Phosphatidylinositol 3-Kinase, Protein Kinase C, and MEK1/2 Kinase Regulation of Dopamine Transporters (DAT) Require N-terminal DAT Phosphoacceptor Sites*

Received for publication, September 18, 2002, and in revised form, March 25, 2003
Published, JBC Papers in Press, March 26, 2003, DOI 10.1074/jbc.M208584200

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The dopamine transporter (DAT) modulates dopamine neurotransmission and is a primary target for psychostimulant influences on locomotion and reward. Selective DAT expression by dopaminergic neurons has led to use of cocaine analog DAT radioligands to assess rates of progression of dopamine neuronal degeneration in Parkinson's disease. We have documented that DAT is a phosphoprotein that is regulated by phosphorylation through pathways that include protein kinase C cascades. We now extend this work using drugs selective for phosphatidylinositol 3-kinase (PI3K), protein kinase C, MEK1/2, p38 kinase, and Ca2+/calmodulin kinase II. We compare the drug effects on wild type DAT to the effects on 20 DAT mutants and a DAT deletion. PI3K and MEK1/2 modulators exert strong effects on DAT expression patterns and dopamine uptake V̇max. PKC principally modulates V̇max. Neither p38 nor Ca2+/calmodulin kinase II agents exert significant influences on wild type DAT. Several mutants and a DAT with an N-terminal deletion display alterations that interact with the effects of kinase modulators, especially S7A for PKC effects; T62A, S581A, and T612A for PI3K effects; and S12A and T585A mutants for MEK1/2 effects. 32P-Labeling studies confirm several of these effects of kinase pathway modulators on DAT phosphorylation. DAT expression and activities can be regulated by kinase cascades that require phosphoacceptor sites most concentrated in its N terminus. These results have a number of implications for DAT regulation and mandate caution in using DAT radioligand binding to infer changes in dopaminergic neuronal integrity after treatments that alter activities of these kinase pathways.

The plasma membrane dopamine transporter (DAT) functions to terminate dopaminergic neurotransmission by re-uptake of synaptic dopamine molecules into presynaptic neurons. Modulators of DAT function thus regulate the intensity and duration of dopaminergic neurotransmission (1, 2). DAT blockade by psychostimulants plays a key role in their rewarding and locomotor-stimulating properties (1, 3, 4). DAT uptake of dopamine-selective neurotoxins such as 6-hydroxydopamine and 1-methyl-4-phenylpyridinium is central to the cell type specificity of these model toxins for parkinsonian neurodegeneration.

DAT is the most selective current marker for selected populations of dopaminergic neurons. It is expressed only in dopaminergic cells, with relatively high levels of expression in substantia nigra pars compacta neurons, intermediate levels in ventral tegmental area dopaminergic neurons, and low levels in accumbus nucleus neurons. These features, and the high affinity with which DAT can recognize cocaine analogs, have led to use of in vitro and in vivo assessments of DAT binding to seek evidence for degeneration of dopaminergic systems (5). Cocaine analog binding to DAT in vitro or in vivo can each reveal reduced DAT binding in Parkinson's disease brains (6–8). The decline in specific DAT binding of 2β-carbomethoxy-3β-(4-[123I]iodophenyl) tropone (123Iβ-CIT) in SPECT studies has correlated well with progression of Parkinson's disease symptoms (7).

DAT functions including its velocity of dopamine transport and its expression on plasma membranes can be regulated by several kinase or phosphatase pathways. DAT activity can be altered by activation of protein kinase C (PKC), Ca2+2, arachidonic acid cascades (9–14), treatment with phosphatase inhibitors, or coexpression with the PKC-activated phosphatase inhibitor KEPl. Most of this regulation changes dopamine uptake V̇max. Increased DAT internalization can also be noted. Few treatments alter the affinity of DAT for cocaine analogs.

We and others have demonstrated DAT phosphorylation. [32P]Orthophosphate labels DAT proteins in expressing COS cells, LLC-PK1 cells (11), and rat brain preparations (15). Increased 32P labeling correlates with decreased V̇max in some experiments. Antiphosphoserine antibodies can recognize DAT in striatal Western blots (16). Despite evidence for functionally important regulation by kinase pathways, little evidence documents which pathways work at which DAT sites to alter phosphoregulation, alter DAT disposition in cells, and/or change DAT dopamine translocation V̇max.

Current DAT modeling supports a 12-transmembrane (TM) topology that localizes potential phosphoacceptor sites to intracellular N- and C-terminal domains and the five putative cytoplasmic loops that connect TM domains (17–21). To identify kinase pathways important for regulation of DAT expression...
and function, we have first characterized the influences on DAT expression and function that are exerted by drugs that change activities in several kinase pathways. To identify which potential phosphoacceptor sites might harbor DAT sequences that are important for DAT regulation, we have assessed modifications of these kinase pathway influences induced by 21 DAT mutations or deletions that alter potential DAT phosphoacceptor sites (Fig. 1). Influences of several kinase pathway agents on DAT functions can be attenuated by removal of several potential DAT phosphoacceptor sites, especially those in its N-terminal domain. We also describe evidence for differential effects of phosphorylation on DAT distribution and function. These data have a number of implications, including cautions in interpreting DAT images obtained after treatments that alter potential phosphoacceptor sites (especially those in its N-terminal domain) and because S261A and T338A are each located on the PEI fragment and because S261A and T338A are each located on the PEI fragment, DNA recombinations of these two cDNA fragments with the corresponding mutations produced the above five combination mutants. N-terminally deleted DAT was prepared by shuttling DNA fragments were confirmed by sequencing.

