Dopamine Transporters are Phosphorylated on N-Terminal Serines in Rat Striatum*

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Dopamine transporters (DATs) are neuronal phosphoproteins that clear dopamine from the synaptic cleft. Activation of protein kinase C (PKC) and inhibition of protein phosphatases by okadaic acid (OA) increase phosphorylation of DAT and lead to concomitant reduction in DAT activity and cell surface expression. Numerous potential sites for phosphorylation are present on DAT, but the sites utilized and their relationship to transport regulation are currently unknown. We used peptide mapping and epitope-specific immunoprecipitation to identify the region of DAT that undergoes phosphorylation in rat striatal tissue. Phosphoamino acid analysis revealed that basal and stimulated samples were phosphorylated primarily on serine. Digestion of $^{32}$PO$_4$-labeled DAT with trypsin, and immunoprecipitation with N- or C-terminal specific antisera, failed to isolate phosphopeptide fragments corresponding to photoaffinity labeled fragments that contain all internal interhelical loops. However, digestion of $^{32}$PO$_4$-labeled DAT with endoproteinase asp-N and immunoprecipitation with an N-terminal antiserum extracted two phosphopeptide fragments from both basal and PKC/OA-stimulated samples, demonstrating that the N-terminal cytoplasmic tail is a major site of phosphorylation. Aminopeptidase treatment of PKC- and/or OA-stimulated DAT cleaved essentially all $^{32}$PO$_4$ label without proteolysis extending past transmembrane domains 1-2, providing further evidence that most phosphorylation sites are near the N-terminus and not in intracellular loops or C-terminal domains. In situ proteolysis of the N-terminal tail indicates that the majority of stimulated phosphorylation sites are N-terminal to an antibody epitope at residues 42-59. Two-dimensional analysis of purified protein produced three tryptic phosphopeptides that may result from phosphorylation of multiple sites, but the fragments did not co-migrate with synthetic
tryptic peptides phosphorylated at serines 2 and 4. These results indicate that most or all of the basal and stimulated phosphorylation of DAT in striatal tissue occurs on one or more residues in a group of six serines clustered near the distal end of the cytoplasmic N-terminus.
INTRODUCTION

The concentration of dopamine in the synaptic and extrasynaptic space is controlled to a great extent by the action of dopamine transporters (DATs\(^1\)), which utilize the energy of Na\(^+\) and Cl\(^-\) ionic gradients to drive reuptake of the neurotransmitter into the presynaptic cell (1). DAT belongs to a family of transporters including those for norepinephrine (NET) and serotonin (SERT) that constitutes the major sites of action for tricyclic antidepressants and psychostimulants (2). Other neurotransmitter reuptake carriers include plasma membrane transporters for glycine (Glyt1) and \(\gamma\)-aminobutyric acid (GAT1), and the synaptic vesicle monoamine and acetylcholine transporters, VMAT and VChT (3,4). These proteins are believed to have a similar structure of twelve transmembrane-spanning domains, cytoplasmically oriented N- and C-termini, and numerous potential phosphorylation sites for various protein kinases.

Recent studies have shown that DATs and other neurotransmitter transporters are phosphoproteins whose functions are acutely regulated by protein kinases, providing a mechanism for temporal and spatial control of synaptic neurotransmitter levels and neural signaling (5-9). Activators of protein kinase C (PKC) and inhibitors of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) cause an increase in DAT phosphorylation with a concomitant reduction in dopamine transport activity (5,10,11). A common theme emerging from these studies is that phosphorylation and functional regulation of transporters are accompanied by changes in their cell surface density (6,7,12-14). Phorbol esters stimulate clathrin-mediated endocytosis of DAT that results in intracellular sequestration and trafficking...
through recycling and degradative pathways (12,13). However, it is not known if phosphorylation of DAT is required for these processes and/or contributes to other aspects of transport regulation or function.

Our current goal is to identify the sites of phosphorylation on DAT and elucidate their functional significance. The presumed intracellular aspects of rDAT contain numerous serines, threonines, and tyrosines, many of which are present in PKC, protein kinase A and Ca\(^{2+}\)-calmodulin dependent protein kinase consensus motifs (Fig. 1). These residues are found in both N- and C-terminal cytoplasmic tails and in all intracellular loops (ILs) except IL1. The large number of potential phosphorylation sites, in conjunction with the possibility of multi-site phosphorylation, has made determination of phosphorylation sites by molecular approaches problematic. Attempts to identify sites contributing to regulation of DAT, GAT1 and Glyt1 using mutagenesis of PKC consensus motifs and/or sites highly conserved throughout the neurotransmitter transporter family have not been successful (14-16), as phorbol ester-induced regulation was maintained after mutagenesis. It is possible that the sites examined were not those involved in phosphorylation, or that multiple sites are involved and mutagenesis of the examined sites was insufficient to produce a result.

In this study we used phosphoamino acid analysis and protease-based peptide mapping strategies to directly identify the region of DAT that undergoes phosphorylation in rat brain tissue. The results show that one or more serines at the extreme end of the N-terminal tail are the major sites of both basal and stimulated phosphorylation. These results have implications for the molecular basis of transport regulation and provide strong direction for mutagenesis approaches.
MATERIALS AND METHODS

Materials – [125I]DEEP and [125I]RTI 82 were synthesized by Dr. John Lever, University of Missouri. Carrier free 32PO4 was from ICN; okadaic acid (OA), 1-oleoyl-2-acetyl-sn-glycerol (OAG), and phorbol 12-myristate, 13-acetate (PMA) were from Calbiochem; endoproteinase asp-N was from Boehringer Mannheim; high and low range Rainbow molecular weight markers were from Amersham; phosphoamino acid standards, Aminopeptidase 1, Carboxypeptidase Y, and TPCK- treated trypsin were from Sigma; phosphopeptide standards were synthesized by Cell Essentials (Boston, MA); and tricine gel reagents were from BioRad. Rats were purchased from Charles River Laboratories and were housed and treated in accordance with regulations established by the National Institutes of Health and the University of North Dakota Animal Care and Use Committee.

