N-terminal Truncation of the Dopamine Transporter Abolishes Phorbol Ester- and Substance P Receptor-stimulated Phosphorylation without Impairing Transporter Internalization*

The structural basis of phosphorylation and its putative role in internalization were investigated in the human dopamine transporter (hDAT). Activation of protein kinase C (PKC) was achieved either directly by treatment with 4α-phorbol 12-myristate 13-acetate (PMA) or by activating the Goα-coupled human substance P receptor (hNK-1) co-expressed with hDAT in HEK293 cells and in N2A neuroblastoma cells. In both cell lines, activation of the hNK-1 receptor by substance P reduced the V_{max} for [3H]dopamine uptake to the same degree as did PMA (~50 and ~20% in HEK293 and N2A cells, respectively). In HEK293 cells, the reduction in transport capacity could be accounted for by internalization of the transporter, as assessed by cell surface biotinylation experiments, and by fluorescence microscopy using enhanced green fluorescent protein-tagged hDAT. In HEK293 cells, hNK-1 receptor activation, as well as direct PKC activation by PMA, was accompanied by a marked increase in transporter phosphorylation. However, truncation of the first 22 N-terminal residues almost abolished detectable phosphorylation without affecting the SP- or PMA-induced reduction in transport capacity and internalization. In this background truncation construct, systematic mutation of all the phosphorylation consensus serines and threonines in hDAT, alone and in various combinations, did also not alter the effect of hNK-1 receptor activation or PMA treatment in either HEK293 or N2A cells. Mutation of a dileucine and of two tyrosine-based motifs in hDAT was similarly without effect. We conclude that the major phosphorylation sites in hDAT are within the distal N terminus, which contains several serines. Moreover, the present data strongly suggest that neither this phosphorylation, nor the phosphorylation of any other sites within hDAT, is required for either receptor-mediated or direct PKC-mediated internalization of the hDAT.

The dopamine transporter (DAT) is situated in the presynaptic membrane of dopaminergic nerve terminals and is responsible for the rapid removal of dopamine released into the synaptic cleft upon neuronal stimulation (1–3). Accordingly, the transporter plays a critical role in regulating the availability of dopamine in the synaptic cleft and thus in modulating the physiological functions of dopamine, including locomotor activity, higher cognitive functions, and neuroendocrine systems (1, 2). Furthermore, the DAT is the principle target for the action of widely abused psychostimulants such as cocaine and amphetamine (1–3). The transporter belongs to the family of Na+/Cl−-dependent transporters, along with transporters for several other neurotransmitters including the norepinephrine (NET), serotonin (SERT), γ-aminobutyric acid (GAT), and glycine (GLYT) transporters (3, 4). This class of transporters is characterized structurally by 12 transmembrane segments, intracellular N and C termini, and a large glycosylated second extracellular loop (3, 4). No high resolution structural information is available for any related transporter, and our insight into the packing of the 12 helices remains quite limited (3, 4).

Given the critical role of the DAT in regulating dopaminergic neurotransmission, it is not surprising that the activity and availability of the DAT at the cell surface is tightly regulated. Most significantly, activation of protein kinase C (PKC) by phorbol esters such as 4α-phorbol 12-myristate 13-acetate (PMA) is known to lead to an acute reduction in the activity of the dopamine transporter (for review, see Refs. 5–7). This has been demonstrated both in synaptosomal preparations (8, 9) as well as in transfected cell lines and Xenopus oocytes (10–13). A similar effect of PKC activation has also been observed for other neurotransmitter transporters within the same class including the SERT, NET, GAT-1, and GLYT-1 (reviewed in Refs. 5–7). Compelling evidence suggests that the PKC-mediated reduction in uptake is the result of an internalization of the transporters rather than a change in the activity of the transporter molecules residing in the membrane (5–7, 11, 13–15). The internalization of the DAT is believed to occur via a clathrin and dynamin-dependent mechanism, resulting in accumulation of the transporter in early endosomes where it co-localizes with transferrin (13).

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‡‡ The abbreviations used are: DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; hDAT, human dopamine transporter; GAT, γ-aminobutyric acid transporter; GLYT, glycine transporter; WT, wild type; EGF, enhanced green fluorescent protein; SP, substance P; NK, neurokinin; HEK293, human embryonic kidney-293; PKC, protein kinase C; PMA, 4α-phorbol 12-myristate 13-acetate; HA, hemagglutinin; PBS, phosphate-buffered saline.
Phosphorylation-independent Internalization of DAT

The activation of PKC leads to a marked increase in phosphorylation of DAT (8–10). It has been hypothesized, therefore, albeit without direct evidence, that serine and/or threonine phosphorylation of the DAT is the molecular event that drives the internalization process. In light of the predicted high specific activity by which these transporters are regulated, it would accordingly be expected that a specific Ser/Thr phosphorylation site(s) could be identified within the intracellular domains of the transporter and that mutation of this site(s) would result in a concomitant impairment of internalization and phosphorylation. Alternatively, the phosphorylation could represent an independent process and phosphorylation of unidentified associated proteins may be the actual trigger of the internalization process.

In the present study, we have investigated the structural basis and putative role of direct phosphorylation of DAT for its acute down-regulation and internalization. Importantly, we examine not only phosphorylation induced by direct PKC stimulation with phorbol esters but also that induced upon activation of a Goq-coupled human substance P (hNK-1) receptor co-expressed with hDAT in HEK293 cells (human embryonic kidney cells) and in N2A neuroblastoma cells. The hNK-1 receptor was chosen as a typical Goq-coupled receptor that stimulates PKC activity via Goq-dependent activation of phospholipase C (16), is expressed in dopaminergic neurons along with DAT (17) and shown to involve in dopamine release (18). Our results show that activation of the co-expressed hNK-1 receptor, as well as direct activation of PKC by phorbol esters, markedly reduced the transport capacity of DAT and that this reduction can be accounted for by rapid internalization of the transporter to an intracellular compartment. Moreover, in agreement with a recent in vivo study of phosphorylation of the rat DAT (19), evidence is obtained that the increase in transporter phosphorylation seen in response to both hNK-1 receptor and PKC activation is caused almost entirely by phosphorylation of the distal N terminus of DAT. Neither receptor-mediated nor direct PKC-mediated internalization of DAT, however, is impaired by truncation of the putative sites of phosphorylation, suggesting that DAT internalization is not dependent on DAT phosphorylation. Subsequent mutation, alone and in combination, of multiple serines and threonines throughout the intracellular domains of the transporter, also had no effect on hNK-1 receptor-mediated or direct PKC-mediated internalization of DAT, thereby providing additional support for the dissociation of internalization and phosphorylation, at least in two heterologous expression systems.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The cDNA encoding the human dopaminergic transporter (hDAT) in pHCCMY (20) was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC). The FLAG-tag sequence (Asp-Tyr-Lys-Asp-Asp-Lys-Asp) (Sigma) was added to the N terminus of the hDAT cDNA using polymerase chain reaction (PCR)-derived mutagenesis using Pfu polymerase and the instructions from the manufacturer (Stratagene, La Jolla, CA). The resulting FLAG-hDAT construct was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The mutations Y335A, Y373A/D387A, D436A, L440A/L441A, and Y575A were generated by PCR-mediated site-directed mutagenesis in the background of this construct (pcDNA3- D436A, D436A/L440A/L441A, and Y575A were generated by PCR-mediated site-directed mutagenesis in the background of pcDNA3 (Invitrogen, Carlsbad, CA). The mutations Y335A (21), D436A/L440A/L441A, and Y575A (22) were checked by restriction enzyme mapping and automated DNA sequencing.

