Reciprocal Phosphorylation and Palmitoylation Control Dopamine Transporter Kinetics*

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The dopamine transporter is a neuronal protein that drives the presynaptic reuptake of dopamine (DA) and is the major determinant of transmitter availability in the brain. Dopamine transporter function is regulated by protein kinase C (PKC) and other signaling pathways through mechanisms that are complex and poorly understood. Here we investigate the role of Ser-7 phosphorylation and Cys-580 palmitoylation in mediating steady-state transport kinetics and PKC-stimulated transport down-regulation. Using both mutational and pharmacological approaches, we demonstrate that these post-translational modifications are reciprocally regulated, leading to transporter populations that display high phosphorylation-low palmitoylation or low phosphorylation-high palmitoylation. The balance between the modifications dictates transport capacity, as conditions that promote high phosphorylation or low palmitoylation reduce transport Vmax and enhance PKC-stimulated down-regulation, whereas conditions that promote low phosphorylation or high palmitoylation increase transport Vmax and suppress PKC-stimulated down-regulation. Transitions between these functional states occur when endocytosis is blocked or undetectable, indicating that the modifications kinetically regulate the velocity of surface transporters. These findings reveal a novel mechanism for control of DA reuptake that may represent a point of dysregulation in DA imbalance disorders.

The dopamine transporter (DAT)4 is a plasma membrane protein that actively transports extracellular dopamine (DA) into presynaptic dopaminergic neurons after transmitter release. Reuptake is the primary mechanism for DA clearance in the brain and controls the spatial and temporal availability of transmitter during neurotransmission (1). DAT is a target for addictive and therapeutic drugs that inhibit uptake or stimulate transmitter efflux and induce effects by increasing extracellular DA levels (2). Transport activity is tightly controlled by many signaling pathways that modulate reuptake in response to specific physiological demands (3–5), and dysregulation of these processes has been postulated to contribute to aberrant transmitter clearance in DA imbalance disorders such as Parkinson disease, schizophrenia, bipolar disorder, and attention deficit hyperactivity disorder (4–7).

Activation of protein kinase C (PKC) has been well characterized to reduce DA transport Vmax and stimulate dynamin- and clathrin-dependent DAT endocytosis (5, 8, 9). Most studies to date have focused on membrane trafficking as the mechanism underlying PKC-induced transport reductions (10–14), but recent findings have shown that significant levels of down-regulation are retained when transporter endocytosis is blocked or impaired (15–17), indicating the presence of a kinetic regulatory mechanism that operates in concert with internalization.

The cytoplasmic domains of DAT contain sites for post-translational modifications that regulate various aspects of its functions (4, 5, 18). DAT undergoes PKC- and protein phosphatase 1-dependent phosphorylation on Ser-7 and other serines near the distal end of the N terminus (19, 20). These resi...

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3 The abbreviations used are: DAT, dopamine transporter; rDAT, rat DAT; DA, dopamine; LLCPK1, Lewis lung carcinoma-porcine kidney cells; 2BP, 2-bromopropionamide; ABE, acyl-biotinyl exchange; BIM, bisindolylmaleimide; ConA, concanavalin A; MMTS, methyl methanethiosulfonate; mAb, monoclonal antibody; OA, okadaic acid; OAG, oleoyl-2-acetyl-sn-glycerol; PAT, palmitoyl acyltransferase; PMA, phorbol 12-myristate 13-acetate; polyclonal antibody; 16: HDPP, bovine; (N-(6-biotinamido)hexyl)-3’-(2’-pyridyldithio)-propionamide; TM, transmembrane domain; ANOVA, analysis of variance.
dues are not necessary for PKC-stimulated endocytosis of the transporter (21, 22), but their role in kinetic regulation of uptake has not yet been investigated. DAT is also palmitoylated on Cys-580 at the membrane-cytoplasm interface of the most C-terminal transmembrane helix (17). Acute inhibition of transporter palmitoylation enhances PKC-stimulated down-regulation and induces significant trafficking-independent decreases in transport $V_{\text{max}}$ (17), also suggesting a role for this modification in catalytic regulation of transport.

Here we investigate the relationship between Ser-7 phosphorylation and Cys-580 palmitoylation of DAT in steady-state transport kinetics and PKC-stimulated down-regulation, finding that these modifications are reciprocally regulated and induce port kinetics and PKC-stimulated down-regulation, and Cys-580 palmitoylation of DAT in steady-state trans-