Metabolic Phosphorylation of DAT – For most experiments phosphorylated DATs were prepared from rat striatal slices metabolically labeled with 32PO4 using procedures adapted from Halpain et al. (17). Male Sprague-Dawley rats (175-300 g) were decapitated and the striata were rapidly removed and weighed. The tissue was sliced into 350 µm slices using a McElvain Tissue Chopper and equivalent amounts of tissue (4-8 slices) were placed into wells of a 12-well culture plate containing oxygenated Krebs-bicarbonate buffer consisting of 25 mM NaHCO3, 125 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 5 mM MgSO4, and 10 mM glucose, pH 7.3. Slices were preincubated for 30 min at 30ÚC, with shaking at 105 rpm, followed by exchange with fresh buffer containing 1 mCi/ml 32PO4 and continued incubation with shaking at 30°C for 90-
120 min. Oxygen (95% O₂: 5% CO₂) was gently blown across the top of the plate during the incubation and test compounds or vehicle were added for the final 30 min. Test compounds (OA, OAG, and PMA) were dissolved at high concentrations in dimethyl sulfoxide (DMSO) followed by dilution in the incubation mixture to a final DMSO concentration of 0.1%. In all experiments control tissue received 0.1% DMSO, which by itself had no effect on the phosphorylation state of DAT. At the end of labeling, tissue slices were transferred to a microcentrifuge tube and centrifuged at 4°C at 800 x g for 4 minutes. The supernatant fractions were removed and 1 ml ice-cold Krebs-bicarbonate buffer was added to the slices. The tissue was disrupted by 4 passages through a 26 gauge needle, samples were centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant fractions were removed from the sedimented membranes.

For *in situ* proteolysis procedures, membranes were resuspended in 50 mM Tris-HCl, pH 8.0, at 50 mg/ml original wet weight, and for immunoprecipitation and gel purification, membranes were solubilized with 0.5% SDS at 20 mg/ml original wet weight. For both procedures, aliquots were removed for protein assay using the Pierce protein assay kit with bovine serum albumin as the standard, and sample volumes were adjusted to equalize protein content. For one experiment phosphorylated DAT was prepared from rat striatal synaptosomes metabolically labeled with \(^{32}\text{PO}_4\) and treated with vehicle, PMA, or OA as previously described (5). Briefly, synaptosomes suspended in KBB were labeled with 1 mCi/ml \(^{32}\text{PO}_4\) at 30 °C for 45 min with drug treatments given for the final 15 min. All phosphorylation and peptide mapping results were verified in 2 or more independent experiments.

**Photoaffinity Labeling and Immunoprecipitation** Rat striatal DATs were photoaffinity
labeled with $^{[125\text{I}]}$DEEP or $^{[125\text{I}]}$RTI 82 as previously described (18) and prepared for in situ proteolysis or gel purification in parallel with $^{32}\text{PO}_4\,$-labeled DATs. Immunoprecipitations of phosphorylated or photoaffinity-labeled samples were performed with rabbit polyclonal antisera 16 or 18 generated against N-terminal amino acids 42-59 (peptide16) or C-terminal amino acids 580-608 (peptide 18) of the rDAT protein sequence (18). Samples were electrophoresed on SDS polyacrylamide gels and dried gels were subjected to autoradiography using Kodak Biomax film for 12-48 hours. The region of the gel containing DAT was excised for processing as described below, and in some experiments radioactivity in excised $^{32}\text{PO}_4\,$-labeled DAT samples was assessed by Cerenkov counting. The identity of novel fragments immunoprecipitated with serum 16 was verified by inclusion of 50 μg/ml peptide 16 or peptide 1 (residues 6-21) during immunoprecipitation. High and low range Rainbow molecular weight markers were used as standards on all gels.

*Phosphoamino Acid Analysis* – Phosphoamino acid analysis was performed using the method of Boyle *et al.* (19). $^{32}\text{PO}_4\,$-labeled striatal slices were treated with vehicle or test compounds, and membranes were solubilized, immunoprecipitated with antiserum 16, and electrophoresed on 8% gels. After drying and autoradiography, the region of the gel containing DAT was excised and protein was eluted overnight at 22°C in 0.1 M ammonium bicarbonate, pH 8.0. Samples were oxidized with performic acid followed by precipitation with trichloroacetic acid and hydrolysis for 2 h at 110°C with 5.7 M HCl. Unlabeled phosphoamino acid standards (p-ser, p-thr, and p-tyr) were dissolved in pH 1.9 buffer (acetic acid 7.8%, formic acid 2.5%) and added to the unknowns at 1 mg/ml. Samples were spotted onto cellulose
thin-layer plates and electrophoresed using a Hunter thin-layer electrophoresis unit at 1.5 kV for 35 minutes at pH 1.9 (acetic acid 7.8%, formic acid 2.5%) in the first dimension, and at 1.3 kV for 20 minutes at pH 3.5 (pyridine 0.5%, acetic acid 5%) in the second dimension. Standards were visualized with ninhydrin and the plates were subjected to autoradiography for 10-14 days.

