Aspartate 345 of the Dopamine Transporter Is Critical for Conformational Changes in Substrate Translocation and Cocaine Binding*  

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The present study elucidated the role of aspartate 345, a residue conserved in the third intracellular loop of all Na+/Cl−-dependent neurotransmitter transporters, in conformational changes of the dopamine (DA) transporter. Asparagine substitution (D345N) resulted in near normal transporter expression on the cell surface but caused extremely low Vmax and Km values for DA uptake, converted the inhibitory effect of Zn2+ on DA uptake to a stimulatory one, and eliminated reverse transport. The cocaine-like inhibitor 2β-carbomethoxy-3β-(4-fluorophenyl)tropane or the selective DA transporter inhibitor GBR12935 bound to D345N with a normal affinity and still inhibited DA uptake potently. However, the mutation reduced the binding capacity of the surface transporter for these two inhibitors by 90% or more. Moreover, the binding activity of D345N can be significantly improved by Zn2+ but not by Na+. These results are consistent with a defect in reorientation of the substrate-binding site to the extracellular side, leading to a loss of the outward-facing conformational state where external DA binds to initiate uptake and the inhibitors bind to initiate uptake inhibition. Alanine or glutamate substitution produced a similar phenotype, suggesting that both the negative charge and the residue volume at position 345 are vital. Furthermore, in intact cells, cocaine potentiated the reaction of the membrane-impermeant sulffhydryl reagent methanethiosulfonate ethyltrimethylammonium with the extracellularly located endogenous cysteines of D345N but not those of wild type, and this potentiation was blocked upon K+ substitution for Na+. Thus, cocaine binding to D345N likely induces a different and Na+-dependent conformational change, which may contribute to its Na+-dependent uptake inhibitory activity.

In the central nervous system, the dopamine (DA)3 transporter (DAT) is found on dendritic and axonal plasma mem-
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**Materials—**The pCIN vector and the pCIN–synthetic human DAT construct (pCIN–DAT) were generous gifts from Dr. Jonathan A. Javitch of Columbia University. [3H]CFT (64.5 Ci/mmol), [3H]1-(2-diphenylmethoxy)ethyl-4-[3-phenylpropyl]piperazine ([3H]GBR 12935; 47.5 Ci/mmol), and [3H]DA (60 Ci/mmol) were from PerkinElmer Life Sciences. [3H]β-Carboethoxy-3-(4-isoprenyl)tropane ([3H]CIT; 64.7 Ci/mmol) was from Toronto Cookson Ltd. (Brussels, UK). Unlabeled CPT was from the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC). Cocaine, GBR12935, benzotropine, m-tyramine, N,N,N,N-tetrais-(2-pyridylmethyl)ethylenediamine were from Sigma. MTSET was from Toronto Research Chemicals (Toronto, Ontario, Canada). Sulfo-NHS-SS-Biotin, NeutrAvidin Plus, mammalian protein extraction reagents, protein inhibitor mixture, and goat horseradish peroxidase-conjugated anti-rabbit antibodies were from Pierce. Rabbit anti-human DAT polyclonal antibody was from Chemicon (Temecula, CA). ChemGlow chemiluminescence substrate solution was from Alpha Innotech (San Leandro, CA). Other chemicals and molecular biology reagents were provided by commercial sources.

**EXPERIMENTAL PROCEDURES**

**Generation of Cell Lines Stably Expressing Wild-type and Mutant DATs—**Mutant transporters were generated by site-directed mutagenesis of the wild-type pCIN–DAT construct as described previously (14). The mutated regions were excised by digestion with appropriate restriction enzymes and subcloned individually or together (double mutant) back into the original pCIN–DAT construct. All mutations were screened by restriction mapping, and confirmed in both directions by dye terminator cycle sequencing the subcloning region (Research Resource Center, University of Illinois). Human embryonic kidney cells (HEK 293, ATCC CRL 1573) were grown in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium supplemented with 10% bovine calf serum and 2 mM t-glutamine at 37 °C and 5% CO2. For stable transfection, cells were seeded into 6-well plates and allowed to grow to 60% confluence. Then each well of cells was transfected with pCIN–DAT construct or pCIN vector using 10 μl of Lipofectamine 2000 (Invitrogen) in 1 ml of serum-free growth medium. Stable pools of HEK-293 cells expressing DATs were selected with geneticin as described previously (14).