**Phosphorylation and Palmitoylation of DAT**

**Experimental Procedures**

**Materials**—[7,8,-$^3$H]DA (45 Ci/mmol) was from PerkinElmer Life Sciences; [9,10-$^3$H]palmitic acid (73.4 Ci/mmol) was from Moravek; $H_2^{32}$PO$_4$ was from MP Biomedicals; Fluoro-Hance fluorographic reagent was from Research Products International; DA was from Research Biochemicals International; phorbol 12-myristate,13-acetate (PMA), oleoyl-2-acetyl-sn-glycerol (OAG), and okadaic acid (OA) were from EMD Millipore; DAT polyclonal antibody 16 (poly16) and monoclonal antibody 16 (mAb 16) were as previously described (23, 24); polyclonal antibodies for Rab5A, Rab7, and Na$^+$/K$^+$ ATPase were from Santa Cruz Biotechnology; those for Rab11 were from BD Biosciences, and for HA were from Covance; X-tremeGENE HP transfection reagent was from Roche Applied Bioscience; methyl methanethiosulfonate (MMTS), HPDP biotin (sulphydryl-reactive (N-(6-(biontinamido)hexyl))-3’-(2’-pyridyldithio)-propionamide), sulfo-NHS-SS-biotin, high capacity NeutrAvidin-agarose resin, protease inhibitor tablets, and bicinchoninic acid protein assay reagent were from Thermo Scientific; PitStop$^2$ was from Abcam Biochemicals; (−)-cocaïne and other fine chemicals were from Sigma. Rats were purchased from Charles River Laboratories. All animals were housed and treated in accordance with regulations established by the National Institutes of Health and approved by the University of North Dakota Institutional Animal Care and Use Committee.

**DAT Phosphorylation**—For experiments in heterologously expressing cells, LLCPK$_1$ cells stably expressing WT or mutant rat DATs (rDATs) were cultured and analyzed for phosphorylation as previously described (22). For DHHC experiments, cells were co-transfected with the indicated plasmids 24 h before the start of the experiment. Plasmids encoding HA-tagged DHHC2 and GST were the generous gift of Dr. Masaki Fukata, National Institute for Physiological Sciences, Japan. DHHA2 negative control with residue Cys-156 changed to alanine to create an inactive protein was created from DHHC2 by site-directed mutagenesis with codon substitution verified by sequencing (Eurofins MWG), and expression of all constructs was verified by anti-HA immunoblotting (not shown). Cells were labeled with phosphate-free medium containing 0.5–1 mCi/ml $^{32}$P for 2 h at 37 °C followed by application of test compounds for the indicated times. Cells were washed, pelleted by centrifugation at 2000 × g for 5 min at 4 °C, and lysed with 0.1% Triton X-100. Lysates were centrifuged at 4000 × g for 2 min, and resulting supernatants were adjusted to contain 0.5% Triton and centrifuged at 20,000 × g for 30 min to remove insoluble material. DATs were immunoprecipitated with poly16 followed by SDS-PAGE and autoradiography (19, 23). For experiments in brain tissue, rat striatal synaptosomes were prepared and labeled with $^{32}$P as previously described (25).

Briefly, synaptosomes prepared in sucrose-phosphate buffer (0.32 m sucrose and 10 mm sodium phosphate, pH 7.4) at 120 mg/ml original wet weight were diluted 4-fold in oxygenated Krebs bicarbonate buffer (25 mm NaHCO$_3$, 125 mm NaCl, 5 mm KCl, 1.5 mm CaCl$_2$, 5 mm MgSO$_4$ and 10 mm glucose, pH 7.3) containing $^{[32P]}$orthophosphate (2 mCi/ml) and $10 \mu$g OA to suppress tonic dephosphorylation. Samples were treated with vehicle (DMSO), 10 μm 2-bromopalmitate (2BP), or 1 μm PMA and incubated at 30 °C for 45 min. Synaptosomes were then placed on ice and washed 3 times with ice-cold Krebs bicarbonate buffer by centrifugation at 17,000 × g for 12 min. Final synaptosomal pellets were solubilized in 100 μl of lysis buffer (60 mm Tris, pH 6.8, 0.5 mm SDS, 10% glycerol, 100 mm DTT, and 3% β-mercaptoethanol with 4 passages through a 26-gauge needle. Insoluble material was removed by centrifugation at 150,000 × g for 20 min, and resulting supernatants were diluted 5-fold for DAT immunoprecipitation with poly16 followed by SDS-PAGE and autoradiography. Total DAT levels in each sample were determined by immunoblotting using mAb 16 (23).

**DAT Palmitoylation**—$[^{3}H]$Palmitate labeling was performed in rDAT-LLCPK$_1$ cells by incubation for 6–18 h with medium containing 0.5 mCi/ml $[^{3}H]$palmitate and treatment with vehicle or test compounds for an additional 60 min. The cells were lysed with radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 125 mm sodium phosphate, 150 mm NaCl, 2 mm EDTA, 50 mm sodium fluoride), and lysates were subjected to immunoprecipitation with poly16 followed by SDS-PAGE, treatment of gels with Fluoro-Hance, and autoradiography.