Cell Culture and Transfection—HEK293 cells were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium with Glutamax I supplemented with 10% fetal calf serum and 0.01 mg/ml gentamicin (all products from Invitrogen) in 5% CO2 in Dulbecco’s modified Eagle’s medium with Earle’s salts with Glutamax-I supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.05 μg/ml streptomycin, and 0.06 μg/ml penicillin G (all products from Invitrogen). For stable expression, the HEK293 or N2A cells were seeded in 100-mm tissue culture plates, grown to ~30% confluence, and subsequently transfected with pcDNA3 or PCIBygro constructs using the LipofectAMINE/Opti-MEM/I (Invitrogen) transfection system. A stably transfected pool was selected with Geneticin (G418) (0.35 mg/ml) or hygromycin (0.35 mg/ml), respectively, as previously described (25). For stable co-expression, a G148-resistant cell line expressing the hNK-1 receptor was transfected with the desired hDAT constructs and stable cell pools expressing both the hNK-1 receptor and the appropriate hDAT construct were obtained by combined selection with hygromycin (0.25 mg/ml) and G418 (0.1 mg/ml).

Dopamine Uptake Experiments—Uptake assays were modified from Girov et al. (26) using 2,5,6-3H)dopamine (7–21 Ci/mmol) (Amersham Biosciences, Little Chalfont, United Kingdom). Transfected cells were plated in poly-L-lysine-coated 24-well dishes (2 × 105 to 4 × 105 cells/well). The cells were grown for 48 h prior to the experiment. On the day of the experiment, the cells were washed once in 500 μl of uptake buffer (7.5 mM HEPES, pH 7.1, with 5 mM Tris base, 120 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1 mM t-arasicid acid, 5 mM d-glucose, and 1 mM amount of the cation-methyltransferase inhibitor 50 mM 3-aminobenzamidine (3-AB) at a final volume of 500 μl. Nonspecific uptake was determined in the presence of 1 mM unlabeled dopamine (Research Biochemicals International, Natick, MA). For determination of non-specific binding (11 different concentrations in triplicate) were added immediately before [3H]dopamine. After 5 min of incubation at 37 °C, the uptake buffer, lysed in 300 μl of 1% SDS, and left 1 h at 37 °C. All samples were transferred to 24-well counting plates (Wallac, Turku, Finland), 600 μl of Opti-phase Hi Safe 3 scintillation fluid (Wallac) was added, and the plates were counted in a Wallac Tri-Lux β-scintillation counter (Wallac).

Binding Assays—Binding assays were carried out essentially as described (27) on whole cells using 125I-labeled substance P (2000 Ci/mmol) (Amersham Biosciences, Little Chalfont, United Kingdom) as radioligand. Transfected cells were plated in poly-L-lysine-coated 96-well dishes (2 × 105 cells/well). The cells were incubated for 48 h prior to the experiment. Binding assays were performed in a final volume of 100 μl of binding buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 5 mM MgCl2, 0.1% bovine serum albumin, and 40 μg/ml bacitracin) with 0.4 μM 125I-labeled substance P plus increasing concentrations of non-labeled dopamine (11 different concentrations in triplicate) were added immediately before [3H]dopamine. After 5 min of incubation at 37 °C, the cells were washed twice with 500 μl of uptake buffer, lysed in 300 μl of 1% SDS, and left 1 h at 37 °C. All samples were transferred to 24-well counting plates (Wallac, Turku, Finland), 600 μl of Opti-phase Hi Safe 3 scintillation fluid (Wallac) was added, and the plates were counted in a Wallac Tri-Lux β-scintillation counter (Wallac). Kinetic analyses of the transporter were performed at 5 μM substance P.

Labeling with Sulfo-NHS-SS-biotin—Biotinylation of cell surface proteins was performed by reaction with the membrane-impermeant maleimido-reactive biotin sulfo-NHS-SS-biotin (Pierce). Transfected HEK293 cells were seeded in poly-L-lysine-coated 100-mm cell culture dishes (Corning, Corning, NY) at 5 × 105 cells/dish and grown for 24 h before the experiment. Cells were stimulated with 200 nM SP, 1 μM PMA, or vehicle for 1 h at 37 °C. The cells were subsequently washed with ice-cold phosphate-buffered saline (PBS)/Ca-Mg, and the cells were then treated with sulfo-NHS-SS-biotin (1 mg/ml) for 40 min in PBS/Ca-Mg, followed by two washes with 100 mM glycine in PBS/Ca-Mg, and incubation with 100 mM glycine for 20 min. The cells were washed with PBS/Ca-Mg and lysed with 3 ml of lysis buffer (25 mM Tris, pH 7.5, with 1 mM EDTA, 5 mM N-ethylmaleimide, 200 μM...
phosphomethylsulfonyl fluoride, and a protease inhibitor mixture tablet (Roche Diagnostics), scraped off, and centrifuged at 1,000 x g for 5 min. The cell pellets were resuspended in 1 ml of solubilization buffer (lysis buffer supplemented with 150 mM NaCl and 1.0% Triton X) and left for 30 min at 4 °C with constant shaking. Lysates were centrifuged at 20,000 x g for 30 min at 4 °C, and the protein concentration in the supernatants was determined using a Bio-Rad D6 protein assay kit. Monomeric avidin beads (175 μl) (Pierce) were added to the samples, 500 μg of total cell protein was used for the Δ1–22 and N’ mutants, whereas 1000 and 1500 μg were used for the for the N + C and ICL mutants, respectively. The volume was adjusted to 1.0 ml with solubilization buffer, and the samples were incubated for 1 h at room temperature. The beads were washed four times with solubilization buffer, before elution with 50 μl of 2X loading buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 0.1% dithiothreitol, and 0.2% bromphenol blue) for 30 min at room temperature. The eluates (25 μl) were resolved by SDS-PAGE (10% acrylamide) and immunoblotted with monoclonal mouse HA antibody (Sigma) diluted 1:2000. Immuno reactive bands were visualized using goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000) and the ECL detection method (Amersham Biosciences). Quantification of bands was performed on a model 300A densitometer in combination with ImageQuant software (Amersham Biosciences) by using film exposures that were in the linear range.