**Surface Biotinylation and Western Blotting—**Cells were washed with ice-cold PBS, pH 7.4, and incubated with Sulfo-NHS-SS-Biotin (1 mg/ml) in PBS for 30 min at 4 °C, followed by incubation with 10 mM glycine in PBS for 10 min. Biotinylated cells were washed with PBS and lysed in mammalian protein extraction reagents supplemented with protein inhibitor mixture for 5 min with gentle shaking. The lysate samples were centrifuged at 14,000 × g for 15 min to remove cell debris. The cleared cell lysates were incubated with NeutrAvidin Plus (100 μl of 50% resins per 100 μg of protein) with end-to-end mixing for 1 h. The resins were extensively washed with PBS, and the biotinylated proteins were eluted with SDS-PAGE sample buffer for 30 min at 37 °C. The bean eluates (from 74 μg of total cell lysates) were separated by 8% SDS-PAGE. The separate proteins were electroelaboratedly transferred to nitrocellulose membranes. Blots were blocked in 5% non-fat dry milk for 1 h in PBS containing 0.05% Tween 20. DAT proteins were detected using the rabbit anti-human DAT polyclonal antibody at 0.5 μg/ml followed by the secondary goat horse radish peroxidase-conjugated anti-rabbit antibody at 0.04 μg/ml. The transporter signal was visualized by using ChemGlow chemiluminescence-conjugate substrate. The images were captured using FluorChem 8800 Image System (Alpha Innotech).

**Measurement of Transport by Rotating Disk Electrode Voltammetry—**The equipment was set up as described previously (15). The DAT-expressing cells were suspended in 300 μl of uptake buffer (without tropolone and ascorbic acid). Transport assays (DA uptake and amphetamine-induced DA efflux) with rotating disk electrode voltammetry (RDEV) were performed as described previously (7, 16) with minor modifications. Briefly, after cell suspensions were placed into the electrochemical cell, a Te-doped glassy carbon rotating disk electrode (1.3 mm diameter, Pine Instrument Co., Grove City, PA) was introduced just below the surface of the solution and rotated with an AFMSRX Analytical Rotator System (Pine Instrument Co.) at 4000 rpm. A potential of 350 mV relative to an Ag/AgCl reference electrode was applied to the working electrode with a Petit Ampere LC-3D potentiostat (Bioanalytical Systems, West Lafayette, IN). DC and disk voltages of the 1350 signal were amplified. Voltammetric signals were acquired at a frequency of 4 Hz through a computer via a PCI-MIO-16XE-50 DAQ interface board controlled by the custom programmed LabVIEW software (National Instruments, Austin, TX). The acquired data were imported into Origin software.
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Results

Surface Expression of Mutated Transporters—To test whether the conserved YRD motif plays a role in transport activity, we introduced several mutations at each of these positions. The neighboring conserved residue, Cys-342, has been found to be important for DAT function (16). For comparison purpose, Cys-342 was also mutated to alanine (C342A). All constructs including the previously generated D345N were transfected using twice the DNA amount reported previously (14) to avoid low surface expression. Cell surface biotinylation followed by Western blot was performed to determine whether the mutants are properly expressed at the cell surface. In biotinylated samples of both WT and the mutants, the antibody against the C terminus of the human DAT primarily labeled a diffuse protein band at 75 kDa (Fig. 1). All mutants appeared to be efficiently delivered to the plasma membrane with a level equivalent to or slightly lower than the wild-type (WT) DAT (Fig. 1). No band was detected in the cell line expressing the pCIN vector alone (data not shown). The efficiency for separation of surface proteins from intracellular proteins was verified by the lack of immunoreactivity in biotinylated samples after re-probing the same blot with the antibody against the intracellular protein calnexin (data not shown).

Mutation of Asp-345 or the Neighboring Residues Alters Kinetic Characteristics of DA Uptake—The uptake of 10 nM [3H]DA was initially examined in each of the mutants (Fig. 2A). As can be seen, replacement of each residue caused a reduction in [3H]DA uptake. At positions 343, nonconservative substitution of the polar tyrosine with the smaller size alanine (Y343A) or the hydrophobic leucine (Y343L) decreased the transport activity by ~50%, and conservative substitution with phenylalanine (Y343F) also did at position 344, replacement of the positively charged arginine by the neutral alanine (R344A) or by the positively charged lysine (R344K) reduced the transport activity to a similar degree, whereas replacement by glutamine (R344Q), an amino acid containing an uncharged polar side chain, affected DA uptake more. A pronounced drop in DA uptake was observed upon mutation at position 345. Removal of the negatively charged aspartate at position 345 with alanine substitution (D345A) reduced DA uptake by 70%. Neutral conservative substitution with asparagine (D345N), which retains both the residue size and carbonyl oxygen, did not significantly attenuate the uptake reduction. The replacement of Asp-345 with glutamate (D345E), which retains the carboxylate functional group but as part of a bulkier side chain, also did not restore transporter activity. Introduction of the R344A mutation into D345A (R344A/D345A) did not show any additional effect on the DA uptake (Fig. 2A).