Analysis of palmitoylation by acyl-biotinyl exchange (ABE) was performed as described (17). For experiments in cells, lysates were prepared as above with 20 mm MMTS added to the lysis buffer to block free thiols. For experiments using brain tissue, rat striatal slices were preincubated in oxygenated Krebs bicarbonate buffer for 30 min at 30 °C with shaking at 105 rpm followed by treatment with vehicle or 1 μm OA plus 10 μm OAG for an additional 30 min. Oxygen (95% O$_2$, 5% CO$_2$) was gently blown across the top of the plate during the incubation. Tissue was homogenized in ice-cold sucrose-phosphate buffer with 15 strokes in a glass/Teflon homogenizer and centrifuged at 3000 × g for 3 min at 4 °C, and the resulting supernatant was centrifuged at 17,000 × g for 12 min. The resulting P2 synaptosomal pellet was resuspended to 50 mg/ml original wet weight.
in ice-cold sucrose-phosphate buffer. The synaptosomal suspension was centrifuged at 20,000 × g for 12 min at 4 °C and solubilized in lysis buffer (50 mM HEPES, pH 7.0, 2% SDS, 1 mM EDTA) containing protease inhibitors and 20 mM MMTS. Cell and synaptosomal lysates were then incubated at room temperature for 1 h with mixing followed by acetone precipitation and resuspension in lysis buffer containing MMTS and incubation at room temperature overnight with end-over-end rotation. Excess MMTS was removed by three sequential acetone precipitations followed by resuspension of precipitated proteins in 200 μl of a buffer containing 4% (w/v) SDS (4SB: 4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4). Each sample was divided into two equal portions that were treated for 2 h at room temperature with 50 mM Tris-HCl, pH 7.4, as control or 0.7 mM hydroxylamine (NH₂OH), pH 7.4, which cleaves the thioester bonds and removes endogenous palmitate groups. NH₂OH was removed by three sequential acetone precipitations followed by resuspension of the precipitated proteins in 240 μl of 4SB buffer. Samples were diluted with 900 μl of 50 mM Tris-HCl, pH 7.4, containing 0.4 mM HPDP biotin to label the liberated sulfhydryl groups and incubated at room temperature for 1 h with end-over-end mixing. Unreacted HPDP biotin was removed by three sequential acetone precipitations followed by resuspension of the final pellet in 75 μl of lysis buffer without MMTS. Samples were adjusted to contain 0.1% SDS by the addition of 50 mM Tris-HCl, pH 7.4, and biotinylated proteins were extracted using NeutrAvidin resin. Proteins bound to the resin were eluted with sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol) containing 100 mM DTT plus 3% β-mercaptoethanol and subjected to SDS-PAGE. DAT was identified by immunoblotting using mAb 16 (23). In all experiments the level of signal intensity from Tris controls was <5% of NH₂OH levels.

**Immunoprecipitation, Electrophoresis, and Immunoblotting—**
Immunoprecipitation of 32P- or [³H] palmitate-labeled DATs was conducted for 2 h at 4 °C using protein A-Sepharose beads cross-linked with poly16 antibody (17, 19), and the precipitated samples were electrophoresed on 4–20% SDS-PAGE gels. Gels were dried and exposed to x-ray film for detection of radioactive activity. For immunoblotting, proteins were subjected to SDS-PAGE, transferred to PVDF membranes, and DATs were detected with mAb 16 as previously described (23). For all experiments, palmitoylated or phosphorylated DAT band intensities were quantified by densitometry using Quantity One (Bio-Rad) software and normalized to the amount of total DAT protein in the sample, and signals for treatment groups were expressed relative to control samples set to 100%. For cell surface expression determination WT or mutant rDAT-LLCPK₁ cells were incubated with the membrane-impermeable biotinylating reagent sulfo-NHS-SS-biotin, and biotinylated DATs were purified from cell lysates (25 μg of protein) by chromatography on NeutrAvidin beads, separated by SDS-PAGE, and quantified by immunoblotting (16). All experiments were performed a minimum of three times with similar results, and values were analyzed for statistical significance using Student’s t test or one way ANOVA as indicated.

**Site-directed Mutagenesis—**S7A, C522A, and C580A DAT mutants used in this study were characterized previously (17, 20). S7A/C580A DAT was produced from a pcDNA 3.0 plasmid containing the DAT C580A cDNA sequence using the Stratagene QuikChange® kit with codon substitution verified by sequencing (Eurofins MWG). For production of stable transformants, LLCPK₁ cells were transfected using X-tremeGENE transfection reagent and 0.5 μg of the mutant plasmid. Transformants were selected 24–48 h later by the addition of 800 μg/ml Geneticin (G418) to the cell culture medium. All mutants were active for [³H]DA transport, expressed at 30–50% that of the WT protein level, and showed ratios of total to cell surface expression that were comparable with that of the WT protein.