**Preparation of Mutant DNAs**

Alanine substitution mutants in the rat DAT cDNA (18) were prepared as described previously (22). Combination mutants S7A/T62A/S261A, S261A/T338A, S7A/T62A/T338A, and S7A/T62A/S261A/T338A were generated as follows. Double mutations S7A/T62A (located on a NotI/BglII fragment) and S261A/T338A (located on a BglII/PspI fragment) were made by annealing two mutagenic oligonucleotides simultaneously to the single strand template during the procedure (22). Because S7A and T62A are each located on the NotI/BglII fragment and because S261A and T338A are each located on the BglII/PspI fragment, DNA recombinations of these two cDNA fragments with the corresponding mutations produced the above five combination mutants. N-terminally deleted DAT was prepared by shuttling a PCR product that carried the NotI/BglII fragment with a deletion of the 12 cDNA coding for Ser-2 to Ser-13 of DAT into the rat DAT (rDAT) cDNA. The two primers used were 5′-GGGGGCGGCCGATGGTGGTGTTGGCCCGGCTAAAGAGTCC-3′ and 5′-GCCCCCGGTAAAGATGCTC-3′; the wild type cDNA was the template. All mutant DNA fragments were confirmed by sequencing.

**Functional Analyses**

Dopamine uptake was assessed in COS cells transiently expressing wild type and mutant DATs in pcDNA3.1+ as described previously (22). Transfected cells were assayed for their abilities to accumulate 5.5 μM [3H]dopamine (initially 10 μM, 56 Ci/mmol (PerkinElmer Life Sciences) adjusted using unlabeled dopamine) with or without treatments with PMA (1 μM) or MAP kinase inhibitors (50 μM) at 37 °C for 30 min. These pretreatments caused only negligible cell detachment from the assay wells. Assessments of protein in the wells before and after treatments revealed no changes. Drugs were also included in the corresponding [3H]dopamine uptake buffers. Kinetic and saturation analyses were used to determine K_m and V_max values as modified from Pfennig and Richelson (23).

**Stable LLC-PK1/rDAT Cell Lines**

Wild type and mutant rDATs- and neo-expressing mammalian plasmids pcDNA3.1/ZL-rDATs (22) were introduced into swine kidney cell line LLC-PK1 (ATCC) by transfection using Effectene kits (Qiagen). After transfection, cells were grown in Dulbecco’s modified Eagle’s medium containing 800 μg/ml geneticin (Invitrogen) for selection of expressing cells. DAT-positive cells were identified by immunohistochemical staining (24). DAT-positive cell lines were obtained by 2–3 rounds of subcloning and maintained in Dulbecco’s modified Eagle’s medium containing 400 μg/ml geneticin (25). In each stable cell line, >95% of the cells showed DAT-positive staining.

**Analyses of Expression**

**Immunohistochemistry**—DAT expression patterns in COS cells were examined by immunohistochemistry as described using antisemurum 16B for every mutant except one (24). For the mutant S45A) located within the peptide against which 16B was raised, the rabbit anti-DAT C-terminal peptide serum 16B (18A) was used.

**Cell Surface Biotinylation**—The surface expression of the wild type DAT proteins was monitored by a biotinylation procedure modified from Ref. 27. 5 x 10^5 LLC-PK1 cells in 6-well plate wells were briefly washed twice with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS/Mg^2+ / Ca^2+). Cell Surface Biotinylation—The surface expression of the wild type DAT proteins was monitored by a biotinylation procedure modified from Ref. 27. 5 x 10^5 LLC-PK1 cells in 6-well plate wells were briefly washed twice with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS/Mg^2+ / Ca^2+). Cell Surface Biotinylation—The surface expression of the wild type DAT proteins was monitored by a biotinylation procedure modified from Ref. 27. 5 x 10^5 LLC-PK1 cells in 6-well plate wells were briefly washed twice with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS/Mg^2+ / Ca^2+).
Ca2+ (pH 8.0). For kinase effector treatments, cells were incubated with drugs at 37 °C for 30 min as indicated in the figure legends. Plates were incubated at 4 °C for 5 min; cells were washed twice with 1 ml of ice-cold PBSMg2+/Ca2+ to remove drugs, and cells were incubated with 0.4 ml of PBSMg2+/Ca2+ containing NHS-Ss-biotin (1.5 mg/ml, Pierce) and Me2SO (0.2%) at 4 °C with gentle agitation for 40 min. NHS-Ss-biotin solution was aspirated, and the remaining NHS-Ss-biotin was quenched by incubating cells with 1 ml of 0.15 M glycine in PBSMg2+/Ca2+ at 4 °C for 20 min. Cells were lysed by adding 50 µl of SDS lysis buffer/well and suspended into an additional 0.45 ml of non-SDS lysis buffer using a cell scrapper. 0.5 ml of the resulting suspension was transferred into an Eppendorf tube and centrifuged at 12,000 × g in a microcentrifuge at 4 °C for 15 min to remove cell debris. The supernatant was incubated for 1 h at 4 °C with 50 µl of streptavidin-sepharose beads (Pierce) with occasional mixing to resuspend the beads. Beads were washed at 4 °C with non-SDS lysis buffer, high salt solution, and then 1 ml of 50 mM Tris-OAc (pH 7.6). Beads were suspended in 30 µl of 100 mM β-mercaptoethanol and incubated at 37 °C for 30 min to release proteins, and 30 µl of 2× SDS sample buffer was added.