Saturation analysis of the DA uptake revealed a decline in the $K_m$ value upon alanine substitution between positions 342 and 345, which was moderate at R344A but progressively decreased from C342A to Y343A to D345A (Fig. 3). With a similar pattern, the $V_{max}$ also declined progressively from C342A to Y343A to D345A (Fig. 3). In general, these mutants were similarly expressed on the cell surface (Fig. 1), and the decline in $V_{max}$ appears to be primarily due to a reduction in transport activity. The close correlation between $K_m$ and $V_{max}$ among all the alanine-substituted mutants (Fig. 3) indicates that mutations at these positions may alter the two parameters primarily via a single mechanism. D345E and D345N mutation reduced the $K_m$ and $V_{max}$ values to a level similar to that caused by D345A mutation (Fig. 3). Again, introduction of the R344A mutation into D345A had little impact (Fig. 3). To gain further insights into the effect of the mutations at position 345 on interactions with substrates, we measured the $K_i$ for various substrates in competing for [3HIDA uptake. The $K_i$ value for a substrate determined from inhibition of transport is thought to reflect the $K_m$ value for the substrate to transport (21). Like DA, the DAT substrates m-tyramine, $\beta$-phenylethylamine, and amphetamine displayed a considerably small $K_i$ value for the mutants (8–20-fold lower than WT, Table 1). A 10–20-fold reduction in $K_i$ was also observed with norepinephrine, a se-
lective substrate for the norepinephrine but a poor one for the DAT (Table I). These effects were generally independent of the properties of the substituted amino acid at position 345 (Table I).

**Mutation of Asp-345 Diminishes Inhibitor Binding but Not Uptake Inhibition**—Consistent with our previous findings (14), all mutations involving position 345 reduced the binding of 4 nM [3H]CFT by 90% or more (Fig. 2B). In contrast, mutations at positions 342, 343, and 344 affected [3H]CFT binding modestly (Fig. 2B). Binding of many inhibitors to the DAT is stimulated by Na+/H+ (19, 22, 23). Thus, we examined Na+/H+ dependence of the binding activity. For WT-expressing cells, a Na+ concentration of 130 mM enhanced [3H]CFT binding by 3.5-fold (Fig. 4A). However, for D345N-expressing cells, the low level of [3H]CFT binding was insensitive to Na+ concentrations up to 130 mM (Fig. 4A). We wondered whether the binding affinity of the mutant for [3H]CFT was too low to allow reliable detection of the Na+ stimulation. Thus, Na+ dependence of the binding of two other radiolabeled inhibitors, [3H]CIT and [3H]GBR12935, was examined. Both radioligands have a binding affinity for DAT ~10-fold higher than that of [3H]CFT (10, 24). To avoid high levels of nonspecific binding, [3H]CIT and [3H]GBR12935 binding assays were conducted in membranes isolated from DAT-expressing cells, and the results were compared with that from [3H]CFT binding to membrane preparations (Fig. 4, B–D). All three radioligands showed very low levels of binding to membranes prepared from D345N-expressing cells. For the cocaine-like inhibitors [3H]CFT and [3H]CIT, a Na+ concentration of 20 mM enhanced their binding to WT membranes by more than 10-fold, but Na+ concentrations up to 300 mM had no effect on their binding to D345N membranes (Fig. 4B and C). For the selective DAT inhibitor [3H]GBR12935, Na+ concentrations up to 80 mM substantially increased the binding to WT membranes but not D345N membranes (Fig. 4D). In contrast, the Na+ dependence of DA uptake was similar for WT and D345N (Fig. 4E). Thus, the loss of the radioligand binding is
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FIG. 3. Kinetic characteristics of DA uptake upon mutation at positions 342–345. There is a correlation between values for $K_\text{m}$ and $V_{\text{max}}$ in mutants with alanine substituted at positions 342–345. The straight line is a linear regression fit to the data points for alanine-substituted mutants. Shown are means ± S.E. of 3–8 experiments performed in triplicate. The horizontal error bar represents S.E. for $V_{\text{max}}$, and the vertical error bar represents S.E. for $K_\text{m}$. Except for R344A, the $K_\text{m}$ and $V_{\text{max}}$ values for the mutants are significantly smaller than WT ($p < 0.05$, Dunnett’s test).