**[^32P]Orthophosphate Labeling and Immunoprecipitation**—Stably transfected HEK293 cells (3.5 x 10⁴) were seeded in poly-D-lysine-coated 25-cm² flasks and grown for 48 h releasing ~ 70% confluence. The cells were washed in phosphate-free medium before labeling at 37 °C for 4 h in phosphate-free medium containing 10 μCi dialyzed fetal calf serum (In vitrogen), 20 mM HEPES, pH 7.2, and 1.0 μCi/[methyl-[^32]P]orthophosphate (Amersham Biosciences). Upon the addition of phosphatase inhibitors, 1 μM okadaic acid and 50 μM NaF, the cells were stimulated with 200 nM SP, 1 μM PMA, or vehicle for 60 min followed by washing in 5 ml ice-cold PBS and subsequent lysis by shaking for 15 min on ice in lysis buffer (25 mM Tris-HCl, pH 7.6, with 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 50 mM sodium pyrophosphate, 50 mM NaF, 1 μM okadaic acid, and 1% Triton X-100) supplemented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture tablet (Roche Diagnostics)). The cell lysates were centrifuged at 20,000 x g for 15 min at 4 °C and precleared with 4 μg/ml mouse IgG and 100 μl of protein G-Sepharose before immunoprecipitation for 1 h at 4 °C using an antibody against the FLAG epitope (2 μg of M2 antibody, Sigma). For immunoprecipitation of the Δ1–22-FLAG-hDAT, 500 μg of total cell protein was used, and, for immunoprecipitation of FLAG-hDAT, 750 μg was used. Samples were resolved by SDS-PAGE (10%) and either transferred into a nitrocellulose membrane and blotted with a biotinylated M2 antibody (Sigma) according to instructions from the manufacturer or exposed to a PhosphorImager screen, quantified using a PhosphorImager (Amersham Biosciences) and analyzed with ImageQuant software (Amersham Biosciences).

**Fluorescence Microscopy**—Stably transfected HEK293 cells were seeded in eight-well Lab-Tek II glass chamber slides (Nalge Nunc International, Naperville, IL). After 36 h, the medium was changed to uptake buffer and the cells were incubated in the presence of indicated substances or vehicle (control) for 30 min at 37 °C. Cells were examined using a confocal microscope (Leica DM IRBE HBC S100 Fluor TCS) microscope (magnification, ×63). The filter sets for the epifluorescence were BP 450–490 nm for excitation of EGFP, and the emitted light passed through a LP 515-nm filter.

**Calculations**—Uptake and binding data were analyzed by nonlinear regression analysis using Prism 3.0 from GraphPad Software, San Diego, CA. One-way analysis of variance followed by Dunnett’s post hoc test was used for statistical comparisons.

**RESULTS**

**Regulation of DAT by Phorbol Esters and Substance P**—The hNK-1 (substance P) receptor, which is a typical Gαq-coupled 7-transmembrane segment receptor (16), was stably expressed in HEK293 cells using the bicistronic vector pCIN4 (23). Expression of the receptor was detected by demonstration of specific high affinity binding of [125I]labeled substance P in a whole cell binding assay (Kd = 0.22 ± 0.04 nM, Bmax = 3.2 ± 1.3 fmol/10,000 cells, n = 4). Specific binding could not be detected in non-transfected cells (data not shown). The effect of hNK-1 receptor activation on hDAT activity in comparison to that of direct PKC activation by phorbol esters was investigated upon transient expression of the hDAT tagged at the N terminus with the FLAG epitope (FLAG-hDAT) in the hNK-1 receptor-expressing cells. Note that the addition of this FLAG tag does not affect the functional properties of the protein.2 In agreement with previous observations, stimulation with the phorbol ester PMA (1 μM for 30 min) resulted in a marked reduction in transporter uptake capacity (~50%) that could be reversed by the PKC inhibitor staurosporine (Fig. 1A). Also as expected, the phosphatase inhibitor okadaic acid reduced uptake capacity, although to a smaller extent (~25%) (Fig. 1A). These responses were compared with those observed in response to the hNK-1 receptor agonist substance P. As shown in Fig. 1A, stimulation with 200 nM substance P for 30 min reduced [3H]dopamine uptake capacity to approximately the same extent as PMA. Importantly, a similar effect of 200 nM substance P was observed for the non-tagged hDAT (data not shown). The combined addition of PMA and substance P as well as the combined addition of okadaic acid and substance P resulted in only marginally higher responses than those observed in response to the individual compounds (Fig. 1A). Addition of the PKC inhibitor staurosporine markedly inhibited the response to substance P, although not as efficiently as it inhibited the response to PMA. This suggests that the inhibitory effect of substance P on hDAT

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2 L. Norregaard, C. J. Loland, and U. Gether, unpublished observation.
is mediated, at least in part, via PKC, consistent with the ability of the hNK-1 receptor to activate G$_q$-dependent pathways.

The acute down-regulation observed in response to substance P treatment of HEK293 cells co-expressing the hDAT and the hNK-1 receptor was further investigated. Incubation of the cells co-expressing FLAG-hDAT and hNK-1 with increasing concentrations of substance P for 30 min caused a dose-dependent reduction in the specific $[^3H]$dopamine uptake with an $EC_{50}$ of 5.5 ± 0.1 nM ($n = 5$) and a maximal inhibition of around 40% (Fig. 1B). Addition of 1 μM selective high affinity hNK-1 receptor antagonist LY303870 (28) essentially eliminated the effect of substance P on $[^3H]$dopamine uptake (Fig. 1B), consistent with the specific action of substance P at hNK-1. Moreover, no effect of substance P was observed when the same experiments were performed on HEK293 cells expressing FLAG-hDAT but not the hNK-1 receptor (data not shown). Finally, to assess the mechanism of the inhibitory effect of substance P and PMA on uptake capacity, saturation uptake experiments with $[^3H]$dopamine were carried out with the Δ1–22FLAG-HA-hDAT construct, which revealed that substance P and PMA profoundly decreased the $V_{\text{max}}$ but did not alter the $K_{\text{m}}$ for $[^3H]$dopamine uptake (Table I).