*In Situ Proteolysis and Immunoblotting* – Striatal membrane suspensions labeled with $^{32}\text{PO}_4$ were subjected to *in situ* proteolysis as previously described (18) followed by analysis of DAT by immunoprecipitation and western blotting. Briefly, membranes were treated with trypsin or endoproteinase asp-N for 10 or 60 min, respectively, at 22°C, sedimented by centrifugation, and the supernatants were transferred to fresh tubes. Membranes were solubilized and subjected to immunoprecipitation with antisera 16 or 18, followed by electrophoresis and autoradiography on 9% SDS-PAGE gels or 8-16% Tris-tricine gradient gels (20). In some experiments, the supernatant fractions were also subjected to immunoprecipitation to assay for fragments released from membranes. For western blotting, trypsin-treated membranes were electrophoresed on 8% SDS-PAGE gels followed by electrophoretic transfer to 0.45 µm PVDF membranes. Dried membranes were blocked with 3% BSA prepared in 10 mM phosphate buffered saline (PBS), pH 7.4 for 1.5 h followed by 5% non-fat dry milk in 10 mM PBS, pH 7.4 for 0.5 h, washed extensively and probed with antiserum 16 diluted 1:100 in 3% BSA-10 mM PBS, 7.4. Blots were washed, then incubated for 1 h at room temperature with goat anti-rabbit IgG linked alkaline phosphatase diluted 1:5000 in a 3% BSA-10 mM PBS solution. After extensive washing, blots were developed colorimetrically using the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue. Dried blots were scanned and quantitated with Molecular Analyst software (BioRad).
**Aminopeptidase and Carboxypeptidase Treatment** – DATs from $^{32}$PO$_4$-labeled striatal synaptosomes or striatal slices were immunoprecipitated, gel purified on 8% SDS-PAGE gels and electroeluted as previously described (21). Aliquots of the electroeluted samples were treated with 1-100 µg/ml of Aminopeptidase 1 or Carboxypeptidase Y prepared in 50 mM Tris HCl, pH 8.0, for 1h at 22°C, followed by addition of SDS-PAGE sample buffer and electrophoresis and autoradiography on 8% SDS-PAGE gels. In some experiments DATs labeled with $[^{125}$I$]$DEEP were treated and analyzed in parallel.

**Phosphopeptide Analysis** – Limit tryptic digestion and phosphopeptide analysis were performed using the method of Boyle et al. (19). $^{32}$PO$_4$-labeled striatal slices were treated with 1 µM OA, and membranes were solubilized, immunoprecipitated with antiserum 16, and subjected to electrophoresis on 8% SDS-PAGE gels. After drying and autoradiography, the region of the gel containing DAT was excised and protein was eluted overnight at 22°C in a solution containing 0.1% SDS, 0.5% 2-mercaptoethanol, and 50 mM ammonium bicarbonate, pH 8.0. The extracted protein was precipitated with trichloroacetic acid in the presence of carrier protein (20 µg RNase A) and the pelleted protein was washed with absolute ethanol. When trypsin was used as the digestive agent, the DAT sample was oxidized prior to digestion using performic acid. The final protein sample was resuspended in 50 µl of 50 mM ammonium bicarbonate, pH 8.3 and incubated at 37°C for 5 hours in the presence of 10 µg TPCK-trypsin follow by a second 10 µg dose of TPCK-trypsin and incubation overnight at 37 °C. After digestion, ammonium bicarbonate was removed by repeated lyophilization and peptides were dissolved in 10 µl running buffer (acetic acid 7.8%, formic acid 2.5%, pH 1.9). A 5 µl aliquot of
this sample was analyzed by electrophoresis and autoradiography on a 15% Tris-tricine gel. The remaining 5 µl aliquot was spotted onto 20 x 20 cm microcrystalline cellulose glass-backed plates and electrophoresed using a Hunter thin-layer electrophoresis unit at 1.0 kV for 35 minutes at pH 1.9 (acetic acid 7.8%, formic acid 2.5%) in the first dimension followed by ascending chromatography in the second dimension (39.3% n-butanol, 30.4% pyridine, 6.1% acetic acid, pH 3.5). Synthetic phosphopeptide standards (10 µg) corresponding to tryptic fragments of the first five amino acids of DAT, [MS(P)K and S(P)K, where (P) indicates phosphorylation of the preceding serine], were included with the unknowns and detected by ninhydrin staining. The standards were analyzed separately and in combination to determine their mobility patterns.

For cyanogen bromide (CNBr) cleavage (22), 32PO4-labeled DAT prepared as above, but without oxidation, was subjected to SDS-PAGE on 8% gels, and electroblotted to a 0.45 µm PVDF membrane. After drying and autoradiography, the region of the membrane containing DAT was excised and feathered by cutting with a razor blade. Immobilized DAT was immersed in 150 µl of 0.25 M CNBr in 70% formic acid. The sample was capped, mixed by vortexing and covered with aluminum foil. The resulting digestion mixture was incubated with agitation at room temperature for 24 h in a chemical fume hood followed by lyophilization and then the addition of 50 µl of purified water and repeated lyophilization. The lyophilized sample was resuspended in 10 µl running buffer (acetic acid 7.8%, formic acid 2.5%, pH 1.9) and subjected to Tris-tricine gel electrophoresis.
RESULTS

Characterization of DAT Phosphorylation in Striatal Slices. Most of the experiments in this study were performed using striatal slices for metabolic phosphorylation of DAT because they can be labeled for much longer times than synaptosomes, and produce highly radiolabeled DAT suitable for additional analyses. Phosphate incorporation into DAT was easily detectable from slices treated with vehicle only, demonstrating the presence of basal phosphorylation, and the level of phosphorylation increased when the tissue was treated with the PKC activator OAG, the PP1/PP2A inhibitor OA, or OA plus OAG (Fig. 2, A and B). This mimics the pattern of DAT phosphorylation found in striatal synaptosomes and cultured cell expression systems (5,10), and indicates the likelihood that the preparations correspond to those that exhibit functional regulation. For most studies slices were stimulated with OA plus OAG to ensure maximal phosphate incorporation, although some analyses were performed using slices or synaptosomes treated individually with test compounds to independently examine kinase and phosphatase effects.