**[³H]DA Uptake—**WT or mutant rDAT-LLCPK₁ cells were grown in 24-well plates to ~80% confluence and rinsed twice with 0.5 ml of 37 °C Krebs-Ringer/HEPES buffer (KRH: 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). Where indicated cells were incubated at 37 °C for 30 min with vehicle or 1 μM PMA or with 250 μg/ml concanavalin A (ConA) for 30 min before the addition of PMA. Uptake was performed in triplicate for 8 min, with nonspecific uptake determined with 100 μM (-)-coca ine. For single point assays uptake was initiated by the addition of 10 nM [³H]DA plus 3 μM DA. For saturation analyses DA was varied from 1 to 30 μM. Cells were rapidly washed 2 times with ice-cold Krebs-Ringer/HEPES buffer and solubilized in 1% Triton X-100, and radioactivity contained in lysates was assessed by liquid scintillation counting. Kinetic values were analyzed by Prism, and Vₘₐₓ values for each form were normalized to surface expression transporter levels determined in parallel for each experiment.

**Subcellular Localization of Phosphorylated and Palmitoylated DATs—**Subcellular fractions were generated by differential centrifugation as described (26). LLCPK₁ cells stably expressing WT rDAT were cultured in 150-mm dishes, grown to ~90% confluence, labeled with ³²P when indicated, and treated with vehicle or 1 μM PMA for 30 min. Cells were washed, scraped, and pelleted by centrifugation at 700 × g for 5 min at 4 °C. Pelleted cells were resuspended in Buffer C (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.8) and homogenized in ice-cold Buffer C with 30 strokes in a Dounce homogenizer. Homogenates were centrifuged at 700 × g to remove nuclei, and post-nuclear supernatants (totals) were centrifuged sequentially at 16,000 × g for 12 min (16,000 × g membranes) and at 200,000 × g for 1 h (200,000 × g membranes). Resulting pellets were resuspended at the original volume in radioimmunoprecipitation assay buffer containing protease inhibitors. Fractions were immunoprecipitated for DAT, Na⁺/K⁺ ATPase, Rab5A, Rab7, and Rab11 or analyzed by ABE for detection of DAT palmitoylation. ³²P-Labeled fractions were immunoprecipitated with poly16 followed by SDS-PAGE and autoradiography for detection of DAT phosphorylation.

**Results**

**C580A DAT Displays Enhanced Phosphorylation on Ser-7—**To determine if phosphorylation of DAT is affected by its level of palmitoylation, we examined the ³²P labeling of C580A DAT, which shows an ~50% reduction in palmitoylation, and C522A
DAT, which shows no reduction in palmitoylation (17) as a specificity control. LLCPK1 cells expressing WT, C522A, or C580A DATs were labeled with $^{32}$P and treated with vehicle or PMA to activate PKC, and equal amounts of DAT were immunoprecipitated and analyzed by SDS-PAGE/autoradiography (Fig. 1A). WT DAT displayed typical levels of basal phosphorylation (263 ± 77% of basal ($p < 0.001$)). C522A DAT showed the same pattern and intensity of labeling, indicating no effect of this residue on phosphorylation. In contrast, $^{32}$P labeling of C580A DAT was enhanced in both basal (263 ± 25% of WT basal, $p < 0.05$) and PMA-stimulated conditions (1154 ± 129% of WT basal, $p < 0.001$ versus C580A basal; $p < 0.01$ versus WT PMA), indicating that lack of palmitoylation on Cys-580 results in enhanced basal and stimulated phosphorylation.

$^{32}$P labeling of DAT reflects modification of all phosphorylation sites, including those in the distal PKC domain and in a membrane proximal proline-directed site (19, 20, 22, 27). Multiple serines in the distal N terminus are phosphorylated, but to date only Ser-7 has been identified as PKC-dependent (20). We thus generated the double mutant S7A/C580A as an initial approach to identify the site of enhanced phosphorylation on C580A DAT (Fig. 1B). Labeling of WT, C580A, and S7A/C580A forms in parallel with $^{32}$P shows PMA-stimulated phosphorylation of WT DAT (329 ± 72% of basal) and enhanced PMA-stimulated phosphorylation of C580A DAT (1178 ± 60% of WT basal; $p < 0.001$ versus WT PMA). PMA-stimulated phosphorylation of S7A/C580A DAT (818 ± 31% of WT basal) was significantly lower than the C580A PMA value ($p < 0.05$), indicating that a major fraction of the increased phosphorylation of C580A DAT occurred on Ser-7. Phosphorylation of S7A/C580A DAT remained elevated compared with the WT protein, however ($p < 0.01$ versus WT PMA), indicating that loss of Cys-580 palmitoylation leads to enhanced phosphorylation of other residues as well.