### Table I

| $V_{\text{max}}$ (means ± S.E.) | $K_{\text{m}}$ (DA) (S.E. interval) | n |
|---------------------------------|-----------------------------------|---|
| pmol/min/10$^6$ cells           | μM                                |   |
| Control                         | 27 ± 2                            | 2.5 (2.3–2.7) | 3 |
| 200 nM SP                       | 7.2 ± 0.8                         | 2.8 (2.3–3.5) | 3 |
| 1 μM PMA                        | 14 ± 4                            | 2.5 (2.1–2.9) | 3 |

### Substance P and PMA Promote Internalization of the hDAT—

The decrease in $V_{\text{max}}$ for $[^3H]$dopamine uptake produced by substance P and PMA could either be the result of sequestration of the transporter from the cell surface or of a decrease in the transport rate of the individual transporter molecules (or a combination of these effects). To investigate this, we fused EGFP to the N terminus of hDAT and stably co-expressed the resulting construct with the hNK-1 receptor in HEK293 cells. Co-expression was verified by measurement of specific $^{125}$I-labeled substance P binding ($B_{\text{max}} = 3.2 ± 1.3$ fmol/10,000 cells, $n = 3$) and specific $[^3H]$dopamine uptake. In the EGFP-hDAT-expressing cells, $[^3H]$dopamine uptake was similar to that observed in FLAG-hDAT-expressing cells (data not shown). Substance P and PMA inhibited $[^3H]$DA uptake in the HEK293 cells co-expressing the EGFP-hDAT and the hNK-1 receptor to an extent similar to that observed in cells co-expressing the FLAG-hDAT and the hNK-1 receptor (data not shown). Fluorescence microscopy of vehicle-treated cells demonstrated a clear fluorescent signal corresponding to the plasma membrane and essentially no intracellular fluorescence (Fig. 2, upper panel). However, a 30-min incubation with either 200 nM substance P or 1 μM PMA resulted in a substantially reduced surface fluorescence and the appearance of punctuate intracellular fluorescence (Fig. 2, middle and lower panels). These data support the hypothesis that the inhibitory effect of substance P on hDAT function, similar to PMA, is caused by rapid internalization of the transporter to an intracellular compartment.

**Mutation of Trafficking Motifs in the hDAT—** Both dileucine motifs ((D/E)XXLL, where D/E is an acidic residue, X any residue, and L a leucine) and tyrosine-based motifs (YXXφ where Y is a tyrosine, X is any amino acid, and φ is a residue with a bulky hydrophobic side chain) are known to play key roles in intracellular trafficking of membrane proteins by mediating critical protein-protein interactions (29, 30). The hDAT contains a dileucine motif in the third intracellular loop (Fig. 3) and two tyrosine-based motifs, one in the second intracellular loop (Tyr-335) and one in the C terminus (Tyr-575). As part of our effort to understand the molecular basis for transporter...
internalization, all three motifs were mutated. Asp-436 was mutated either alone (D436A) or together with the two leucines (D436A/L440A/L441A, called DLL in Fig. 4), whereas Tyr-335 and Tyr-575 were mutated one at a time to alanine (Y335A and Y575A). The mutants were all transiently expressed in HEK293 cells stably expressing the hNK-1 receptor. In all four mutants (D436A/L440A/L441A, D436A, Y335A, and Y575A), 200 nM SP and 1 \( \mu \)M PMA inhibited \( [3H] \)dopamine uptake to the same extent as observed in the WT (Fig. 4). It should be noted that in Y335A the overall specific uptake was reduced substantially (to \( \approx 4\% \) of the WT level, Table II). In agreement with our previously published results, addition of Zn\(^{2+}\) partially restored uptake in the Y335A mutant (21). However, the relative effect of substance P and PMA in the presence of Zn\(^{2+}\) on uptake in cells expressing the Y335A mutant was the same as in the absence of Zn\(^{2+}\) (data not shown). Altogether, these data indicate that the trafficking motifs present in hDAT are not critical for either PKC- or hNK-1 receptor-mediated hDAT-internalization.

Mutation of Putative Phosphorylation Sites in the hDAT—It is the prevailing perception that phosphorylation of specific serine and/or threonine residues is required for PKC-dependent hDAT internalization. We decided to undertake a systematic mutagenesis approach both to test this hypothesis and to identify the possible specific site or sites responsible for the internalization process. As shown in the two-dimensional representation of hDAT in Fig. 3, DAT contains multiple serines and threonines in the predicted intracellular domains. In several cases, these residues are part of consensus sequences for PKC phosphorylation (Ser-7, Ser-53, Thr-62, Ser-261, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31), cGMP-dependent protein kinase phosphorylation (Ser-53, Thr-62, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31), cGMP-dependent protein kinase phosphorylation (Ser-53, Thr-62, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31), cGMP-dependent protein kinase phosphorylation (Ser-53, Thr-62, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31), cGMP-dependent protein kinase phosphorylation (Ser-53, Thr-62, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31), cGMP-dependent protein kinase phosphorylation (Ser-53, Thr-62, Ser-262, Thr-339, Ser-517, Ser-586, and Thr-613) (31).

**FIG. 3.** Schematic two-dimensional representation of the human dopamine transporter. All enlarged circles indicate residues that were mutated to alanines. Black circles with white letter indicate mutated Ser/Thr residues that are part of phosphorylation consensus sites (31). Dark shaded circles with white letter indicate Ser/Thr residues that were mutated but that are not part of classical consensus sites for phosphorylation. Light shaded circles with black letter indicate residues that are part of putative trafficking motifs (tyrosine-based and dileucine motifs) (29, 30).