TABLE 1

<table>
<thead>
<tr>
<th>Inhibitors, nM</th>
<th>WT</th>
<th>D345A</th>
<th>D345E</th>
<th>D345N</th>
<th>R344A/D345A</th>
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<tr>
<td>CPT</td>
<td>38 ± 5</td>
<td>123 ± 15$^a$</td>
<td>73 ± 7$^a$</td>
<td>52 ± 5</td>
<td>83 ± 14$^a$</td>
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<tr>
<td>Cocaine</td>
<td>247 ± 23</td>
<td>594 ± 45$^a$</td>
<td>851 ± 110$^a$</td>
<td>557 ± 83$^a$</td>
<td>345 ± 21</td>
</tr>
<tr>
<td>GBR12935</td>
<td>229 ± 28</td>
<td>200 ± 30</td>
<td>196 ± 38</td>
<td>216 ± 32</td>
<td>209 ± 39</td>
</tr>
<tr>
<td>Benztropine</td>
<td>287 ± 34</td>
<td>528 ± 124</td>
<td>493 ± 54</td>
<td>340 ± 44</td>
<td>411 ± 34</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td>41 ± 3</td>
<td>110 ± 15$^a$</td>
<td>132 ± 11$^a$</td>
<td>76 ± 9</td>
<td>92 ± 13$^a$</td>
</tr>
<tr>
<td>Dopamine ($K_\text{m}$)</td>
<td>622 ± 62</td>
<td>42 ± 5$^a$</td>
<td>28.3 ± 2.3$^a$</td>
<td>63 ± 5$^a$</td>
<td>34 ± 5$^a$</td>
</tr>
<tr>
<td>$m$-Tyramine</td>
<td>450 ± 77</td>
<td>33 ± 4$^a$</td>
<td>42 ± 7$^a$</td>
<td>66 ± 17$^a$</td>
<td>40 ± 1$^a$</td>
</tr>
<tr>
<td>$\beta$-Phenylethylamine</td>
<td>1466 ± 234</td>
<td>50 ± 9$^a$</td>
<td>84 ± 8$^a$</td>
<td>100 ± 16$^a$</td>
<td>58 ± 18$^a$</td>
</tr>
<tr>
<td>β-Amphetamine</td>
<td>132 ± 26</td>
<td>11.8 ± 1.0$^a$</td>
<td>14.0 ± 2.6$^a$</td>
<td>17 ± 3$^a$</td>
<td>15 ± 3$^a$</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>6380 ± 1095</td>
<td>311 ± 62$^a$</td>
<td>532 ± 103$^a$</td>
<td>751 ± 163$^a$</td>
<td>352 ± 15$^a$</td>
</tr>
</tbody>
</table>

$^a$ p < 0.05 versus wild type (Dunnett’s test).

not due to a defect in Na$^+$ binding/action but more likely due to a lack of intrinsic binding activity.

Because of the low binding activity of Asp-345 mutants, attempts to measure the binding parameters were not successful in most cases. Only 4 of 12 experiments with D345N allowed plotting a Scatchard graph and fitting parameters for CFT binding to intact cells. The results revealed that D345N mutation reduced the $V_{\text{max}}$ by more than 90% without a statistically significant effect on the $K_\text{d}$ (Fig. 5A). A similar conclusion was obtained from saturation analysis of binding to membranes with increasing concentrations of [3H]GBR12935 (Fig. 5B). The $K_\text{d}$ values for GBR12935 binding to both WT and D345N were comparable with those reported in the literature (20, 24).

In separate experiments, CFT binding to WT cells and the broken cell membranes was performed under identical conditions. The $K_\text{d}$ (nM) was 16.4 ± 1.5 for cells and 20.7 ± 1.7 for membranes. The $B_{\text{max}}$ (pmol/mg) was 3.95 ± 0.36 for cells and 11.2 ± 0.4 for membranes when normalized with respective cell protein and membrane protein. In these experiments, the membrane protein was only 30% of total cell protein. If cell binding is corrected by taking into account this 30% value, the $B_{\text{max}}$ for cells is 13.2 pmol/mg, not different from that for membrane preparations. Furthermore, DA, which is relatively membrane-impermeant, was equally potent in inhibiting CFT binding to cells and membranes (data not shown). In confocal imaging experiments with a rat monoclonal antibody against the N terminus of the DAT (Chemicon), both WT and D345N were almost exclusively expressed on the cell surface (data not shown). Thus, under our conditions, we consider that measurements with intact cells and broken cell membranes principally reflect binding to surface transporters.

