PKC dependent. (A) rDAT LLC-PK$_1$ cells were metabolically labeled with $^{32}$PO$_4$ and pre-incubated for 5 minutes with or without 10 µM bisindoylmaeimide 1 (BIM) followed by addition of 10 µM METH for an additional 10 minutes. Samples were subjected to immunoprecipitation, SDS-PAGE and autoradiography of DAT. Upper panel: Autoradiograph of representative experiment, lanes correspond to treatments indicated directly below on histogram. The order of some lanes was altered for consistent data presentation. Lower panel: Summary of DAT phosphorylation levels (means ± S.E. of 4 independent experiments performed in triplicate). *, p<0.05 relative to basal by ANOVA. (B) rDAT LLC-PK$_1$ cells were pre-treated for 10 min with or without 10 µM BIM or 1 µM H89 followed by addition of 10 µM METH or 1 nM PMA for an additional 10 minutes. Cells were washed to remove drug and assayed for $[^3]$H]DA uptake. Results shown are means ± S.E. of 3 independent experiments performed in triplicate. ***, p<0.001 relative to control by ANOVA.

Figure 8. METH-induced DAT phosphorylation but not down-regulation requires N-terminal serines. (A) LLC-PK$_1$ cells expressing wt or $\Delta^{21}$ rDAT were metabolically labeled with $^{32}$PO$_4$ and treated with 10 µM METH for 10 min or 10 µM PMA for 30 min followed by immunoprecipitation, SDS-PAGE and autoradiography of DAT. The upper panel shows an autoradiograph of a representative experiment. The lower panel shows an immunoblot of wt and $\Delta^{21}$ rDAT proteins from whole cell lysates. The order of some lanes was altered for consistent data presentation. (B) LLC-PK$_1$ cells expressing $\Delta^{21}$ rDAT were treated with 10 µM METH or 10 nM PMA for 10 min. Cells were washed to remove drug and assayed for $[^3]$H]DA uptake. Results shown are means ± S.E. of 3 independent experiments performed in triplicate. ***, p<0.001 relative to control by ANOVA.
Figure 2
Figure 3
Figure 4.
Figure 5

A. 

B. 

% Basal Phosphorylation

Time (min)

(2) (3) (2) (4) (2)

(2)
Figure 7

A. 

B. 

[Graph showing % Basal Phosphorylation and [H] DA Uptake (% Control) with bars for different conditions: BIM, METH, H89, METH, PMA]
Figure 8

A.

B. 

[\text{[^{3}H]}DA Uptake (% Control)]

- Control
- METH
- PMA

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Psychoactive substrates stimulate dopamine transporter phosphorylation and down regulation by cocaine sensitive and protein kinase C dependent mechanisms
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