**Western Blotting**—Proteins in the SDS sample buffer were heated at 80 °C for 10 min, resolved by 10% SDS-PAGE, and transferred to PVDF membranes (25). DAT was recognized with primary antibody 16B diluted 1:2000 and secondary anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:5000, using an ECL plus Western blotting detection system (Amersham Biosciences). To provide a control, protein phosphatase type 1 (PP1, the catalytic subunit of serine/threonine phosphatase) in LLC-PK1 cells was detected using the rabbit anti-PP1 sera (Calbiochem) diluted 1:1000. For PP1 assays with whole cells, LLC-PK1 cells were washed twice with 1 ml of PBS to remove the medium, dissolved in 1× sample buffer, and heated at 80 °C for 10 min before analyses using 10% SDS-PAGE and Western blotting.

**Immunofluorescence Assay**—DAT/LLC-PK1 cells were grown on coverslips. After fixation in 2% paraformaldehyde, cells were permeabilized with PBS containing 0.1% Triton X-100, 1 mM MgCl2, 0.1 mM CaCl2, and blocked with augmented TBS (TBS with 2% skim milk powder, 0.2% Triton X-100, 1 mM MgCl2, 0.1 mM CaCl2, 0.01% sodium azide) for 1 h at room temperature. Blocking solution was removed; cells were rinsed once with TBS (TBS containing 0.2% Triton X-100, 1 mM MgCl2/0.1 mM CaCl2) and cells were incubated with the primary anti-DAT serum 16B diluted 1:16 diluted 1:2,000 in TBS for 1 h. The primary antibody was aspirated; cells were washed 4 times with TBS for 7 min per incubation, and cells were incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG (H & L), Molecular Probes, Eugene, OR, diluted 1:200 in TBS) for 1 h. Secondary antibodies were removed; cells were washed 4 times with TBS (7-min incubations each) and once with PBS, and coverslips were mounted onto slides using the ProLong Antifade kit (Molecular Probes) and allowed to air-dry in darkness for overnight. Coverslips were sealed with Clarion nailpolish, and fluorescence images were obtained via a Zeiss LSM 410 Invert Laser Scan Microscope system, at excitation/emission of 488/515. z-sections were obtained at a half of the cell height.

**32P Labeling of Wild type and Mutant DAT Proteins**

LLC-PK1 cells stably expressing wild type and mutant rDAT cDNAs were grown to near-confluence in 6-well plates, rinsed once with 1 ml of phosphate-free Dulbecco’s modified Eagle medium, and incubated in the same medium for 1 h. Cells were trypsinized twice with 0.05% trypsin/0.53 mM EDTA, and the medium was removed; cells and incubated at 30 °C for 90 min in labeling Krebs-Ringer-Henseleit medium containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 25 mM NaHCO3, 5.6 mM glucose, 1.3 mM CaCl2, pH 7.3, 1 µM okadaic acid, and 1 mM/ml [32P]orthophosphorus. Cells were then treated for 30 min with either 0.2% Me2SO or 50 µM concentrations of test drug in the same medium. For the PKC activator PMA, 25 nM, 2% Me2SO. Labeling medium was removed; cells were rinsed once with 1 ml of ice-cold labeling Krebs-Ringer-Henseleit containing 1 µM okadaic acid, and cells were scraped into ice-cold sucrose-phosphate (SP) buffer containing 10 mM sodium phosphate, pH 7.4, 0.32 M sucrose, 1 µM okadaic acid, and 1/5 protease inhibitor set I.

Cells were homogenized by passing through 22-gauge needles 10 times; lysate was centrifuged at 700 × g for 10 min; supernatants were centrifuged at 325,000 × g for 20 min, and pellet membrane proteins were solubilized in 500 µl of solubilization buffer containing PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM diosyrdium pyrophosphate, 1 µM okadaic acid, and 1× protease inhibitor set I by resuspension and rotation of the tubes at 4 °C for 1 h. 10 µl of a 50% protein A-Sepharose CL 4B bead slurry was added to solubilized samples; samples were rotated at 4 °C for 1 h and 5 µl of the suspension set aside for SDS-PAGE and Coomassie Blue protein estimations. The remaining slurry was incubated with 20 µl of anti-DAT/protein A-Sepharose CL 4B bead slurry, which was prepared by rotating protein A-Sepharose CL-4B beads with rabbit anti-rDAT serum 16B (Pierce). Slurry preparations were incubated with 50 µl of streptavidin-agarose bead slurry or with solubilization buffer only and rotated at 4 °C for 2 h. Beads were washed with 3× 500 µl of solubilization buffer and proteins released from beads by 10-min 65 °C incubations in 30 µl of SDS sample buffer, and each sample was resolved on two parallel gels of 7% SDS-PAGE. One of each gel pair was dried for autoradiographic analysis, and proteins on the second parallel gel were transferred to a PVDF membrane for quantitation using India ink staining. LLC-PK1 proteins on PVDF membrane were used as a control for the amount of proteins loaded on the gels.