To determine which amino acids were phosphorylated under our labeling and stimulation conditions, $^{32}$PO$_4$-labeled DAT samples were prepared from striatal slices that were treated with vehicle, OA or OAG. DATs were purified by immunoprecipitation and gel electrophoresis, and subjected to phosphoamino acid analysis on two-dimensional thin-layer plates. The results show that in slices treated individually with vehicle, OA, or OAG, DATs undergo phosphorylation primarily on serine (Fig. 3), as did tissue treated with OA and OAG together (not shown). A low level of phosphothreonine was consistently observed in all samples, but phosphotyrosine was not detected, even with longer film exposures. We have not attempted to
detect phosphotyrosine on DAT by immunoblotting with anti-phosphotyrosine antibodies.

*Immunoprecipitation of Trypsin Fragments.* Our initial attempts to map phosphorylation sites utilized procedures that have been successful in identifying sites of photoaffinity label attachment (18,21). For these experiments $^{32}$PO$_4$-labeled DATs prepared from OA/OAG treated slices were proteolyzed with trypsin, which cleaves at lysine and arginine residues, and the digests were immunoprecipitated with antibodies 16 and 18 specific for N- and C-terminal domain epitopes (Fig. 1). When performed on DATs photoaffinity-labeled with $^{[125]}$IDEEP or $^{[125]}$IRTI 82, these procedures produce easily detectable peptide fragments of 45 and 32 kDa that correspond to large portions of the N- and C-terminal halves of the protein (Fig. 4, right two lanes). The 45 kDa fragment (arrow a) contains epitope 16 in the N-terminal tail and extends through extracellular loop 2 (EL2), while the 32 kDa fragment (arrow b) begins in EL2 and extends through epitope 18 in the C-terminal tail (18,21). The 14 kDa fragment (arrow c) is a product of the 45 kDa fragment that retains epitope 16 but terminates in IL1. However, while antisera 16 and 18 immunoprecipitated these photoaffinity labeled fragments and non-proteolyzed $^{32}$PO$_4$-labeled DAT (left two lanes), no convincing phosphopeptide fragments were extracted by either of these antisera (middle two lanes), or by antisera 15 and 5 (not shown) that recognize other epitopes in the N-terminal tail and EL2 (18). This suggested that tryptic proteolysis separated the phosphorylated residues from the antibody epitopes or generated phosphopeptide fragments too small to be analyzed with these techniques. Since the 45 and 32 kDa photoaffinity labeled fragments collectively contain all the transmembrane domains and
internal interhelical loops of DAT (18), the inability to generate comparable \(^{32}\text{PO}_4\)-labeled fragments is strong evidence that serines 261, 333, 421, 428, and 504 in internal loops 2, 3, 4, and 5 (Fig. 1) are not major phosphorylation sites.

**Immunoprecipitation of Asp-N Fragments.** Because these results indicated that the bulk of the \(^{32}\text{PO}_4\) radiolabeling was occurring on residues in the cytoplasmic tails that were being proteolytically separated from antibody epitopes, we sought to minimize this possibility by digesting DAT with endoproteinase asp-N (asp-N), which cleaves on the N-terminal side of aspartic acid residues. In contrast to the numerous trypsin sites present in these domains, the N-terminal tail of rDAT has no aspartates upstream of epitope 16, while two aspartates in the C-terminal tail are downstream of serines 581 and 585, and most of epitope 18 (Fig. 1). For this experiment membranes prepared from basal and OA/OAG stimulated \(^{32}\text{PO}_4\)-labeled striatal slices were digested with asp-N, followed by solubilization and immunoprecipitation with antisera 16 or 18. Figure 5 shows that asp-N treatment cleaved a substantial portion (~80%) of the protein and produced strongly labeled phosphopeptide fragments of about 19 and 16 kDa from both basal and stimulated tissue (Fig. 5A, arrow). Both fragments were immunoprecipitated with serum 16, and precipitation was blocked by inclusion of peptide 16 but not an irrelevant peptide (Fig. 5B), verifying the presence of epitope 16. These results therefore provide positive identification of the N-terminal cytoplasmic tail of DAT as a major site of both basal and OA/OAG-stimulated phosphorylation.

The masses of the asp-N fragments suggest that they are not generated by proteolysis at D68 or D79 in or near TM1 (Fig. 1), which would produce fragments of ~7-8 kDa. No fragments of
this mass were found in either treated membranes (Fig. 5) or asp-N supernatants (not shown), indicating that asp-N access to D68 and D79 during in situ proteolysis is prevented by the lipid bilayer or the tertiary structure of the protein. The next aspartates in the DAT sequence are in EL2, at positions 174 and 191 (Fig. 1). The masses of the 16 and 19 kDa fragments are consistent with cleavage at one or both of these residues or other aspartates in EL2, and the fragments therefore contain the N-terminal tail, TM 1-3, EL1, IL1 and part of EL2. However, since there are no serines in IL1, the N-terminal tail serines must represent the sites of phosphorylation of these fragments.

Aminopeptidase Analysis. We did not find phosphopeptide fragments in asp-N digestion experiments using C-terminal antibody 18 for immunoprecipitation (not shown). Nevertheless, since some full-length DAT protein remained after asp-N treatment, we could not exclude the possibility that some of the labeled sites were present outside the NH₂ tail. We further investigated this issue by treating ³²PO₄⁻ labeled gel purified DAT with aminopeptidase (AP) and carboxypeptidase (CP), which non-specifically cleave inward from protein N- or C-termini. Figure 6A shows that AP treatment removed essentially all ³²PO₄ from OA/OAG treated DAT while CP had no obvious effect. To monitor the extent of AP-induced proteolysis in conjunction with phosphorylation site loss we examined the effect of AP on DATs labeled with [¹²⁵I]DEEP in parallel with ³²PO₄-labeled sample. AP digestion of OA/OAG treated ³²PO₄-labeled DAT produced dose-dependent hydrolysis of essentially all ³²PO₄ from the protein with the reciprocal appearance of radiolabel at the gel dye front, while the same treatment of
[125I]DEEP-labeled DAT produced a loss of ~5-7 kDa in mass (Fig. 6B), consistent with proteolytic digestion of most of the NH₂ tail. [125I]DEEP is incorporated in DAT in TMs 1-2 (18), and the retention of most of the photolabel on the AP-treated protein demonstrates that proteolysis did not progress past this region.