S7A DAT Displays Enhanced Palmitoylation on Cys-580—We then performed the reverse experiment to determine if Cys-580 palmitoylation is affected by Ser-7 phosphorylation. LLCPK1 cells expressing WT, S7A, C580A, or S7A/C580A rDATs were labeled with $[^3]H$palmitate, and equal amounts of DAT were immunoprecipitated and analyzed by SDS-PAGE/fluorography (Fig. 1C). WT DAT displayed a tonic level of $[^3]H$palmitate labeling that reflects steady-state palmitate turnover, whereas S7A DAT, which shows ~50% reduction in phosphorylation (20), shows strongly enhanced $[^3]H$palmitate labeling (219 ± 34% relative to WT; $p < 0.05$). Parallel analysis of C580A and S7A/C580A DATs to investigate the site of increased palmitoylation showed that C580A DAT labeling was reduced to about half the WT level (57 ± 10% of WT; $p < 0.001$), consistent with our previous findings (17). Palmitoylation of S7A/C580A DAT was similarly reduced relative to the WT protein (55 ± 26% of WT; $p < 0.01$) and was significantly reduced relative to S7A DAT ($p < 0.05$ versus S7A), indicating that the enhanced palmitoylation seen on S7A DAT occurred on Cys-580.

DAT Palmitoylation Is Regulated by Phosphorylation Modulators—These results demonstrate that prevention of Ser-7 phosphorylation or Cys-580 palmitoylation by mutagenesis leads to enhancement of the alternate modification. To determine if these changes are indirect effects of mutagenesis or are specific to the modifications, we then determined if similar changes would result from pharmacological modulation of the modifications in the WT protein. For analysis of rDAT palmitoylation changes in LLCPK1 cells we performed...
Using ABE we found that palmitoylation of WT DAT was negatively regulated by PKC in both cells and brain tissue, suggesting this as a possible mechanism linking palmitoylation to PKC-dependent regulation of transport.

PKC regulation of palmitoylation was mediated by phosphorylation to PKC-dependent regulation of transport. Protein palmitoylation is negatively regulated by PKC in both cells and brain basal (Fig. 2B). These results demonstrate that DAT palmitoylation is negatively regulated by PKC in both cells and brain tissue, suggesting this as a possible mechanism linking palmitoylation to PKC-dependent regulation of transport.

Because Ser-7 is a known PKC target on DAT, we examined the palmitoylation responsiveness of S7A DAT to determine if PKC regulation of palmitoylation was mediated by phosphorylation of this site or via an independent mechanism (Fig. 2C). Analysis of DAT palmitoylation by ABE showed that OA/OAG induced a 15 ± 5% reduction in palmitoylation (p < 0.05 versus basal) (Fig. 2B). These results demonstrate that DAT palmitoylation is negatively regulated by PKC in both cells and brain tissue, suggesting this as a possible mechanism linking palmitoylation to PKC-dependent regulation of transport.

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DAT Phosphorylation Is Regulated by Palmitoylation Modulators—We then sought to determine if pharmacological modulation of DAT palmitoylation would alter its phosphorylation state. Protein palmitoylation is controlled by protein acyltransferases (PATs) that catalyze palmitoyl addition and acyl protein thioesterases and protein palmitoyl thioesterases that catalyze palmitate removal (28), but there are few specific pharmacological activators or inhibitors available for the multiple known PATs, acyl protein thioesterases, and protein palmitoyl thioesterases. To assess the effects of reduced palmitoylation on DAT phosphorylation, we thus used the nonspecific PAT inhibitor 2BP, which rapidly reduces DAT palmitoylation in rat striatal synaptosomes by ~50% without changing protein levels (17). Using [32P]-labeled rat striatal synaptosomes, we found that acute 2BP application did not change total DAT protein but caused an increase in DAT phosphorylation (154 ± 20% of basal, p < 0.05) that was comparable to the stimulation induced in parallel samples by PMA (Fig. 3A). As 2BP has no known ability to stimulate PKC or other kinases (29), this indicates that the increased DAT phosphorylation results from acute suppression of the palmitoylation state.

To examine the effects of enhanced DAT palmitoylation on phosphorylation levels, we co-expressed rDAT-LLCPK1 cells with the PAT enzyme DHHC2, which we recently identified as one of a subset of neuronal PATs that enhance palmitoylation of DAT on Cys-580 (30). Our findings show that co-expression of DHHC2 reduced the [32P] labeling of DAT to 84 ± 4% that of control (p < 0.01), whereas co-expression of DHHA2, the inactive form of the enzyme that does not increase DAT palmitoylation (30), does not affect [32P] labeling (120 ± 11%; p > 0.05 versus DHHC2) (Fig. 3B). Together these results support the reciprocal regulation of DAT phosphorylation level by pharmacological modulation of Cys-580 palmitoylation in both cells and brain tissue.

Ser-7 and Cys-580 Differentially Regulate Steady-state DA Transport Capacity—Previous studies have shown that DA transport V_{max} is reduced by conditions that stimulate phosphorylation of the DAT distal N terminus (8, 9) and by inhibition of Cys-580 palmitoylation (17), suggesting that transport is decreased by phosphorylation of Ser-7 and other N-terminal
Ser-7 and Cys-580 Differentially Control Kinetic Down-regulation of Transport—We then used WT, S7A, and C580A DATs to investigate the role of Ser-7 phosphorylation and Cys-580 palmitoylation in PMA-induced transport down-regulation (Fig. 5). In untreated cells, where DATs can undergo constitutive and regulated endocytosis (Fig. 5A), WT DATs showed a typical level of down-regulation (79 ± 1% of control; p < 0.001), and C580A DATs showed a statistically greater level of down-regulation (73 ± 2% of C580A control, p < 0.001; p < 0.01 versus WT PMA), consistent with our previous results (17). In contrast, S7A DATs showed less down-regulation (89 ± 3% of S7A control; p < 0.001) than either the WT (p < 0.001 versus WT PMA) or C580A forms (p < 0.001 versus C580A PMA).