**Results**

**Basal [3H]Dopamine Uptake Activity**—Cells expressing wild type DAT displayed robust uptake of radioiodelabeled dopamine. Cells that expressed 18 of the 21 DAT mutants displayed uptake activities ≥80% of wild type values (Table I, first two columns). Val-14-deleted DAT displayed about one-fourth of this activity (n = 5). S333A and S421A mutants decreased uptake to less than 10% of wild type values. Conversely, S12A DAT had uptake activity 120% of wild type values.

**Influences of Kinase Effectors on [3H]Dopamine Uptake**

**Vmax and Kinetic Analyses**—Quantitation of dopamine uptake, calculation of data fitting, and t tests or ANOVA were followed by Tukey’s multiple comparison tests used Prism version 3 programs (GraphPad Software, San Diego). Mutation effects on drug-induced Vmax reductions are defined as the differences between Vmax reductions identified in mutant and wild type DATs (see figure legends).

**Influences of Kinase Effectors on [3H]Dopamine Uptake**

**Vmax in Wild Type DAT**—PMA, U0126, LY294002, SB202190, and KN-62 were tested for effects on dopamine uptake in cells expressing wild type DAT using saturation analyses. Treatment with 50 µM LY294002, an IP3-kinase inhibitor, reduced Vmax by 61%. Treatment with 10 µM of the PKC activator PMA at 37 °C for 30 min reduced dopamine uptake Vmax by 29%. Treatment with 50 µM of U0126, a MEK1/2 inhibitor, reduced Vmax by 26%. Neither the p38 kinase inhibitor SB20219 nor the Ca2+/CaMII inhibitor KN-62 exerted any significant effect on overall Vmax (Fig. 2A). Examination of Eadie-Hofstee plots revealed that SB20219 treatments displayed trends toward reducing uptake at higher dopamine concentrations. Lack of KN-62 effects reduced interest in the pathways influenced by this effector.

**Expression of DAT and DAT Mutants in COS Cells**—Wild type DAT expressed transiently in COS cells displayed most immunoreactivity in or near plasma membranes and perinuclear compartments. All but 3 of the 21 alanine substitution and N-terminal truncation DAT mutants displayed similar expression patterns. Whereas Val-14 displayed reduced overall expression, Val-14 immunoreactivity localized well to plasma membranes. T383A, located in the third intracellular loop,
DAT Phosphorylation Mutants

Mutational influence on alteration of dopamine uptake activity by protein kinase effectors

All the uptake assays were performed with single concentrations of [1H]dopamine.

| DATα   | Kinase effectors | LY294002 | PMA | U0126 | SB203580 |
|--------|------------------|----------|-----|-------|-----------|
| N-terminal |                  |          |     |       |           |
| WT     |                  | 100      | 67  | 73    | 92        | 97       |
| V14β   |                  | 24β      | 22β | 76    | 166β      | 248β     |
| S2A    |                  | 99       | 65  | 71    | 85        | 119β     |
| S4A    |                  | 99       | 55  | 71    | 103β      | 131β     |
| S7A    |                  | 81       | 69  | 85β   | 91        | 113β     |
| S12A   |                  | 121      | 55β | 75    | 129β      | 159β     |
| S13A   |                  | 75       | 49β | 70    | 134β      | 150β     |
| S21A   |                  | 90       | 48β | 63β   | 133β      | 142β     |
| S45A   |                  | 91       | 48β | 73    | 151 β     | 156β     |
| T62A   |                  | 64       | 71  | 79    | 78β       | 105      |
| S253A  |                  | 76       | 69  | 62β   | 94        | 103      |
| S261A  |                  | 70       | 68  | 79β   | 90        | 99       |
| S332A  |                  | 82       | 64  | 72    | 87        | 116      |
| S333A  |                  | 6β       | 72  | 58β   | 74β       | 111      |
| T338A  |                  | 70       | 67  | 78β   | 52β       | 110      |
| S421A  |                  | 8β       | 91  | 60    | 112       | 110      |
| S428A  |                  | 94       | 71  | 71    | 93        | 105      |
| S504A  |                  | 96       | 69  | 69β   | 98        | 107      |
| C-terminal |              |          |     |       |           |
| S581A  |                  | 106      | 75  | 74    | 97        | 111      |
| S585A  |                  | 72       | 61  | 61β   | 86        | 100      |
| T358A  |                  | 58       | 69  | 78β   | 105β      | 107      |
| T612A  |                  | 108      | 74  | 74    | 108β      | 105β     |

α Wild type (WT) and alanine substitution mutant DATs. Mutated residues located in the N or C termini are indicated ‘N-terminal’ or ‘C-terminal’ (see Fig. 1).

β Data are presented in percentage of the uptake activity in the wild type DAT. The substrate concentration used was 2.5 μM [3H]dopamine in the presence of the kinase effectors after the drug treatment (at ≥ 37 °C for 30 min (see “Materials and Methods” for details). Mutational attenuation of PMA effect under “Results” is calculated as 100 × (1 – (100 – % for mutant) / 100 – 73% for WT).

γ Protein expression was low as indicated by the immunoreactivity only 20-30% of the wild type DAT, based on immunohistochemical staining assays.

δ ANOVA is p < 0.01. WT; n = 2-4, each in duplicate. Italics indicate mutational enhancements of drug effects.

ε t test result is p < 0.01, compared with WT; n = 2-11, each in duplicate.

η t test result is p < 0.001 compared with WT; n = 2-4, each in duplicate.

ζ t test result is p < 0.05 compared with WT; n = 2-4, each in duplicate.