To assess the location of sites phosphorylated independently in response to PKC activation and phosphatase inhibition, we performed a similar analysis on DATs prepared from 32PO₄ labeled striatal synaptosomes treated separately with PMA or OA (Fig. 6C). Both treatments stimulated DAT phosphorylation relative to vehicle-treated controls (left three lanes). AP treatment removed most of the 32PO₄ label from both stimulated samples (middle three lanes) with the small loss of mass indicated by analysis of [125I]DEEP-labeled DATs (right two lanes), showing that phosphate incorporation induced by independent PMA and OA treatments occurs on the same general N-terminal domain as that seen for the combination OA/OAG treatment. Thus, the aminopeptidase results confirm the N-terminal localization of both PKC and OA-induced phosphorylation sites on DAT and provide additional evidence that significant 32PO₄ incorporation does not occur in the internal loops or C-terminal tail. Several serines are present in the NH₂ tail, whether the specific sites phosphorylated in response to separate and combined PKC and OA treatments are the same, different, or overlapping remains to be ascertained. Although the phosphorylation intensity of the basal sample (shown for comparison to stimulated samples) is too low to be analyzed in this experiment, the N-terminal localization of basal sites is clearly shown in asp-N digestion experiments in Figure 5.
In Situ Proteolysis. There are eight serines in the rDAT N-terminal tail, a cluster of six near the N-terminus at residues 2, 4, 7, 12, 13, and 21, and two more internally at positions 45 and 64 (Figs. 1 and 7C). We used an in situ proteolysis approach based on the position of antibody epitope 16 at residues 42-59 to distinguish between these groups of serines. For this experiment, membranes from OA-stimulated 32PO4-labeled striatal slices were treated with increasing concentrations of trypsin, and serum 16 was used to either western blot or immunoprecipitate aliquots of each sample to independently assess the retention of the antibody epitope and the 32PO4 label. The western blot of these samples shows only a negligible loss of epitope 16 immunoreactivity on the full length protein compared to untreated controls (Figs. 7A, bottom, and 7B), while even the lowest doses of trypsin removed over 75% of the 32PO4 label (Figs. 7A, top, and 7B). This indicates that serines 45 and 64, which are within or C-terminal to epitope 16 (Fig. 7C), are not the major phosphorylation sites, and that most of the 32PO4 label is found N-terminal to the epitope. Separation of phosphorylated residues from the epitope could occur by cleavage at one or more of several potential tryptic proteolysis sites present between the first six serines and epitope 16 (Fig. 7C). This result is also compatible with those shown in Figure 4, as 32PO4-labeled fragments comparable to those labeled by [125I]DEEP would presumably be produced and precipitated with serum 16 if DAT underwent substantial phosphorylation on serines 45 or 64. The small fraction of 32PO4 label remaining on the protein after in situ treatment with trypsin could indicate that a minor amount of phosphorylation occurs on these more internal serines, or could indicate a pool of DATs inaccessible to cleavage.
One- and Two-Dimensional analysis of Purified DAT Fragments. We have also subjected
OA-stimulated phosphorylated DAT to limit digestion with trypsin and cyanogen bromide
(CNBr). Extensive proteolysis of immunopurified $^{32}\text{PO}_4$-labeled DAT with trypsin produced
three strongly labeled peptide fragments with masses of about 18, 4, and 1.5 kDa, and some less
intensely labeled bands (Fig. 8A). The calculated masses of fragments resulting from complete
tryptic proteolysis of the DAT N-terminus (not including mass potentially provided by
phosphate incorporation) is shown in Fig. 8C. We do not know if the 1.5 and 4 kDa fragments
represent independent or overlapping phosphopeptides generated from digestion at these sites.
Taking into account the limitations of gel electrophoresis for molecular mass estimation, the
mass of the smallest fragment is reasonably compatible with various combinations of complete
or incomplete digestion of the N-terminus, while the 4 kDa fragment is likely to represent an
incomplete digest, for example, a fragment encompassing residues 1-35 or 20-60. The 18 kDa
fragment is likely to result from digestion at residues outside the N-terminal tail, and was
probably obtained in this experiment but not others because the acid precipitation used during
sample preparation denatured the protein in a way that prevented proteolysis at sites that were
cleaved in other preparations.

An aliquot of the trypsin digest analyzed in panel A was subjected to two-dimensional thin-
layer electrophoresis and chromatography (Fig. 8B), which produced three intensely labeled
spots and some minor spots that correspond to the bands on the Tris/tricine gel. This sample was
electrophoresed in the presence of synthetic phosphopeptides corresponding to trypsin fragments
from the first five residues of the DAT N-terminus [MS(P)K and S(P)K, where (P) indicates
phosphorylation of the preceding serine], but the major $^{32}$PO$_4$-labeled fragments did not co-
migrate with either peptide. This result may be evidence that serines 2 and 4 are not
phosphorylated, but would also occur if DAT failed to undergo cleavage at lysines 3 and 5, or if
other modifications such as acetylation were present on the endogenous peptides. A minor
$^{32}$PO$_4$-labeled spot partially overlapped the serine 4 phosphopeptide standard, possibly representing a
stoichiometrically minor amount of phosphorylation at S4 or a minor amount of production of
this fragment.