Consistent with a near-normal binding affinity (Fig. 5A), in [3H]DA uptake inhibition experiments with Asp-345 mutants, CFT, and cocaine showed only a 2–3-fold reduction in their potency to inhibit [3H]DA uptake (Table I). The reduction in the uptake inhibitory potency was modest for methylphenidate and did not reach a statistical significance for benztropine. For GBR12935, the $K_\text{d}$ value was significantly higher than the $K_\text{d}$ value for binding to membranes (Fig. 5B) but comparable with that found in human striatum membranes (25). This discrepancy is likely due to differences in assay buffer (uptake buffer versus Tris-HCl buffer) and DAT preparations (cells versus membranes). As shown for the binding $K_\text{d}$ (Fig. 5B), the $K_\text{d}$ of GBR12935 in inhibiting DA uptake was virtually the same for WT and Asp-345 mutants (Table I). Different from the binding assays where Na$^+$ stimulation was observed in WT but not D345N, Na$^+$ enhanced the potency for cocaine to inhibit [3H]DA uptake in both WT and D345N (Fig. 6, A and B). However, at D345N, Na$^+$ was less effective in enhancing cocaine potency. Thus, at 10 mM Na$^+$, the potency of cocaine in inhibiting DA uptake already reached a maximum in WT (Fig. 6A) but remained significantly weaker in D345N, resulting in a
At higher concentrations, Zn2+ significantly greater stimulation on the binding of [3H]CFT to presence of 5 mM NaCl or 0.5 nM [3H]CIT (V—345 Mutants). The kinetic characteristics (small endogenous Zn2+ whereas it reduced the 3-fold for D345E and by 2-fold for other Asp-345 mutants, reached a maximum between 10 and 100 M Zn2+ A mutant, D345A, and D345E (data not shown). Furthermore, Zn2+ enhanced both DA uptake and CFT binding at Asp-345 mutants—The kinetic characteristics (small Vmax and Km) of DA uptake at Asp-345 mutants are reminiscent of that reported for Y335A (15). An intriguing change in Y335A is the reversal of the Zn2+ effect on DA uptake. Occupancy of the endogenous Zn2+-binding site of WT DAT partially inhibits DA uptake, presumably via restricting, but not completely preventing, movements associated with DA translocation (26, 27) and favoring an outward-facing conformation of the transporter. In contrast, mutation of Tyr-335 converts the inhibitory effect of Zn2+ on DA uptake to a stimulatory one. The explanation for this observation is that mutation of Tyr-335 causes DAT to accumulate in an inward-facing conformational state, and Zn2+ attenuates this trend (15). We reasoned that if Asp-345 mutation-induced alterations in DA uptake and radiogold binding result from the same mechanism, i.e., accumulation of the transporter in an inward-facing conformational state, and Zn2+ would be capable of improving not only the DA uptake but also the CFT binding. Indeed, in D345N, Zn2+ neither inhibited DA uptake but instead increased it (Fig. 7A). Furthermore, Zn2+ showed significantly greater stimulation on the binding of [3H]CFT to cells expressing D345N (Fig. 7B), D345A, and D345E (data not shown) than to WT-expressing cells. Both effects of Zn2+ reached a maximum between 10 and 100 μM, concentrations for Zn2+ binding to the endogenous Zn2+ site of the DAT (26, 27). At higher concentrations, Zn2+ become inhibitory to DA uptake and lost its stimulatory effect on CFT binding. In further saturation analysis of [3H]DA uptake with D345A, D345E, D345N, and R344A/D345A, 10 μM Zn2+ increased the Vmax by 3-fold for D345E and by 2-fold for other Asp-345 mutants, whereas it reduced the Vmax of WT by 40% (Fig. 8A). In the presence of 10 μM Zn2+, the Km value for DA uptake was modestly increased, but this effect was not significantly different between WT and the mutants (Fig. 8B). 10 μM Zn2+ also doubled the potency for cocaine to inhibit DA uptake, and this effect was similar for WT and Asp-345 mutants (Fig. 8C). In saturation analysis of CFT binding to cells in the presence of 10 μM Zn2+ (n = 3), compared with control values (see Fig. 5A), Zn2+ reduced Kd similarly at D345N (24.1 ± 2.4 versus 11.3 ± 2.1 nM, p < 0.05) and WT (18.2 ± 2.9 versus 8.18 ± 0.40 nM, p < 0.05), 2-fold for each cell line. It increased Bmax also by 2-fold (0.30 ± 0.03 versus 0.65 ± 0.17 pmol/mg, p < 0.05) at D345N but not at WT (4.1 ± 0.27 versus 3.77 ± 0.35 pmol/mg). Thus, the greater stimulation of Zn2+ on CFT binding to D345N cells is due to an increase in Bmax.