η ANOVA is p < 0.001, compared with WT; n = 2-11, each in duplicate.

T595A, located in the C terminus (Fig. 1), both displayed mildly reduced COS cell expression. T338A displayed fiber-like DAT staining patterns in both COS and stably expressing LLC-PK1 cells. The S12A mutant that displayed uptake activity greater than wild type values also displayed DAT immunostaining of greater intensity that was found in cells expressing wild type DAT (immunohistochemical staining data not shown).

Influences of Kinase Effectors on Cell Surface Expression—
Kinase effects on reducing cell surface DAT expression of DAT protein were also explored using cell surface biotinylation assays. DAT was easily detected by Western blotting analysis among proteins accessible to NHS-SS-biotin treatments of intact DAT-expressing LLC-PK1 cells. NHS-SS-biotin-accessible DAT amounts were reduced in proteins extracted from cells pretreated with either LY294002 or U0126 (Fig. 2B). Cell surface biotinylated DAT protein was decreased by 45% after LY294002 treatments and by 20% after U0126 treatments after normalization for protein loading, findings consistent with the magnitudes of V_max reductions identified for these variants (Fig. 2A). However, PMA treatments did not appear to influence DAT surface expression (Fig. 2E), even though they reduced V_max values.

Control experiments included observations that under these assay conditions the NHS-SS-biotin did not have access to the intracellular control protein FP answered (Fig. 2C). When PVDF membranes used for Western blots (Fig. 2B) were stained with India ink (Fig. 2D), lane-to-lane differences in protein staining were no greater than 20%. When we compared India ink staining intensities to amounts of known control proteins electrophoresed similarly and blotted onto similar PVDF membranes, staining intensities were linear over protein ranges of at least 2-100 ng.

Treatments with the IP3-kinase inhibitor LY294002 reduced dopamine uptake activity by 33% in wild type DAT in single concentration experiments and reduced V_max values to 19% of control values in kinetic Eadie-Hofstee analyses. In kinetic analyses, T62A, S581A, and T612A attenuated these LY294002 effects by 92, 80, and 77%, respectively (Fig. 3). Studies of 11 mutations revealed no evidence for interaction with LY294002 effects. Val-14, S12A, S13A, S21A, and S45A enhanced drug effects in single concentration experiments (Table I, 3rd column). Treatments with the PKC stimulator PMA reduced DAT uptake activity to 73% of wild type values in single substrate concentration experiments and to 71% in Eadie-Hofstee analyses. Mutations S7A, T62A, S261A, T338A, and T595A attenuated PMA effects by 43, 24, 25, 19, and 19%, respectively (Table I, 4th column). Kinetic analyses revealed that the S7A mutant reduced PMA effects on V_max by 70% but that these variable data did not reach statistical significance (Fig. 3). Eleven mutations did not influence PMA effects. Mutations S21A, S253A, S333A, S504A, and S585A each enhanced PMA effects by 9-14%, leading to greater PMA-induced reductions in DAT V_max values than those found in wild type DAT (Table I, 4th column).

Each of five combination mutants S7A/T62A/S261A, T62A/S261A/T338A, S7A/S261A/T338A, S7A/T62A/S261A/T338A expressed well in COS cells. Even the most poorly expressing of these mutants, S7A/T62A/S261A/T338A, displayed most of its DAT immunoreactivity associated with plasma membranes and less than 30% in perinuclear zones. None of these combination mutants differed from wild type DAT in responses to PMA treatments assessed in single concentration experiments (data not shown).

Treatments with the MEK1/2 inhibitor U0126 reduced uptake of wild type DAT to 92% of control values in single concentration experiments and reduced V_max values to 74% of wild type values in Eadie-Hofstee analyses. Ten mutations failed to influence this U0126 effect. T62A, T338A, and S333A each augmented U0126 effects in single concentration experiments, allowing the drug to reduce uptake from 56 to 85% of wild type values. Eight mutants displayed uptake activities that were higher than wild type values after U0126 treatments, including Val-14 (166%), S4A (103%), S12A (129%), S13A (134%), S21A (133%), S45A (151%), T595A (105%), and T612A (108%; Table I, 5th column). Kinetic analyses revealed that mutations S12A, T595A, S13A, T612A, S4A, S45A, and S21A reversed drug effects on uptake V_max by 141, 103, 97, 75, 70, 53, and 43% of values found in the wild type DAT, respectively (Fig. 3).