We also digested $^{32}$PO$_4$-labeled DAT with CNBr, which cleaves proteins on the C-terminal
side of unoxidized methionine (M) residues. This treatment produced one distinct
phosphopeptide of about 12 kDa (Fig. 8A), a mass that corresponds to cleavage of the DAT N-
terminal sequence at residues M106 or M111 in TM2 (Fig. 1). We did not observe a $^{32}$PO$_4$-
labeled peptide fragment of ~1 kDa which would correspond to cleavage of DAT at M11 (Fig. 1,
Fig. 8C, arrow). This result could indicate that serines 2, 4, and 7 are not phosphorylated,
consistent with the results of the two-dimensional thin layer analysis of the tryptic fragments,
although again, this result would also be obtained if cleavage at M11 did not occur.

**DISCUSSION**

Although DAT has now been characterized as a phosphorylated protein in several studies,
many questions remain regarding its properties and significance. For example, it is not known if
basal phosphorylation is mediated by PKC or other kinases, or if PKC-induced phosphorylation
is due to direct phosphorylation of DAT by PKC or by activation of an intermediate kinase. It is
also not known if OA-stimulated phosphorylation represents accumulation of basal phosphorylation via direct inhibition of DAT phosphatase(s) or if OA stimulates phosphorylation by an indirect mechanism. Multiple electrophoretic mobility isoforms of DAT can be visualized on SDS gels, this is most easily observed in immunoblots (23), but is also apparent at times by photoaffinity labeling and phosphorylation. We do not know if these electrophoretic mobility isoforms are caused by heterogeneous phosphorylation, glycosylation, or other factors. Western blotting of immunoprecipitated $^{32}$PO$_4$-labeled DAT demonstrates substantial overlap between both signals$^2$, indicating that most bands visualized by immunostaining are phosphorylated. However, in some but not all experiments PMA or OA stimulation results in an upward shift of the phosphorylated band or concentration of $^{32}$PO$_4$ radiolabeling near the trailing edge of the DAT complex (5), but we do not know if this indicates preferential phosphorylation of some isoforms. Clarification of these issues will clarify our understanding of the role of phosphorylation in DAT physiology and function.

The goal of this study was to determine which regions of DAT undergo phosphorylation in brain tissue. For this purpose we characterized DAT phosphorylation in rat striatal slices, finding that phosphate incorporation was stimulated by PKC activators and PP1/PP2A inhibitors as in synaptosomes and cells, and was sufficiently robust for additional sample manipulation. These preparations were therefore suitable for phosphopeptide mapping analyses with correlation to our previous findings of DAT functional regulation.

For all basal and stimulated conditions we found that DAT is phosphorylated primarily on serine, indicating the potential for these residues to be major determinants of DAT properties.
regulated by kinases and phosphatases. In addition, a low level of phosphothreonine was consistently observed, and many threonines are found within PKC consensus sequences that are highly conserved throughout the monoamine transporter family (2). Recent studies have reported the presence of phosphotyrosine on GAT1 and the functional regulation of DAT and GAT1 by tyrosine kinase inhibitors (9,24,25). We found no detectable phosphotyrosine from DATs prepared under the described conditions, but did not employ treatments such as tyrosine kinase activators or phosphotyrosine phosphatase inhibitors specific for generation or preservation of phosphotyrosine.

Several lines of evidence demonstrate that the N-terminal tail of DAT is the major, and possibly sole, domain that undergoes basal, PKC-induced and OA-induced phosphorylation in rat striatum. Basal and OA/OAG-stimulated \( ^{32} \text{PO}_4 \)-labeled asp-N fragments were immunoprecipitated with N-terminal antisera 16, and aminopeptidase but not carboxypeptidase removed PKC- and/or OA-stimulated \( ^{32} \text{PO}_4 \) label from DAT without proteolysis extending past TMs 1-2. In conjunction with the inability to immunoprecipitate tryptic or asp-N phosphorylated fragments with C-terminal antisera, these results indicate that the N-terminus of DAT is the primary site of basal, PKC-stimulated, and OA-stimulated \( ^{32} \text{PO}_4 \) incorporation.

*In situ* proteolysis of the NH\(_2\) tail strongly implicates the region N-terminal to antibody epitope 16 (residues 42-59), as opposed to more internal regions, as the major site of stimulated phosphorylation. The distal end of the NH\(_2\) tail contains six serines, some closely spaced, within
the first twenty one residues. The stoichiometry of DAT phosphorylation is unknown, and
within this cluster of serines we do not know which combination of sites is utilized. PKC
consensus motifs are present at S7 and S21, although it is not known if PKC phosphorylates
DAT directly or acts by an indirect mechanism that would lead to phosphorylation at non-
canonical sites. We also do not know if phosphorylation induced by PKC activators and
phosphatase inhibitors occurs on the same, different, or overlapping sites within this domain.
Limit digestion of phosphorylated DAT with trypsin produced two low molecular weight
phosphopeptides, but because of the number of serines, lysines, and arginines in the N-terminal
tail we cannot determine if the fragments represent distinct or overlapping peptides. The major
endogenous phosphopeptides did not co-migrate with synthetic phosphopeptides representing
tryptic digests of the first five rDAT residues containing serines 2 and 4, potential evidence that
these sites are not phosphorylated. Digestion with CNBr also failed to generate a phosphorylated
fragment consistent with cleavage at M11 that would contain serines 2, 4, and 7. Taken together,
all of our results are compatible with phosphorylation of DAT occurring on some combination of
serines 12, 13, and 21, but evidence from additional strategies will be required to definitively
identify the sites utilized. Our current efforts are directed towards the use of mutagenesis and
mass spectrometry to address these questions.