Mutation of Asp-345 Impairs Reverse Transport as Measured by RDEV—To address whether mutation of Asp-345 affects reverse transport, we examined amphetamine-induced DA efflux using RDEV. This approach allows us to monitor the process of DA preloading and efflux in a single experiment with high time resolution at 37 °C. A saturating concentration (10 μM) of amphetamine failed to induce any DA efflux from D345N-expressing cells (data not shown). However, this could be due to the slow uptake rate of DA at D345N, which compromises the DA preloading level. Taking advantage of the opposite effect of Zn2+ on DA uptake by WT and D345N, we added Zn2+ before starting the DA preloading so that the amphetamine-induced DA efflux could be tested under identical DA preloading level. Fig. 9 illustrates the experimental strategies. In the absence of Zn2+, the uptake velocity of 1 μM DA was very slow in D345N (Fig. 9B), compared with WT (Fig. 9A), which caused incomplete DA preloading. Zn2+ (30 μM) reduced the rate of DA uptake by WT (compare Fig. 9, A with C) but increased that by D345N (compare Fig. 9, B with D), allowing preloading of the two cell lines with the same amount of DA within the same period (~210 s). After the extracellular DA concentration reached the steady state, 10 μM amphetamine was added. It was found that amphetamine induced a rapid increase in the extracellular concentration of DA in WT-ex-
pressing cells (Fig. 9C) but not in D345N-expressing cells (Fig. 9D). We tested DA uptake and amphetamine-induced DA efflux in the presence of various concentrations of Zn$^{2+}$. As observed in [$^3$H]DA uptake, from 0 to 30 μM, Zn$^{2+}$ inhibited DA uptake at WT but stimulated it at D345N. However, the inhibitory effect of Zn$^{2+}$ on DA uptake by WT was significantly stronger in RDEV than in [$^3$H]DA assays, as shown by a 5-fold reduction in the initial uptake rate at 10 μM Zn$^{2+}$ (19.2 ± 1.46 pmol/mg/s for control and 3.84 ± 0.72 pmol/mg/s with Zn$^{2+}$). This may reflect the impact of different assay temperature (37 versus 25 °C) and read-out (extracellular clearance of DA versus intracellular accumulation of radioactivity) between RDEV and [$^3$H]DA assays. From 30 to 100 μM, Zn$^{2+}$ had no further effect on the initial rate of DA uptake (1 μM) at either WT or D345N, and this rate was not significantly different between WT and D345N (Fig. 10A). However, over this range of Zn$^{2+}$, the initial rate of amphetamine-induced DA efflux was substantially slower at D345N than WT (Fig. 10B).

If the initial efflux rate of DA is proportional to the initial uptake rate of the inducer, the observed difference in the initial rate of amphetamine-induced DA efflux between WT and D345N might be partially related to their different uptake kinetics. To exclude this possibility, we measured the kinetic parameters for DA uptake in the presence of 30 μM Zn$^{2+}$, a concentration at the plateau level for Zn$^{2+}$ to inhibit uptake by WT and to stimulate uptake by D345N in RDEV (Fig. 10A). At this concentration, Zn$^{2+}$ reduced the uptake V$_{max}$ for WT from 30.8 ± 2.4 to 3.01 ± 0.57 pmol/mg/s, whereas it increased the uptake V$_{max}$ for D345N from 0.58 ± 0.07 to 2.38 ± 0.12 pmol/mg/s, resulting in no significant difference in uptake V$_{max}$ between WT and D345N (n = 3). Previous studies with RDEV suggest that DAT transports various substrates with similar V$_{max}$ (7). Thus, the uptake V$_{max}$ for amphetamine would be expected to be also similar for WT and D345N at 30 μM Zn$^{2+}$. In addition, at 30 μM Zn$^{2+}$, the difference in DA uptake K$_m$ between WT and D345N (691 ± 77 versus 361 ± 54 nM), which is negligible in the presence of saturating concentrations of amphetamine. Thus, at 30 and 100 μM Zn$^{2+}$, the more than 4-fold difference in amphetamine-induced DA efflux between WT and D345N is most likely due to differences in reverse transport itself. However, at 10 μM Zn$^{2+}$, the different uptake kinetics may have an impact on amphetamine-induced DA efflux, because the initial uptake rate for D345N remained significantly slower than that for WT (Fig. 10A).