To determine if these down-regulation differences arise from effects on endocytosis, we then examined the responses of the mutants when internalization was blocked. Several studies have shown by both surface biotinylation and confocal microscopy that stimulated internalization of DAT in heterologous expression systems and cultured neurons can be prevented with inhibitors of clathrin-mediated endocytosis including clathrin and dynamin siRNA (14, 31, 32), dynamin dominant negative suppression (16, 33), and ConA (16, 33).

For these experiments we used acute ConA to inhibit DAT endocytosis, as this treatment has no effect on the total expression, resting surface levels, or basal transport activity of DAT (33). With ConA pretreatment the magnitude of PKC-induced down-regulation for all DAT forms was significantly less than the levels observed in control cells (WT ConA versus WT control, p < 0.001; S7A ConA versus S7A control, p < 0.05; C580A ConA versus C580A control, p < 0.001) (Fig. 5B), consistent with reduction of down-regulation due to suppression of endocytosis. The extent of this reduction was similar for all three DAT forms, suggesting that in control conditions each of the transporters undergoes a similar level of PKC-induced endocytosis. Although further work is needed to more fully evaluate this issue, this interpretation is consistent with findings that transporter internalization does not require N-terminal phosphorylation (21) and suggests that Cys-580 palmitoylation also does not play a major role in this process.

Down-regulation of WT DAT in the presence of ConA (88 ± 1% of control, p < 0.001 versus WT basal) was about half the level seen in control conditions, which is consistent with our previous results (16) and indicates that transport reductions in control conditions occurred via relatively equal contributions from trafficking and non-trafficking processes. Strikingly, in ConA-treated cells, S7A DAT showed a complete absence of PMA-induced down-regulation (99 ± 4% of S7A basal, p > 0.05; p < 0.001 versus WT PMA; Fig. 5B), indicating that in control conditions most of its transport reduction occurred via endocytosis and that its lesser overall down-regulation was due to reduced kinetic regulation. In contrast, down-regulation of C580A DATs in ConA-treated cells (82 ± 3% of C580A basal, p < 0.001) remained greater than both WT (p < 0.05 versus WT PMA) and S7A DATs (p < 0.001 versus S7A PMA), indicating that its greater transport reduction in both control and ConA-treatment conditions was due to enhanced kinetic down-regulation. The rank order of kinetic down-regulation of these forms (C580A > WT > S7A) thus correlates positively with the
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Phosphorylation and Palmitoylation Are Present on Surface Transporters—Kinetic regulation of uptake by phosphorylation and palmitoylation requires that the modifications be present on surface transporters. To investigate this, rDAT-LLCPK1 cells expressing the indicated DAT forms were treated with vehicle or 1 μM PMA for 30 min and assayed for [3H]DA uptake. Data are presented as % basal (mean ± S.E.) for each form. ***p < 0.001 PMA versus basal for each form; ##, p < 0.01; ###, p < 0.001 PMA versus PMA for indicated combinations. n = 19 (WT), 7 (S7A), 15 (C580A). B, LLCPK1 cells expressing the indicated DAT forms were treated with 250 μg/ml ConA for 30 min followed by the addition of vehicle or 1 μM PMA for 30 min and assayed for [3H]DA uptake. Data are presented as % basal (mean ± S.E.) for each form. ***p < 0.001 PMA versus basal for indicated forms; #, p < 0.05; ###, p < 0.001 PMA versus PMA for indicated combinations; †, p < 0.05; ††, p < 0.01; PMA of indicated form in B versus PMA of corresponding form in A. n = 19 (WT), 7 (S7A), 13 (C580A). Statistical analyses were performed by ANOVA with a Tukey post-hoc test.

Discussion

In this study we identify a previously unknown mechanism for regulation of DA transporters via a kinetic process that is driven by reciprocal levels of Ser-7 phosphorylation and Cys-580 palmitoylation. Fig. 7 and Table 1 summarize the numerous findings from this study and previous reports that support this concept. In this model the DAT population is driven toward increased phosphorylation and reduced palmitoylation by pharmacological or receptor-mediated activation of PKC, inhibition of protein phosphatase 1, inhibition of PATs, and C580A mutation (Refs. 8, 9, 17, and 19 and Figs. 1–3), whereas the population is driven toward reduced phosphorylation and increased palmitoylation by inhibition of PKC, overexpression of PATs, and S7A mutation (Refs. 8 and 9 and Figs. 1–3). In addition, DAT phosphorylation is increased in a PKC-dependent manner by binding or transport of amphetamines (22) and

level of Ser-7 phosphorylation and negatively with the level of Cys-580 palmitoylation and parallels the order of steady-state kinetics of these forms.