32P labeling analyses further demonstrated the importance of these DAT phosphoacceptor sites for effects of several different kinase effectors in studies of the seven most interesting mutants. LY294002 and U0126 treatments each reduced wild type DAT 32P labeling by almost half (Fig. 4). Mutants in Ser-12, Ser-13, Thr-62, Ser-581, Thr-595, and Thr-612 all at-
Effect of treatments with protein kinase effectors, LY294002, PMA, U0126, SB202190, and KN-62 on wild type DAT: $[^3]$Hdopamine uptake $V_{max}$ and cell surface expression. A, dopamine uptake $V_{max}$ values are expressed as percentages of values obtained without drug treatments or with treatment by 4$\alpha$-PDD as an inactive control for PMA effects. Data derive from two experiments, each in duplicate, using COS cells transiently expressing DAT. CTL, control. B, Western blot detection of cell surface biotinylated DAT. DAT was stably expressed in LLC-PK1 cells and surface-biotinylated. Biotinylated proteins from $2.5 \times 10^5$ cells were loaded onto each lane, resolved by 10% SDS-PAGE, transferred onto PVDF membrane, and detected by an ECL plus Western blotting detection system (see "Materials and Methods"). Upper bands represent the remaining DAT-streptavidin complex. CTL, control. Kinase effectors were LY294002, PMA, and U0126. 4$\alpha$-PDD provided a negative control for PMA experiments and produced a band similar to that for PMA (data not shown). C, Western blot detection of PP$\alpha_1$ in whole cells but not among cell surface-biotinylated proteins. The anti-PP$\alpha_1$ rabbit antibody recognized the swine PP$\alpha_1$ expressed by LLC-PK1 cells. Protein from $3 \times 10^6$ for whole cells and $2.5 \times 10^6$ cells subjected to cell surface biotinylation was loaded. D, India ink detection of cell surface-biotinylated proteins and molecular weight markers (kDa) on the PVDF membrane corresponding to the presence of LY294002 revealed peak uptake values at 10 $\mu$M, whereas the S7A mutant displayed modest evidence for interaction with drug effects. U0126 increased $V_{max}$ values in S4A, S13A, and S21A and produced trends toward increased $V_{max}$ values in S4A, S21A, and S2A (Fig. 5). N-terminal mutants displayed modest evidence for interaction with drug effects. U0126 increased $K_m$ by 30% only in S12A, and SB202190 increased it by 20% each in S12A, S4A, and S13A and produced trends toward increased $V_{max}$ values in S4A, S21A, and S2A (Fig. 5).

Saturation kinetics of dopamine uptake in wild type DAT in the presence of LY294002 revealed peak uptake values at 10 $\mu$M $[^3]$Hdopamine and then significant declines to 34% of peak values in uptake with increasing dopamine concentrations (to 50 $\mu$M). There were smaller trends toward similar effects for U0126 (66%) and SB202190 (79%, Fig. 6). Kinetic analyses also revealed several mutant effects on affinity for $[^3]$Hdopamine. T62A and T595A decreased $K_m$ by $\sim$50% for dopamine uptake. N-terminal mutants displayed modest evidence for interaction with drug effects. U0126 increased $K_m$ by 30% only in S12A, and SB202190 increased it by 20% each in S12A and S21A.

$\beta$-CIT Effects and Interaction with LY294002—Wild type DAT/LLC-PK1 cells were treated with $\beta$-CIT (0.1 and 50 $\mu$M), LY294002 (50 $\mu$M), and $\beta$-CIT plus LY294002 (50 $\mu$M) at 37 °C for 30 min, and cellular DAT localizations were examined by immunofluorescence confocal microscopic and cell surface biotinylation methods (Fig. 7). 0.1 and 50 $\mu$M $\beta$-CIT treatments increased plasma membrane DAT immunostaining. LY294002-treatment virtually abolished this PMA effect (Fig. 4).

Treatments with the p38 MAP kinase inhibitor SB202190 did not significantly influence overall dopamine uptake $V_{max}$ values in wild type DAT, although it displayed a nonsignificant trend toward producing effects at higher concentrations (see below). Fourteen mutations failed to change this SB202190 effect. In single substrate concentration experiments, SB202190 treatments increased uptake by 150, 62, 53, 45, 39, 35, 21% in Val-14, S12A, S13A, S21A, S45A, S4A, and S2A, respectively (Table I, last column). Kinetic analyses revealed that SB202190 treatments significantly increased $V_{max}$ values by 54, 18, and 17% in S12A, S4A, and S13A and produced trends toward increased $V_{max}$ values in S4A, S21A, and S2A (Fig. 5).

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$\beta$-CIT Effects and Interaction with LY294002—Wild type DAT/LLC-PK1 cells were treated with $\beta$-CIT (0.1 and 50 $\mu$M), LY294002 (50 $\mu$M), and $\beta$-CIT plus LY294002 (50 $\mu$M) at 37 °C for 30 min, and cellular DAT localizations were examined by immunofluorescence confocal microscopic and cell surface biotinylation methods (Fig. 7). 0.1 and 50 $\mu$M $\beta$-CIT treatments increased plasma membrane DAT immunostaining. LY294002-treatment virtually abolished this PMA effect (Fig. 4).

Treatments with the p38 MAP kinase inhibitor SB202190 did not significantly influence overall dopamine uptake $V_{max}$ values in wild type DAT, although it displayed a nonsignificant trend toward producing effects at higher concentrations (see below). Fourteen mutations failed to change this SB202190 effect. In single substrate concentration experiments, SB202190 treatments increased uptake by 150, 62, 53, 45, 39, 35, 21% in Val-14, S12A, S13A, S21A, S45A, S4A, and S2A, respectively (Table I, last column). Kinetic analyses revealed that SB202190 treatments significantly increased $V_{max}$ values by 54, 18, and 17% in S12A, S4A, and S13A and produced trends toward increased $V_{max}$ values in S4A, S21A, and S2A (Fig. 5).

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treatment increased intracellular DAT staining intensities. Treatments with \( \beta \)-CIT plus LY294002 (50 μM each) resulted in an intermediate pattern of DAT localization (Fig. 7). Cell surface biotinylation assays also indicated that treatments with 0.1 μM \( \beta \)-CIT increased biotinylated DAT by 12%, 50 μM LY294002 decreased it by 40%, and 0.1 μM \( \beta \)-CIT plus 50 μM LY294002 decreased it by 25% (Fig. 7, last panel; band intensities were normalized as described in Fig. 2). Treatments with 50 μM \( \beta \)-CIT decreased DAT cell surface biotinylation by 67%; high \( \beta \)-CIT concentrations may have interfered with biotinylation.