Under RDEV conditions, Zn$^{2+}$ itself did not induce DA efflux, and unlike reported by a superfusion study with [$^3$H]substrates.

![Saturation analysis of [$^3$H]inhibitor binding at D345N. A, [$^3$H]CFT binding to cells. Cells were incubated with 4 nM [$^3$H]CFT and various concentration of unlabeled CFT (0–300 nM); B, [$^3$H]GBR12935 binding to membranes. Cell membranes were incubated with increasing concentrations of [$^3$H]GBR12935 (0.26–15 nM). Shown are Scatchard plots. The solid straight line represents the best fit chosen by the Ligand program. Each panel shows a representative experiment performed in triplicate. The parameters in each panel are means ± S.E. of 4 experiments for CFT binding and of 3 experiments for GBR12935. *, p < 0.05 versus WT (t test).](http://www.jbc.org/).
Fig. 6. Mutation of Asp-345 retains Na\(^+\) potentiation of cocaine potency in inhibiting \[^{3}H\]DA uptake. A, WT; B, D345N; C, D345A; D, R345AD345A. Cells were incubated with 10 nm \[^{3}H\]DA in the presence of various concentrations of cocaine (0–30 \(\mu\)M). Na\(^+\) concentrations were varied by isotonically replacing NaCl with NMDG-Cl. Shown are means ± S.E. of 4–6 experiments performed in triplicate. *, \(p < 0.05\) versus the value at 130 mM Na\(^+\) (Dunnett's test).

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(28), it had no significant effect on the DA efflux induced by 10 \(\mu\)M amphetamine (data not shown). The discrepancy is likely due to methodological differences between RDEV and superfusion designs and will be addressed in the future.

Cocaine Induces Different Conformational Changes at WT and D345N—To further assess the conformational status of D345N, we investigated the reactivity of endogenous cysteines in the extracellular portions of WT and D345N with the membrane-impermeant sulfhydryl reagent MTSET. Application of 3 mM MTSET to DAT-expressing cells for 10 min modestly increased \[^{3}H\]CFT binding (Fig. 11B), which is consistent with modification of Cys-90 and Cys-306 on the extracellular loops (29, 30). This stimulation was similarly observed in cells expressing WT, C342A, and D345N (Fig. 11B), suggesting that in intact cells, the intracellular Cys-342 does not react with MTSET and D345N mutation does not alter the reactivity of Cys-90 and Cys-306. Co-presence of DA (100 \(\mu\)M) during MTSET treatment had no effect on this MTSET stimulation. For

Fig. 7. Concentration-dependent effect of Zn\(^{2+}\) on \[^{3}H\]DA uptake and \[^{3}H\]CFT binding upon mutation of Asp-345. Cells were incubated with 10 nM \[^{3}H\]DA (A) or 4 nM \[^{3}H\]CFT (B) in the presence of various concentrations of Zn\(^{2+}\). Shown are means ± S.E. of 4 experiments performed in triplicate. *, \(p < 0.05\) versus the value in the absence of Zn\(^{2+}\) (Dunnett’s test).
WT and C342A cells, the stimulatory effect of MTSET on \([^{3}H]CFT\) binding was also not changed by the co-presence of cocaine (10 \(\mu M\)). However, for D345N cells, this stimulation was significantly potentiated by cocaine (Fig. 11B). Moreover, this effect was blocked by substitution of K\(^+\) for Na\(^+\) in the reaction buffer, although K\(^+\) itself had little effect on MTSET modification (Fig. 11D). The data suggest that cocaine binding at D345N caused a conformational change different from that at WT and that this conformational change requires Na\(^+\) to occur. Consistent with a different and ion-sensitive conformational change induced by cocaine, with K\(^+\) substitution for Na\(^+\), cocaine enhanced the inhibitory effect of MTSET on the DA uptake by WT significantly but not that by D345N (Fig. 11, A and C). A similar conclusion could also be reached for DA when inspecting the effect of DA on MTSET inhibition of DA uptake (Fig. 11, A and C).