**FIG. 4.** Mutation of a dileucine motif and two tyrosine-based motifs does not affect regulation of DAT by substance P or PMA. The data represent the relative \( [3H] \)dopamine uptake observed upon stimulation with substance P (200 nM) or PMA (1 \( \mu \)M) for 30 min at 37°C in percentage of uptake in untreated cells (mean ± S.E. of three to five experiments performed in triplicate). The experiment was performed in HEK293 stably expressing hNK-1 and transiently expressing either FLG-hDAT (WT in figure) or indicated mutants (Table I) generated in the background of this construct. The decrease in \( [3H] \)dopamine uptake as compared with untreated control cells was significant for all four mutants \( (p < 0.01) \).
339, Ser-517) (31), cAMP-dependent kinase phosphorylation (Ser-517) (31), and calmodulin-dependent protein kinase II phosphorylation (Thr-613) (31). An analysis of the hDAT sequence using the NetPhos prediction server (www.cbs.dtu.dk/services/NetPhos), which produces neural network predictions for serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins, also confirmed that several sites are highly likely to become phosphorylated. Notably, four serines/threonines and one tyrosine displayed a probability larger than 0.8 for being a phosphorylation site (Ser-53, Ser-517, Ser-586, Thr-613, and Tyr-593).

In our mutagenesis strategy, we initially generated a construct in which the first 22 N-terminal residues were deleted and substituted with the FLAG tag followed by a HA tag (Δ1–22FLAG-HA-hDAT) (22). This construct displayed uptake properties similar to the full-length DAT (Tables I and II and Ref. 22). In the background of Δ1–22FLAG-HA-hDAT, we generated eight constructs containing various Ser/Thr substitutions and a single tyrosine substitution (Table II). Overall, we mutated all remaining serines and threonines in the N terminus (N’), all serines and threonines plus Tyr-593 in the C terminus (C), and four serines/threonines in the loops, which are part of PKC consensus motifs (ICL). These mutants were combined in different ways (N’+C, N’+C, N’+ICL, and C+ICL).

In addition, we generated a construct in which eight PKC consensus sites were simultaneously mutated (XPK8). All the mutants displayed specific [3H]dopamine uptake upon transient transfection into the HEK293 cells stably expressing the hNK-1 receptor (Table II). As a general trend, increasing the number of mutated residues in the transporter resulted in lower uptake capacity (Table II). The transporter was particularly sensitive to mutations in the intracellular loops. Accordingly, it was not possible to generate a transporter entirely devoid of Ser/Thr residues, but only the construct with eight PKC consensus sites mutated (XPK8). This mutant transporter displayed a substantially reduced uptake capacity (Table II).

Importantly, surface biotinylation experiments provided evidence that this reduction was caused by a lowered surface expression rather than a functional change of the mutant transporters residing in the membrane (Fig. 6 and data not shown).

The mutant transporters were assayed for their sensitivity to hNK-1 receptor and PKC activation. As shown in Fig. 5, like FLAG-hDAT, all nine mutants displayed a strong inhibition of [3H]dopamine uptake in response to both 200 nM substance P and 1 μM PMA (Fig. 5). We even observed a tendency to an enhanced response to PMA in the mutants containing substitutions of residues in the intracellular loops (ICL, N+ICL, C+ICL, and XPK8) (Fig. 5). In the ICL mutant, for example, PMA reduced uptake more than 70% in contrast to ~50% for the WT. Likewise, PMA reduced uptake in XPK8 by ~65% despite the fact that eight consensus sites for PKC phosphorylation were simultaneously mutated (Fig. 5). Assuming that the observed reduced uptake capacity for each mutant reflects internalization of the transporter, these data argue against the hypothesis that phosphorylation of specific serines and threonines is required for internalization promoted by either direct

**TABLE II**

| Abbreviation | Full name of transporter | $V_o$ % |
|--------------|--------------------------|--------|
| WT | FLAG-hDAT | 100 ± 10 |
| Δ1–22 | Δ1–22FLAG-HA-hDAT | 71 ± 2 |
| N’ | Δ1–22FLAG-HA-hDAT T43A/S44A/S53A/T62A/G74A:S53A/T62A | 36 ± 7 |
| C | Δ1–22FLAG-HA-hDAT Y593A/S582A/S586A/T613A | 43 ± 8 |
| N’+C | Δ1–22FLAG-HA-hDAT S44A/S53A/S53A/T62A/S582A/S586A/T613A | 18 ± 6 |
| ICL | Δ1–22FLAG-HA-hDAT T43A/S44A/S53A/T62A/G74A:S53A/T62A/Y593A/S582A/S586A/T613A | 15 ± 4 |
| N’+ICL | Δ1–22FLAG-HA-hDAT S53A/T62A/S586A/S582A/S586A/T613A | 16 ± 10 |
| C+ICL | Δ1–22FLAG-HA-hDAT S262A/T339A/S517A/S582A/S586A/T613A | 10 ± 5 |
| XPK8 | Δ1–22FLAG-HA-hDAT S53A/T62A/S262A/T339A/S517A/S582A/S586A/T613A | 1.5 ± 0.4 |
| Y575A | FLAG-hDAT-Y575A | 12 ± 3 |
| Y35A | FLAG-hDAT-Y35A | 3.7 ± 1 |
| D436A | FLAG-hDAT-D436A | 13 ± 3 |
| DLL | FLAG-hDAT-D436A/L440A/L441A | 31 ± 5 |

**Fig. 5. Mutation of multiple Ser/Thr residues including all nine PKC consensus sites does not affect regulation of DAT by substance P and PMA.** The data represent the relative [3H]DA uptake observed upon stimulation with substance P (200 nM) or PMA (1 μM) for 30 min at 37 °C in percentage of uptake in untreated cells (mean ± S.E. of three to five experiments performed in triplicate). The experiment was performed in HEK293 stably expressing hNK-1 and transiently expressing either Δ1–22FLAG-HA-hDAT or indicated mutants generated in the background of this construct (Table I). The decrease in [3H]DA uptake as compared with untreated control cells was significant for all four mutants ($p < 0.01$).
PKC activation or by activation of the G-protein-coupled hNK-1 receptor. In addition, the data suggest that phosphorylation of Tyr-593 is also not critical for this process.

Direct Evidence for PMA- and Substance P-induced Internalization of the Mutant Transporters—Next, we wished to verify that the inhibition of uptake observed in response to PMA and substance P in the mutants described above did reflect an actual sequestration of the transporter from the surface of the cell. Therefore, we carried out cell surface biotinylation experiments on selected mutants using the membrane-impermeant biotinylation reagent sulfo-NHS-SS-biotin (sulfosuccinimidyl-NHS-biotin) to assess whether stimulation with PMA and substance P would alter the amount of hDAT protein in the plasma membrane. As shown in Fig. 6, the data from the biotinylation experiment substantiated the observations with EGFP-tagged transporter (Fig. 2) by providing clear biochemical evidence that the reduced uptake seen upon treatment of the cells with substance P and PMA could be explained by sequestration of the transporter from the cell surface (Fig. 6). The data also showed that neither removal of the serines in the distal N terminus (∆1–22FLAG-HA-hDAT), additional mutation of several N-terminal serines and threonines (N), mutation of PKC consensus sites in the loops (ICL), nor the combined mutation of several N-terminal and all C-terminal serines/threonines plus Tyr-593 affected sequestration of the transporter from the cell surface. For all four analyzed constructs, substance P reduced surface expression ~40%, whereas PMA reduced surface expression ~60–70%. These results correspond well to the observed inhibition of uptake for these mutant transporters (Fig. 5).