Phosphorylation and Palmitoylation of DAT—Kinetic regulation of uptake by phosphorylation and palmitoylation requires that the modifications be present on surface transporters. To investigate this, rDAT-LLCPK1 cells treated with vehicle or PMA were subjected to subcellular fractionation for analysis of DAT phosphorylation and palmitoylation. Post-nuclear supernatants were subjected to differential centrifugation to generate fractions enriched in plasma membranes (16,000 × g pellets) or endosomes (200,000 × g pellets) (26). Immunoblotting showed enrichment of the plasma membrane marker Na^+/K^+-ATPase in the 16,000 × g membranes and the early endosomal marker Rab5A in the 200,000 × g membranes, confirming the separation (Fig. 6). Markers for late endosomes (Rab7) and recycling endosomes (Rab11) were also enriched in the 200,000 × g membranes (not shown). Most of the DAT immunoreactivity was present in the 16,000 × g membranes (82 ± 2% of total) compared with the 200,000 × g membranes (18 ± 2% of total), consistent with numerous confocal microscopy studies showing the majority of DAT expression at the cell surface of cultured neurons and heterologously transfected cells (34–36) including rDAT-LLCPK1 cells. For phosphorylation analysis, cells were labeled with ^32P, and DATs from each fraction were immunoprecipitated. Both basal and PMA-stimulated phosphorylation of DATs were clearly evident in the plasma membrane-enriched fraction, supporting the occurrence of regulated phosphorylation in this compartment. We also isolated ^32P-labeled DATs by cell-surface biotinylation (not shown), further supporting this result. Phosphorylated transporters were also present in the endosome-enriched fraction, suggesting a possible role for this modification in additional functions. ABE analysis showed that palmitoylated transporters were highly enriched in the 16,000 × g membranes, strongly supporting the presence of phosphorylation and palmitoylation on surface transporters.

5 J. D. Foster and R. A. Vaughan, unpublished data.
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Fig. 6. Subcellular localization of phosphorylated and palmitoylated DATs. rDAT-LLCPK1 cells with [32P]-labeling where indicated were treated with vehicle or 1 μM PMA for 30 min. Cells were homogenized, and post-nuclear supernatants (Total) were subjected to differential centrifugation to produce 16,000 x g membranes and 200,000 x g membranes. Fractions were immunoblotted for Na+/K+/ATPase, Rab5A, or DAT, immunoprecipitated for detection of DAT-32P labeling, or subjected to ABE (NH2-OH-treated samples shown) for detection of DAT palmitoylation. Results are representative of three-four independent experiments performed in duplicate. Where present, vertical white dividing lines indicate the rearrangement of lane images from the same immunoblot or autoradiogram.

Reduced via an unknown mechanism by botulinum neurotoxin C-mediated proteolysis of syntaxin 1A (37). Importantly, transporter function is connected to these modifications, as conditions that promote high phosphorylation or low palmitoylation reduce transport Vmax and enhance PKC-induced down-regulation (Refs. 8, 9, 17, and 22 and Figs. 4 and 5), and conditions that promote low phosphorylation or high palmitoylation (30, 37, Figs. 4 and 5) increase transport Vmax and suppress PKC-induced down-regulation.

Several observations now support the occurrence of these transport capacity alterations in both cell systems and brain tissue when endocytosis is blocked or cannot be detected (Table 1). In LLCPK1 cells, ConA treatment blocks PMA-induced DAT endocytosis but does not eliminate transport down-regulation (16), and significant levels of down-regulation are also retained in cells subjected to dynamin dominant negative inhibition (16), and significant levels of down-regulation are also retained in cells subjected to dynamin dominant negative inhibition (16) or treatment with the clathrin inhibitor PitStop2.5 In rat striatal synaptosomes prepared in hypertonic sucrose, which inhibits DAT phosphorylation and palmitoylation levels are also not known. Reciprocity between phosphorylation and palmitoylation has been found for other proteins, and for some this occurs via steric hindrance of the opposite modification. Examples of this include the β2-adrenergic receptor in which palmitoylation of residues Ser-345 and Ser-346, which modulate the ability of Cys-341 sterically inhibits PKC-mediated phosphorylation (37, 43, 44). The equilibrium between these conformations determines the rate of forward transport and also impacts cocaine analog binding, which is favored by the outwardly facing form, and substrate efflux, which is thought to be favored by the inwardly facing form (45–48).