To our knowledge these results are the first to demonstrate phosphorylation of a
neurotransmitter transporter on the N-terminal domain. A recent study reported that serine to
glycine mutagenesis of residues S262 in IL2, and S586 and T613 in the COOH-tail of human
DAT (corresponding to S261, S585, and T612 of rDAT), resulted in the loss of PMA-stimulated
phosphorylation (16). These results directly conflict with our results that positively demonstrate
the presence of phosphorylation sites on the NH₂-tail after stimulation by PMA alone or with OA/OAG together, and show no evidence for PKC-stimulated phosphorylation in IL2 or the COOH-tail. The discrepancies could result from methodological differences, as the mutagenesis study examined human DAT expressed in a cell line, while our mapping was done using the rat isoform phosphorylated in striatal tissue. The positions of serines and threonines in the NH₂ tail of rat and human DAT are very similar, with serines in both isoforms at positions 2, 4, 7, 12, 13, and 45, and threonines at positions 43, 46, and 62. Differences are present at residues 21 (S in rat, P in human), 44 (N in rat, S in human), 48 (N in rat, T in human), 53 (T in rat, S in human) and 64 (S in rat, G in human). Alternatively, the mutations may have induced secondary effects on protein conformation that indirectly prevented N-terminal domain phosphorylation.

The potential for similarities of our results to phosphorylation sites and PKC dependent regulation of other plasma membrane neurotransmitter transporters is not known. The N-terminal tail of SERT has 7 serines and 10 threonines, some within PKC consensus sites, but phosphorylation site analysis of SERT has not yet been reported. Interestingly, NET, which displays PKC-dependent and independent functional regulation (26), has three threonines but no serines in its N-terminal domain. Our results differ from the phosphorylation pattern found for synaptic vesicle acetylcholine and monoamine transporters, which undergo phosphorylation exclusively on C-terminal tail residues (27,28). In the case of the VACHT, mutation of serine 480 resulted in altered intracellular sorting that may impact neurotransmitter packaging (29). This difference in phosphorylation patterns of plasma membrane and vesicular neurotransmitter transporters may indicate the potential for these classes of proteins to be subject to different
molecular mechanisms of kinase-induced regulation.

The finding that basal, PKC-stimulated, and OA-stimulated DAT phosphorylation all occur on N-terminal serines is compatible with basal phosphorylation resulting from tonic PKC and PP1/PP2A action which may function to define baseline dopamine clearance. DAT dephosphorylation in the brain is extremely robust and may serve to tightly regulate the DAT phosphorylation state and functional activity (30). DATs are found in complexes with PP2A (31) and undergo in vitro dephosphorylation by PP1 (32), and regulation of basal and stimulated DAT phosphorylation may occur through physiological control of these phosphatases as well as through activation of kinases.

At present, the function of DAT phosphorylation is not known. A correlation exists between the dose and kinetics of PKC- and OA-induced DAT phosphorylation, down-regulation, and transporter internalization (5,10-13,33). Transporter phosphorylation could serve as a marker for recruitment of endocytic adaptor proteins in a manner analogous to that of G protein coupled receptors (34). Other potential functions of phosphorylation could include recognition by other targeting proteins or involvement in an autoregulatory mechanism. For example, the N-terminus of GAT1 interacts with the SNARE protein syntaxin 1 in a PKC-specific manner that results in regulation of GABA transport (35-37), although it is not known if this is mediated by GAT1 phosphorylation. Recent evidence also indicates the potential for DAT and SERT to exist as dimers or oligomers (38,39), a process controlled for some proteins by phosphorylation (40). Our current efforts are aimed at mutating N-terminal DAT serines to assess their usage as phosphorylation sites and examine their involvement in down-regulation and other processes.
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FOOTNOTES

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1 The abbreviations used are: DAT, dopamine transporter; NET norepinephrine transporter; SERT, serotonin transporter; DA, dopamine, VMAT, vesicular monoamine transporter; VAChT, vesicular acetylcholine transporter; TM, transmembrane domain; EL, extracellular loop, IL, intracellular loop; asp-N, endoproteinase asp-N; AP, aminopeptidase 1; CP, carboxypeptidase; [125I]DEEP, [125I]-1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl]piperazine; [125I]RTI 82, [125I]-3β-(p-chlorophenyl)tropane-2β-carboxylic acid, 4’-azido-3’-iodophenylethyl ester; OA, okadaic acid; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PMA, phorbol 12-myristate, 13-acetate; SNARE, soluble N-ethylmaleimide-sensitive factor-attachment protein receptor; TPCK, N-tosyl phenylalalanine chloromethyl ketone; PVDF,
polyvinylidene difluoride.

2 R.A. Vaughan, unpublished results

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FIGURE LEGENDS

Fig. 1. Schematic diagram of the rat dopamine transporter showing antibody epitopes and potential phosphorylation and proteolysis sites. Intracellularly-oriented serines that represent potential phosphorylation sites are indicated with arrows designated with the residue number. Epitopes for antisera 16 and 18 are outlined and labeled. Aspartic acid (D) and methionine (M) residues near the N- and C-termini that represent potential cleavage sites for endoproteinase asp-N and cyanogen bromide are enclosed and shaded. Numerous potential sites of trypsin proteolysis at lysine (K) and arginine (R) residues in the N- and C-termini are present but not highlighted.