The hDAT Is Phosphorylated in Its Distal N Terminus—To further substantiate the conclusion that hDAT phosphorylation is not required for internalization, we performed direct phosphorylation assays in the transfected HEK293 cells. Specifically, we wished to exclude the remote possibility that phosphorylation of one or more of the few remaining non-consensus site serines and threonines that we did not mutate might be responsible for PMA- and substance P-induced internalization. We compared control cells expressing the hNK-1 receptor, cells co-expressing the hNK-1 receptor and FLAG-hDAT, and cells co-expressing the hNK-1 receptor and ∆1–22FLAG-HA-hDAT. The cells were labeled for 4 h with [32P]orthophosphate before stimulation for 60 min with 200 nM substance P or 1 μM PMA in the presence of 1 μM okadaic acid. The transporter was subsequently immunoprecipitated from the cell extracts with an antibody directed against the FLAG epitope and analyzed by SDS-PAGE and phosphoimaging (Fig. 7A). For the FLAG-hDAT, basal phosphorylation is markedly enhanced both upon stimulation with PMA and substance P (Fig. 7A). The phosphorylated FLAG-hDAT is seen as a broad band with an apparent mass of ~100 kDa, which represents the mature, fully glycosylated monomeric form of the transporter (22). As expected, the band was not observed in the HEK293 cells solely expressing the hNK-1 receptor (control in Fig. 7A). Notably, the phosphorylated band was also not observed upon truncation of the 22 N-terminal residues (∆1–22FLAG-HA-hDAT). Parallel Western blotting analysis of the same immunoprecipitated cell extracts verified the presence of FLAG-hDAT in quantities similar to ∆1–22FLAG-HA-hDAT (Fig. 7B). Taken together, these data suggest that one or more of the serines present in the first 22 N-terminal residues of the transporter represent the major target for phosphorylation of the hDAT.
and ICL were verified by detection of specific \([3H]DA\) uptake in FLAG-hDAT and the mutants. The standard error is smaller than the size of the symbols.

One-phase exponential association). Note that the apparent absence of fmol/10,000 cells, mean

the transfected cells. In /H9004

/22FLAG-HA-hDAT, both constructs displayed a similar response to substance P and PMA after 30 min of stimulation (Fig. 5). However, this does not exclude the possibility that phosphorylation of the distal N terminus could play a role in the rate at which the response develops. Accordingly, we performed time-course experiments of the response to substance P on both FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT. As illustrated in Fig. 8, the rate of the responses to substance P was essentially identical in the two constructs with \(t_1/2\) values of 9 (7–16) min and 12 (10–16) min (means of \(n = 3\); S.E. interval) for FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT, respectively. It should be noted that, because of the rapidity of the response, reliable determinations at early time points were rather difficult explaining the relatively large error bars (Fig. 8).

Both WT and Mutants Are Regulated by PMA and Substance P in N2A Neuroblastoma Cells—Finally, we wanted to investigate whether receptor-mediated and PKC-mediated regulation of DAT is independent of serine and threonine phosphorylation not only in HEK293 cells but also in a neuronally derived cell line. Accordingly, the hNK-1 receptor was stably expressed in the human neuroblastoma cell line N2A followed by transient co-expression with FLAG-hDAT and selected hDAT mutants. Expression of hNK-1 was assessed by radioligand binding using \(^{125}\text{I}\)-labeled substance P (\(B_{\text{max}} = 2.4 \pm 0.9 \text{ fmol/10,000 cells, mean} \pm \text{S.E.; } n = 3\)). Transient expression of FLAG-hDAT and the mutants \(\Delta1–22\)FLAG-HA-hDAT, \(N^c + C\), and ICL were verified by detection of specific \([3H]DA\) uptake in the transfected cells. In \(\Delta1–22\)FLAG-HA-hDAT, \(N^c + C\), and ICL, \([3H]DA\) uptake values were 80 \(\pm\) 28, 62 \(\pm\) 15, and 29 \(\pm\) 6.8\%, respectively (means \(\pm\) S.E.; \(n = 3\)) of that observed for FLAG-hDAT (0.14 \(\pm\) 0.04 pmol/min/10\(^5\) cells, mean \(\pm\) S.E.; \(n = 3\)). As shown in Fig. 9, the response to both PMA and substance P was somewhat smaller in the transfected N2A cells (\(-20\%\) reduction in uptake versus \(40–60\%\) in HEK293 cells) but still reproducible and highly significant for the mutants tested (FLAG-hDAT, \(\Delta1–22\)FLAG-HA-hDAT, \(N^c + C\), and ICL) (Fig. 9). The almost identical effects of substance P and PMA in the mutants as compared with those in the WT support the conclusion that regulation via a G protein-coupled receptor and/or PKC is independent of alteration in Ser/Thr phosphorylation of hDAT in a neuronally derived cell line as well as in HEK293 cells.

**FIG. 8.** Time course of substance P-mediated regulation of FLAG-hDAT compared with \(\Delta1–22\)FLAG-HA-hDAT. HEK293-hNK-1 cells transiently transfected with FLAG-hDAT (A) or \(\Delta1–22\)FLAG-HA-hDAT (B) were incubated at 37 °C in the presence of 200 nM substance P for the indicated amounts of time before performing \([3H]DA\) uptake. The data are shown as percentage of maximum inhibition of uptake (\(t = 60\) min) (means \(\pm\) S.E. of \(n = 3\)). The \(t_1/2\) for FLAG-hDAT is 9 (7–16) min and 12 (10–16) min for \(\Delta1–22\)FLAG-HA-hDAT (means of \(n = 3\); S.E. interval), non-linear regression analysis using one-phase exponential association). Note that the apparent absence of error bars for time points 30 and 60 min is a result of the fact that the standard error is smaller than the size of the symbols.