Although Ser-7 is far in primary sequence from the TM domains and separated physically from the core active site, its mutation reduces cocaine analog binding affinity and Zn2+ stimulation of binding (20), supporting its ability to impact the protein conformational equilibrium. The transition of DAT from the occluded to the inwardly facing conformation involves major rearrangements of the inner segment of TM1 (49, 50) and loss of interactions between N-terminal intracellular gate residues Arg-60 and its gating partners (4, 51). It is possible that Ser-7 is positioned near these regions and that its phosphorylation state impacts these or other intracellular domain conformational changes that occur during transport. Alternatively, phosphorylation of Ser-7 may regulate transport indirectly by affecting interactions of the N terminus with binding partners, as supported by our finding that botulinum neurotoxin C-induced proteolysis of syntaxin 1A leads to reduced DAT phosphorylation and enhanced transport Vmax (37). The mechanism for transporter regulation by palmitoylation is also likely to be indirect, as TM12, which contains Cys-580, is located outside the transporter core structure and does not contribute directly to the substrate active site (4, 52). Palmitoylation of TM12 may affect its tilt or orientation to indirectly impact the active site, alter interactions occurring between TM domains during the transport cycle, or affect transporter oligomerization or binding partner interactions.

The mechanisms underlying the reciprocal regulation of DAT phosphorylation and palmitoylation levels are also not known. Reciprocity between phosphorylation and palmitoylation has been found for other proteins, and for some this occurs via steric hindrance of the opposite modification. Examples of this include the β2-adrenergic receptor in which palmitoylation of residues Ser-345 and Ser-346, which modulate the ability of the receptor to interact with Gα (53), and the AMPA receptor where palmitoylation of residues Ser-811 interferes with phosphorylation of Ser-816 and Ser-818, which modulate receptor surface levels (54). DAT differs from these proteins in that Ser-7 and Cys-580 are far apart in primary sequence, although it is not known if they are in three-dimensional proximity. Another possibility is that DAT phosphorylation and palmitoylation could occur in distinct membrane microdomains. DAT is distributed between cholesterol-rich membrane raft and nonraft domains, with those found in rafts possessing the highest pro-
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FIGURE 7. Reciprocal regulation of DAT by phosphorylation and palmitoylation. Schematic representation of DAT Ser-7 phosphorylation and Cys-580 palmitoylation cycles showing populations possessing high phosphorylation and low palmitoylation (left) or low phosphorylation and high palmitoylation (right), indicated by the size of blue phosphorylation (P) and red palmitoylation (PAL) symbols. Increased phosphorylation and/or decreased palmitoylation was induced by activation of PKC (PMA, OA, Substance P), inhibition of protein phosphatase 1 (OA), or inhibition of PATs (2BP), whereas decreased phosphorylation and/or increased palmitoylation was induced by inhibition of PKC (BIM, staurosporin) or overexpression of PATs. Proteins are also driven in the directions indicated by S7A and C580A mutations, binding, or transport of amphetamines (AMPH) or treatment with botulinum neurotoxin C (BoNT/C). Conditions that increase phosphorylation or suppress palmitoylation reduce transport $V_{\text{max}}$ or enhance down-regulation, and conditions that reduce phosphorylation or increase palmitoylation increase transport $V_{\text{max}}$ or suppress down-regulation (Table 1). PPT, protein palmitoyl thioesterase.

TABLE 1
Studies showing the presence of transport up- or down-regulation when DAT endocytosis was blocked or when surface levels did not change.

| Treatment/condition | Transport reducing conditions | Transport increasing conditions |
|---------------------|-------------------------------|-------------------------------|
| **Phosphorylation modulators** | ↑ phosph | ↓ palm | ↓ uptake | ↓ phosph | ↑ palm | ↑ uptake |
| PMA (8,9,16, 19,20, 21, 22) | PMA (Fig. 2) | PMA (8,9,16a, 21, 22) | BIM (8,9) | BIM (Fig. 2) | BIM (down-reg) (9) |
| OAG (9) | OA (Fig. 2) | OA (Fig. 2) | Stauro (8,9) | Stauro (down-reg) (8,9) |
| SubstanceP (21) | OA (9,19) | OA (9, 21) | BonT/C (37) | BonT/C (37) |
| OA (9,19) | AMPH (22) | AMPH (22) | AMPH (22) |
| **Palmitoylation modulators** | 2BP (Fig. 2) | 2BP (17) | 2BP (17a) | DHHHC2 (30) | DHHHC2 (30a) |
| Mutations | C580A (Fig. 1) | C580A (17, Fig. 1) | C580A ↓ $V_{\text{max}}$ (Fig. 4a) (down-reg) (17a) | DHHHC2 (30) |
| S7A (20) | S7A (Fig. 1) | S7A ↑ $V_{\text{max}}$ (Fig. 4a) (down-reg) (5) |

* Studies showing the presence of transport up- or down-regulation when DAT endocytosis was blocked or when surface levels did not change.
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alterations, and targeting defects that have been linked to diseases such as cancer, heart disease, and diabetes (65–67), suggesting that similar conditions could contribute to DA imbalances via dysregulation of WT transporters and that these enzymatic inputs could represent therapeutic targets for DA disorders or drug addiction.

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