**DISCUSSION**

The current data document that agents that influence PI3K, PKC, and MEK1/2 kinase pathways can regulate DAT activi-
ties including its expression and DA uptake $V_{\text{max}}$, while providing little evidence to support $\text{Ca}^{2+}$/calmodulin kinase II roles. Each of these drugs might display relative rather than absolute specificities. Nevertheless, the pattern of the current results appears to indicate that there are specific effects of these kinase pathways that are likely to be influenced in vivo through impact of neurotransmitters, trophic factors, and other agents on single-TM domain receptors, seven-TM domain receptors, ligand-gated ion channels, and perhaps even nuclear receptors expressed by dopaminergic neurons. Much of this regulation could well involve the N- and C-terminal domains of DAT in pathway-specific fashions. The current data are also consistent with observations reported recently from other laboratories (29, 30).

Several kinase pathway modulators may exert principal effects on DAT cellular distribution. LY294002 effects on cell surface expression provide strong parallels with the effects of compounds on dopamine uptake $V_{\text{max}}$, and contrast with the failure of LY294002 to affect DAT $K_{m}$. PKC alters cell surface expression of multidrug resistance and glucose transporters (31, 32). Mutation sites effective in attenuating these PI3K effects are more concentrated in C- than in N-terminal DAT domains. Signals for cellular redistribution of DAT could thus be found in C-terminal phosphoacceptor sites, as recently identified for glucose transporters (33). It is interesting that high dopamine concentrations reduced uptake velocities in the presence of LY294002 (Fig. 6). Among other explanations, such observations might result from direct effects of high dopamine concentrations by occupying all the monomers at the same time in the oligomerized DAT molecules. The idea is that DAT monomers have cooperation in translocating dopamine molecules and the translocation would not happen when all the monomers are occupied in the presence of LY294002. Another explanation could be that high dopamine concentrations also alter DAT features including phosphorylation/conformations so that IP3 kinase mediates more significant effects on DAT cellular redistribution. At the high concentrations of dopamine required for these effects to be manifest, less specific oxidative/reductive and other effects cannot also be excluded. However, complete nonspecificity is excluded by the failure of cells to demonstrate reduced uptake of 50 $\mu$M dopamine in the absence of drugs or in the presence of U0126 or SB202190.

Other kinase pathway modulators, including the PKC agent PMA, exert their principal effects on DAT $V_{\text{max}}$ with little impact on cellular distribution patterns (but see Ref. 34 for PMA effects on canine cellular DAT expression). Most brain DAT immunoreactivity is associated with plasma membranes, suggesting that PKC pathways may be good candidates for in vivo regulation (35). The mutations that attenuate PMA effects most strongly are located in the N terminus (two), cytoplasmic loops (two), and C terminus (one) of DAT. Some of these influences could well arise through direct alterations in DAT phosphorylation in vivo, as documented here in vitro. PMA treatments are well documented to alter the amount of phospho-DAT in the current and prior experiments (7, 11). N-terminal DAT sequences contain phosphoacceptor sites well positioned for such regulation, and the S7A deletion dramatically reduces the ability of PMA to enhance DAT phosphorylation. PKC can act to regulate through the N-terminal domains of other proteins, such as cardiac $\text{Ca}^{2+}$ channel subunits (36). However, PKC might also regulate DAT activity more diffusely and/or more indirectly (37). Ten Ser/Thr DAT mutations from S7A to T595A each provide individually modest reductions in PMA effects on transport. Neither the Val-14 mutant nor a combination mutant incorporating each of the four mutants$^3$ that are most important individually for this effect (Table I) provides any evidence that combinations of these small effects add up to the entire effect. PMA could alter effects of PKC on other proteins, including PKC-dependent phosphatase inhibitors (see below), providing plausible alternative pathways for PKC effects on DAT function.

The MEK1/2 agent U0126 displays effects similar to PI3K agents; it reduces plasma membrane DAT expression by $\sim$20%,

$^3$ Z. Lin and G. R. Uhl, unpublished observations.
a magnitude similar to the size of effects on DAT $V_{\text{max}}$. Four N-terminal and one C-terminal mutants decrease this effect on $V_{\text{max}}$. These data again underscore the predominant influences of N- and C-terminal sites for kinase regulation.

Initial studies with SB202190 revealed little influence on dopamine uptake $V_{\text{max}}$ activity with modest effects on uptake at higher dopamine concentrations. However, SB202190 increased uptake and $V_{\text{max}}$ values in most N-terminal mutants (Table I and Fig. 5). In our in vitro system, KN-62 had no effect on DAT activity, effects contrasting with significant reported KN-62 effects on DAT activity in rat synaptosomes (14). There is thus less evidence for p38 MAP kinase, Ca$^{2+}$/calmodulin kinase II, or related kinase effects on DAT regulation than for the PKC, PI3K, and MEK1/2 pathways.