**FIG. 9.** Regulation of DAT and selected Ser/Thr mutants expressed in N2A cells by substance P and PMA. N2A-hNK-1 cells transiently expressing the indicated constructs (Table I) were stimulated with substance P (200 nM) or PMA (1 \(\mu\)M) for 30 min at 37 °C before measurement of \([3H]DA\) uptake. The data shown are the relative \([3H]DA\) uptake expressed as a percentage of uptake in untreated cells (mean \(\pm\) S.E.; \(n = 3\) experiments performed in triplicate). The decrease in \([3H]DA\) uptake as compared with untreated control cells was significant for all constructs tested (*, \(p < 0.05\); ***, \(p < 0.01\)). No effect of substance P was observed when the same experiments were performed on N2A cells not expressing hNK-1 (data not shown).

**Similar Kinetics of Substance P-mediated Down-regulation of FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT**—Despite the major difference in phosphorylation of FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT, both constructs displayed a similar response to substance P and PMA after 30 min of stimulation (Fig. 5). However, this does not exclude the possibility that phosphorylation of the distal N terminus could play a role in the rate at which the response develops. Accordingly, we performed time-course experiments of the response to substance P on both FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT. As illustrated in Fig. 8, the rate of the responses to substance P was essentially identical in the two constructs with \(t_1/2\) values of 9 (7–16) min and 12 (10–16) min (means of \(n = 3\); S.E. interval) for FLAG-hDAT and \(\Delta1–22\)FLAG-HA-hDAT, respectively. It should be noted that, because of the rapidity of the response, reliable determinations at early time points were rather difficult explaining the relatively large error bars (Fig. 8).

**Discussion**

Considerable evidence has previously indicated a key role of PKC in regulating the activity and availability of DAT at the cell surface (reviewed in Refs. 5–7). In agreement with this, we show here both in transfected HEK293 cells and N2A neuroblastoma cells that activation of PKC by phorbol esters elicits a marked decrease in hDAT transport activity and that this decrease results from rapid sequestration of the transporter from the cell surface. In addition, we demonstrate in the two cell lines that DAT activity can be regulated by the \(\text{G}_{\alpha}\)-coupled hNK-1 (substance P) receptor, which is known to be expressed in dopaminergic neurons (17). Like direct activation of PKC by PMA, activation of the hNK-1 receptor by substance P caused a marked reduction in uptake and a concomitant internalization of the hDAT.

The acute down-regulation of the hDAT observed in response to PKC activation was accompanied by a parallel increase in hDAT phosphorylation (Fig. 7) in concurrence with earlier studies in heterologous cells and rat synaptosomes (8–12). Likewise, we found that activation of the hNK-1 receptor expressed in the same cells enhanced phosphorylation of hDAT (Fig. 7). Truncation of the 22 N-terminal residues, which contains several serines that might be phosphorylated, nearly abolished detectable phosphorylation without affecting functional regulation by PKC and the hNK-1 receptor (Fig. 7). To address the question whether phosphorylation below the level that we could detect still played a role in the truncation mutant (\(\Delta1–22\)FLAG-HA-hDAT), we performed systematic mutagenesis of multiple serines and threonines in the predicted intracellular domains of the transporter. None of the mutants, including one in which eight PKC consensus sites were simultaneously mutated and another without any serines or threonines in the entire N and C termini, displayed a significantly altered fractional response to substance P or PMA in...
either HEK293 cells or N2A cells (Figs. 4 and 5). Several of the mutants did, however, display decreased overall uptake capacity as compared with that of the WT (Table II), but this was a result of lowered surface expression of the mutant transporters rather than of altered function of the transporter molecules in the plasma membrane, as indicated from surface biotinylation experiments of selected mutants (Fig. 6 and data not shown). It should be noted that a few non-consensus serines and threonines, such as, e.g., Ser-333 and Ser-334 (Fig. 3), is the critical driving force for the internalization observed. Hence, altogether our data provide strong evidence that phosphorylation is not required for internalization of the transporter. Furthermore, the data suggest that N-terminal serines are the major phosphorylation sites in the hDAT. Obviously, we cannot exclude the alternative possibility that the N terminus is not directly phosphorylated but instead is critical for phosphorylation of other sites in the transporter. It is therefore particularly interesting that a recent in vivo study supports the conclusion that serines in the N-terminal cytoplasmic tail of the DAT represent the major sites of phosphorylation in response to PKC activation (19). In this study, enzymatic digestion of $^{32}$PO$_4$-labeled DAT was carried out followed by immunoprecipitation of the resulting peptide fragments with N- or C-terminal specific antibodies (19). Specifically, proteolytic cleavage with endoproteinase Asp-N resulted in isolation of phosphorylated N-terminal fragments (19). Moreover, it was found that brief aminopeptidase treatment removed all detectable phosphorylation from the DAT (19).

Because the present study was carried out using epitope-tagged constructs in heterologous expression systems, it is necessary to be somewhat cautious in extrapolating our conclusions to the in vivo situation. However, the fact that that we were able to achieve the same result in both HEK293 cells and in the neuronally derived N2A cells does support that our data are of general importance. Moreover, as mentioned above, our data agree fully with recent in vivo data providing evidence for phosphorylation of the distal N terminus (19). In addition, we have no evidence that the addition of the N-terminal FLAG epitope to the full-length transporter affects its function or regulation. Expression and uptake are essentially identical, as are the PMA- and substance P-induced acute reduction in uptake capacity of both the non-tagged and the FLAG-tagged transporter (data not shown). Another critical issue when using heterologous expression is that the expression levels achieved may not correspond to that found in native tissue. Without the use of inducible promoters, it is unfortunately difficult to accurately titrate expression. It is interesting to note, however, that despite substantial differences in expression levels several mutants displayed the same sensitivity to PMA and substance P, indirectly suggesting that the level of expression is not highly critical for the observed effects. Finally, we cannot rule out the remote possibility that the use of a heterologous expression system masks more subtle effects of phosphorylation. For example, phosphorylation under in vivo conditions, although not essential in itself, might enhance the likelihood of internalization when associated with other factors. Furthermore, it should be considered that both the measurements of internalization and phosphorylation are rather slow measurements that may not reveal minor kinetic differences, which could be critical in vivo.