Fig. 2. DAT phosphorylation in striatal slices. Rat striatal slices labeled with $^{32}$PO$_4$ were treated with vehicle, 10 µM OAG, 10 µM OA, or 10 µM OA plus 10 µM OAG, followed by immunoprecipitation of DAT with serum 16, SDS-PAGE, and autoradiography. A. Quantitation of DAT phosphorylation. Immunoprecipitated DAT bands were excised and counted for Cerenkov radioactivity. The data from three independent experiments are normalized, averaged, and expressed as the ratio of $^{32}$PO$_4$-incorporation relative to the basal sample ± S.E. B. Autoradiogram of phosphorylated DAT. Equal amounts of sample from treated and untreated tissue were subjected to immunoprecipitation, electrophoresis and autoradiography. $[^{125}$I]$^{1}$ DEEP-labeled DATs were immunoprecipitated and electrophoresed in parallel as a control. Molecular weight standards for all gels are shown in kDa.
Fig. 3. **Phosphoamino acid analysis of striatal DATs.** 32PO₄-labeled striatal slices were treated with vehicle (no treatment), 10 μM OA, or 10 μM OAG, and DATs were purified by immunoprecipitation and gel electrophoresis. Autoradiographs displayed the phosphorylation pattern demonstrated in Fig. 1A. DAT bands were excised, eluted, and subjected to acid hydrolysis. Cerenkov counting of the hydrolysates showed cpm of 41, 90, and 129 in the basal, OAG and OA samples, respectively. Aliquots of the hydrolysates were mixed with phosphoamino acid standards and amino acids were separated by two-dimensional electrophoresis on thin-layer cellulose plates. Plates were subjected to autoradiography and phosphoamino acid standards were visualized with ninhydrin (dotted circles). S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

Fig. 4. **Tryptic proteolysis and epitope specific immunoprecipitation of phosphorylated and photoaffinity-labeled DAT.** Striatal slices were labeled with 32PO₄ and stimulated with 10 μM OA plus 10 μM OAG, or were photoaffinity labeled with [125I]DEEP or [125I]RTI 82 as indicated. DATs were gel purified, treated with or without trypsin, and immunoprecipitated with antiserum 16 or 18 as indicated at the top of the lanes, followed by electrophoresis and autoradiography on 14% SDS-PAGE gels. Full-length DAT is present at ~80 kDa, arrows a and c denote the positions of 45 and 14 kDa [125I]DEEP-labeled fragments that immunoprecipitate with antiserum 16, and arrow b denotes a 32 kDa [125I]RTI 82-labeled fragment that immunoprecipitates with antiserum 18. df, gel dye front.
Fig. 5. **Immunoprecipitation of asp-N phosphopeptide fragments with antiserum 16.**

A. Membranes from striatal slices labeled with $^{32}$PO$_4$ and treated with or without 10 µM OA plus 10 µM OAG were subjected to proteolysis with the indicated concentration of endoproteinase asp-N for 1h at 22 °C. The membranes were sedimented, immunoprecipitated with antibody 16, and analyzed by electrophoresis and autoradiography on an 8-16% Tris/tricine gel. The arrow on the right denotes the position of immunoprecipitated phosphopeptide fragments at approximately 16 and 19 kDa. B. Asp-N fragments prepared from basal and OA/OAG-stimulated $^{32}$PO$_4$-labeled striatal slices were immunoprecipitated with serum 16 in the presence of no addition, 50 µg/ml peptide 16 or 50 µg/ml peptide 1, as indicated.

Fig. 6. **Aminopeptidase treatment of $^{32}$PO$_4$ and [I$^{125}$]DEEP labeled DATs.** DATs from $^{32}$PO$_4$-labeled striatal slices treated with 10 µM OA plus 10 µM OAG (A and B) or $^{32}$PO$_4$-labeled striatal synaptosomes treated with vehicle, 10 µM PMA or 1 µM OA (C), were prepared by immunoprecipitation and gel purification. A. DATs were treated with the indicated doses of Aminopeptidase 1 or Carboxypeptidase Y for 1h at 22°C followed by electrophoresis and autoradiography on an 8% SDS-PAGE gel. B. $^{32}$PO$_4$ or [I$^{125}$]DEEP -labeled DATs were treated with the indicated doses of aminopeptidase, followed by electrophoresis and autoradiography on an 8% gel. C. $^{32}$PO$_4$ or [I$^{125}$]DEEP-labeled DATs were treated with or without aminopeptidase as indicated, followed by electrophoresis and autoradiography.
Fig. 7. **Analysis of phosphorylation sites by in situ proteolysis.** A. $^{32}$PO$_4$-labeled membranes prepared from striatal slices treated with 1 µM OA were incubated with the indicated concentrations of trypsin at 22°C for 10 min. Following proteolysis, samples were divided into two aliquots: one set was immunoprecipitated with serum 16 to assess the retention of $^{32}$PO$_4$ on the protein (top panel), the other set was immunoblotted with antibody 16 detected colorimetrically to assess the retention of the epitope (bottom panel). B. Quantitation of band densities relative to control samples after immunoprecipitation (open circles) or immunoblotting (filled circles). C. Sequence of rDAT NH$_2$-tail up to the approximate point of entry into TM1, showing relative positions of epitope 16 and serine, lysine, and arginine residues. Reference residue numbers are indicated above the sequence, serines are indicated in bold, spaces after lysine and arginine residues indicate potential tryptic proteolysis sites and resulting fragments.

Fig. 8. **Tryptic and CNBr phosphopeptide fragments.** A. $^{32}$PO$_4$-labeled DATs from striatal slices treated with 1 µM OA were isolated by immunoprecipitation and gel purification. TCA-precipitated samples were extensively digested with trypsin or CNBr as indicated and analyzed by electrophoresis and autoradiography on 15% Tris-tricine SDS gels. B. An aliquot of the trypsin digest shown in panel A was subjected to two-dimensional thin-layer separation in the presence of two synthetic phosphopeptide standards corresponding to tryptic fragments from the first five residues of DAT (sequences indicated at right). Plates were subjected to autoradiography and phosphopeptide standards were visualized with ninhydrin (dotted ovals). C.
Sequence of the rDAT NH₂-tail showing predicted tryptic fragments (separated by spaces) with calculated masses denoted below each sequence. Reference residue numbers are indicated above the sequence and serines are indicated in bold. The arrow indicates the position of methionine 11.
A

Enzyme Dose, μg/ml

B

Aminopeptidase, μg/ml

C

Aminopeptidase, μg/ml
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