Interpretation of effects, or lack of effects, on DAT function and expression should be informed by considerations of the differences between the cellular expression systems studied here and the in vivo brain environment. Among these differences may be the complements of other molecules, including both phosphorylating and dephosphorylating enzymes found in these expression systems versus those found in dopaminergic neurons in brain. We have identified recently KEPI and GBP1, members of a family of PKC-dependent inhibitors of the major brain protein phosphatases 1 and 2A, which can thus modulate phosphorylation in a PKC-dependent fashion and alter DAT activities when coexpressed.4 DAT can coimmunoprecipitate with PP2A (35), suggesting a role for this phosphatase in DAT phosphorylation, and can display C-terminal interactions with PICK proteins whose expression alters DAT clustering (38). Conceivably, phosphorylation on DAT C-terminal residues could alter these sorts of interactions and even contribute differentially to changes in cellular localization noted in different cell contexts.

DAT-expressing dopaminergic neurons express G/Gx-coupled G-protein-coupled receptors, single TM domain receptor kinases, and ligand-gated channels (39–43) that are well positioned to modulate the signaling pathways studied here. D2 and D3 receptors can alter functions of MAP kinase activities in renal cells, and D3 modulates PKC activities by actions on PI3K and MAP kinases, for example. Drugs that modulate activities of broad classes of tyrosine kinase receptors can modulate activities of DAT when expressed in Xenopus systems (44).

Combined observations using drugs and mutants can help to parse the otherwise complex picture of DAT regulation through many cellular information pathways. The N terminus of DAT appears to represent a remarkably dominant domain for much DAT regulation. Influences of N-terminal mutations are observed on alterations of dopamine uptake activity induced by PI3K inhibitor, PKC activator, and MAP kinase inhibitor drugs (Fig. 3 and Table I). Each of the eight serine or threonine mutants in the DAT N terminus modulates DAT responses to at least one of the drug treatments examined here: S7A, S21A, and T62A for PMA; S12A, S13A, S21A, and S45A for LY294002; S4A, S12A, S13A, S21A, S45A, and T62A for U0126; and S2A, S4A, S12A, S13A, S21A, and S45A for SB202190. Each of these mutated residues, except S21, is highly conserved in monoamine transporters (Fig. 1). This N-terminal influence is also manifest by the effects of the Val-14 deletion on responses to several of the drugs. By comparison, 75% of the 12 (Ser/Thr) mutations made in other DAT domains modulate DAT responses to at least one drug treatment: S253A, S261A, S333A, T338A, S504A, S585A, and T595A for PMA; and S333A, T338A, T595A, and T612A for U0126, with the second highest density residing in the C terminus of DAT. The relatively high concentration of N-terminal mutations that create functional influences, however, does not appear to indicate that each pathway is influenced in a similar fashion by these mutations. In fact, mutations of the N-terminal serines Ser-4, Ser-12, Ser-13, Ser-21, and Ser-45 influence regulation of DAT activities by PI3K, MEK1/2, and p38 kinase in different directions. Each of the mutants enhances the inhibition of PI3K by LY294002, attenuates the inhibition of MEK1/2 by U0126, but increases the uptake activity when the p38 kinase is inhibited by SB202190 (Table I and Figs. 3 and 4).

Different Ser/Thr mutations influence DAT activity likely through different mechanisms. Mutations at least in Ser-7, Ser-12, Ser-13, Thr-62, Ser-581, Thr-595, and Thr-612 reduce phosphorylation of DAT by PKC, MEK1/2, and PI3K pathways (Fig. 4). This indicates either that these Ser/Thr residues are the direct phosphoacceptor sites or that some of the residues are not the direct phosphoacceptor sites, but mutations in these residues indirectly reduce phosphorylation of DAT. Our current data do not differentiate these two possibilities. Mutations in Ser-12, Ser-13, Thr-2, Ser-581, Thr-595, Thr-612, and possibly in Ser-4, Ser-21, and Ser-45 remove/affect the phosphoacceptor sites, which decreases phosphorylation of DAT and makes the cell surface expression of DAT less sensitive to regulation by kinase pathways. S7A mutation, in this assay system, appears to abolish the phosphorylation of DAT induced by p38, preventing DAT from down-regulation of uptake activity in the plasma membrane by the PKC pathway (Figs. 3 and 4). In either case, these mutations help DAT maintain uptake activity in response to treatments of the kinase effectors (Fig. 3).

Results with PI3K and MEK1/2 kinase pathway modulators mandate special caution when using DAT radioligand binding after treatments that alter kinase pathway activities to infer changes in dopaminergic neuronal integrity. The cocaine analog used for much of this imaging, [3H]β-CIT, can display pseudo-irreversible interactions with DAT-expressing systems in vivo, whereas the "bound" ligand may not necessarily reflect the total DAT pool. [3H]β-CIT binding could be altered by PI3K and MEK1/2 kinase pathways, providing an especial caution for interpretation of results obtained after chronic administration of DRD2 family agonists, for example.

Acknowledgments—We thank Cheryl Evans, Suxia Li, Masanari Itokawa, and Jian-Ping Gong for technical assistance. We also thank Donna Walther for synthesis of DNA oligonucleotides and Dr. Tony Shippenberg for discussions of MAP kinase pathway effects on DAT.

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By guest on July 18, 2018

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Phosphatidylinositol 3-Kinase, Protein Kinase C, and MEK1/2 Kinase Regulation of Dopamine Transporters (DAT) Require N-terminal DAT Phosphoacceptor Sites
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J. Biol. Chem. 2003, 278:20162-20170.
doi: 10.1074/jbc.M209584200 originally published online March 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209584200

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