Consistent with the present data, the simultaneous muta-
tion of Ser-262, Ser-586, and Thr-613 to glycine in the human DAT was recently reported not to affect PKC-mediated regulation of DAT activity (32). The authors also studied phosphorylation of the triple mutation in comparison to the WT (32). They found that the PMA-induced increase in phosphorylation apparently was absent in the mutant, although basal phosphorylation in the absence of PMA was preserved or even increased (32). Consequently, it was concluded that direct phosphorylation of the DAT is not responsible for PKC-mediated regulation of the transporter (32). Although we have reached the same conclusion based on our present data, their phosphorylation data differ substantially from both our data and the data from the very recent study of rat striatal tissue (19). The apparent discrepancy with the observations by Chang et al. (32) may relate to differences between the phosphorylation assay protocols. Thus, Chang et al. measured phosphorylation over an extended time frame of 12 h (32). Upon 30 min of stimulation with PMA, the cells were incubated with $^{32}$PO$_4$ for 12 h before the level of phosphorylation was assessed (32). In contrast, in both our protocol and that of Vaughan and co-workers (19), the cells were precinated with $^{32}$PO$_4$ followed by PMA stimulation for 30–60 min and immediate assessment of phosphorylation. It is highly likely that adaptive processes together with the concerted action of both kinases and phosphatases over 12 h may cause profound changes in the transporter phosphorylation state; accordingly, it may be difficult to judge the relationship between the acute effect of PKC activation and phosphorylation measured over such a long time period.

In addition to DAT, several other Na+/Cl⁻-dependent neurotransmitter transporters have been shown to be regulated by PKC-dependent mechanisms. These include SERT (33, 34), NET (35, 36), GAT-1 (37), and GLYT-1 (38, 39). Activation of PKC is generally found to inhibit transporter uptake, and for SERT, NET, and GAT-1, PKC activation also leads to sequestration of the transporters from the cell surface to an intracellular compartment (33, 35–37). In SERT, as in DAT, PKC activation causes direct phosphorylation (34), which has led to the prevailing assumption that phosphorylation of the transporter is critical for PKC-mediated down-regulation. This assumption was further supported by a close association between the time course of down-regulation of SERT and phosphorylation (34). In SERT, the effect of PKC activation can be modulated by substrates (40). The substrates serotonin and amphetamine caused retention of the transporter on the surface upon PKC activation, whereas no effect was observed in the presence of inhibitors such as cocaine, citalopram, and imipramine (40). Interestingly, the effect of the substrates was directly reflected in the level of phosphorylation; in response to phorbol esters the level of SERT phosphorylation increased, but in the presence of substrates this increase was abolished (40). These data are consistent with a critical role of SERT phosphorylation in regulating the subcellular localization of the transporter and accordingly its activity. However, no thorough mutagenesis study aimed at identifying the critical phosphorylation sites in SERT has been carried out; thus, it is in principle possible that, at least in transfected cells, the alteration in direct phosphorylation of SERT is secondary and is not essential for transporter internalization. Accordingly, it is possible that phosphorylation is dependent upon internalization and that the suppression of phosphorylation by substrates is because of suppression of trafficking. It is also possible that phosphorylation may have distinct roles in different transporters. Indeed, the N and C termini, which contain the majority of the potential phosphorylation sites, are poorly conserved among the different transporters within the family (1, 3). SERT and DAT also clearly differ in the effect of substrate and inhibitors on
their PKC-mediated down-regulation. In contrast to the SERT, DAT substrates such as amphetamine and dopamine have been reported not to affect PKC-mediated internalization in transfected MDCK cells (13). In the same cells as those used in the present study (HEK293), however, it has recently been shown that substrates by themselves promote internalization of the transporter with dopamine causing a modest effect and amphetamine displaying the strongest effect (41).

Only little is known about the mechanisms in vivo that govern the modulatory effects of PKC on the activity of DAT and related transporters. A growing number of reports suggest the critical function of co-expressed G protein-coupled receptors (37). In the NET, it has for example been shown that activation of muscarinic receptors in SK-N-SH cells inhibits NET uptake capacity, causes internalization of NET, and increases phosphorylation (36). This hNET response was, like the present hDAT response to substance P, only partially reversed by the PKC inhibitor staurosporine in contrast to the responses to PMA, suggesting that both PKC-dependent and -independent pathways may be involved in regulating the function of this class of transporters. Of further interest, activation of the metabotropic glutamate receptor 5 (mGluR5) has recently been shown to increase phosphorylation (36). This hNET response was, like the hNET uptake capacity, causes internalization of NET, and in- 

creased phosphorylation. Importantly, previous studies have shown that this receptor, like the DAT, is expressed in dopaminergic neurons and that substance P and related tachykinins can mediate dopamine release directly and/or indirectly (17, 18). Interestingly, it is not clear from the studies performed whether one or more of the very few proteins known to be phosphorylated by PKC or the hNK-1 receptor. It remains to be determined whether such motifs in the DAT sequence did not alter regulation by PKC or the hNK-1 receptor. It remains to be determined what the molecular trigger of the internalization process needs to be identified, and, second, the functional role of N-terminal phospho-

ylation remains to be determined. With regard to the mo-

lecular trigger of the internalization process, we hypothesize that upon phosphorylation another protein either dissociates from or binds to the transporter. In the present paper we could not find evidence that proteins known to mediate internaliza-

tion via interaction with dileucine- or tyrosine-based trafficking motifs are involved in this process, because the mutation of such motifs in the DAT sequence did not alter regulation by PKC or the hNK-1 receptor. It remains to be determined whether one or more of the very few proteins known to be associated with DAT and the closely related transporters SERT and NET may play a role in the internalization process (44–46). Of these, the interaction with PP2Ac, which is best described for SERT, is of particular interest. It has been reported not only that the SERT exists in a complex with PP2Ac in transfected cells and in native tissue, but also that the interaction may be regulated by PKC (44). Although PP2Ac was shown to interact with DAT, the functional consequences of this interaction were not investigated. Thus, clarification of a putative role of PP2Ac in regulating DAT trafficking awaits further studies.

Recently an interaction between the PDZ domain-containing “scaffolding” protein PICK-1 and the C-terminal PDZ-binding sequence of DAT has been reported (45). The association between the DAT and PICK-1 provides the first glimpse of how DAT may be targeted to the presynaptic membrane, but also introduces an entirely new paradigm to this class of transporters; via PDZ domain interactions, the transporters may be part of large multipoprotein complexes containing a broad spec-

trum of cellular proteins. Given that several hundred different proteins are known to contain PDZ domains, we would predict that DAT likely interacts with other PDZ domain-containing proteins in addition to PICK-1. The identification of yet unknown proteins within such multiprotein complexes, as well as unraveling putative interactions with other PDZ domain-containing proteins may provide a path toward understanding the molecular mechanisms underlying DAT internalization as well as insight into the still unknown role of N-terminal phosphorylation.

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