**DISCUSSION**

In the present study, we show that Asp-345 of the human DAT, located in the third putative cytoplasmic loop, plays a critical role in both forward and reverse transport of DA as well as in actions of DAT inhibitors. Analysis of multiple substitutions at positions 345 demonstrates that the replacement of the original aspartate residue rather than the introduced residue is responsible for the observed phenotype. Clearly, both the negative charge and the volume of the side chain are vital. However, the side chain of Asp-345 does not seem to have a direct contact with that of its neighboring residues, because substitutions for Cys-342, Tyr-343, or Arg-344 exerted relatively smaller impact on transporter activities. Despite a lack of evidence for direct interactions, mutation of the CYRD domain encompassing positions 342–345 showed a similar trend in reducing \(K_m\) for DA uptake, and except for position 344, this trend is accompanied with a decline in \(V_{max}\). It is possible that the neighboring residues play a certain role in properly positioning the side chain of Asp-345, and thus mutation of them affects the function of Asp-345. Interestingly, the uptake prototype of Asp-345 mutants (small \(V_{max}\) and \(K_m\), and reversal of Zn\(^{2+}\) inhibition) resembles that reported for mutation of Tyr-335 on the same intracellular loop (15). However, uptake inhibition by various DAT inhibitors was largely preserved upon mutation of Asp-345 (current study) but severely damaged upon mutation of Tyr-335 (15), which also argues against a direct contact between the two residues. It is likely that like Tyr-335, Asp-345 is a part of a network of the intramolecular interactions (15), which provides the structural basis for appropriate conformational interconversions during the transport cycle (see below).

Three lines of evidence indicate that mutation of Asp-345 arrests the DAT in a conformation that cannot readily assume the outward-facing state, regardless of whether it binds substrates or not. First, all the Asp-345 mutants displayed extremely low \(V_{max}\) and \(K_m\) values for DA uptake. The former is far below expected from their surface expression. Such kinetic characteristics for DA uptake are consistent with a defect in
the reorientation of the empty transporter. According to kinetic rate equations (31), $V_{\text{max}} = \frac{C_1 f_1 f_2}{f_1 + f_2}$, and $K_m = \frac{K_s (f_1 + f_2)}{f_2}$, with $C$ being the transporter density, $K_s$ the binding affinity for substrates, and the other parameters, the rate constants for conversion of the outward-facing empty transporter to the inward-facing one ($f_1$), for reorientation of the empty transporter from inward-facing to outward-facing ($f_\mu$), and for the inwardly orientation of the substrate-transporter complex ($f_2$). For catecholamine transporters, substrate uptake can be accelerated by the presence of intracellular substrates, which is thought to arise from the transporter returning in the substrate-loaded form (32, 33). The trans-acceleration implies that reorientation of the empty transporter ($f_\mu$) is the rate-limiting step in substrate uptake. When $f_\mu \ll f_\mu$, the rate equation for $V_{\text{max}}$ reduces to $Cf_1 f_2 / (f_1 + f_2)$, and $K_m$ reduces to $K_s (f_1 + f_2) / f_2$. It can be easily seen that impairment of a single step, i.e. the return of the empty transporter ($f_\mu$), would reduce both $V_{\text{max}}$ and $K_m$ values. The present study cannot completely exclude a contribution of the mutation-induced increase in the substrate binding affinity (decrease in $K_s$) to the small $K_m$. However, because Asp-345 is conserved throughout the family of the Na$^+$/Cl$^-$-dependent transporters, it seems less likely that the mutation would promote the formation of a binding site with high affinity for all the tested phenethylamine substrates, regardless of their selectivity. Second, the mutation reverted the effect of Zn$^{2+}$ on DA uptake from inhibition to stimulation, which is consistent with a shift in conformational distribution away from the outward-facing state. It has been proposed that the Zn$^{2+}$ ions, by occupying the endogenous Zn$^{2+}$-binding site of the DAT and constraining the transporter

Fig. 9. Time course for RDEV measurements of DA uptake and amphetamine-induced DA efflux in the absence and presence of Zn$^{2+}$. A, WT in the absence of Zn$^{2+}$; B, D345N in the absence of Zn$^{2+}$; C, WT in the presence of 30 μM Zn$^{2+}$; D, D345N in the presence of 30 μM Zn$^{2+}$. Zn$^{2+}$ was added at 0 min (i.e. 1 min prior the addition of DA) if present. The arrow in each panel denotes the addition time of DA (1 μM) or amphetamine (AMP, 10 μM). Shown is a representative experiment performed on the same batch of cells.

Fig. 10. Initial rates of DA uptake and amphetamine-induced DA efflux in the presence of various concentrations of Zn$^{2+}$. The protocol applied is depicted in Fig. 9, C and D. A, initial rate of DA uptake; B, initial rate of amphetamine-induced DA efflux. Shown are means ± S.E. of 6–8 experiments. *, $p < 0.05$ versus WT (t test).
movements, increases the chance of DAT staying in an outward-facing like conformation (15, 26, 27). Thus, Zn$^{2+}$/H11001 is capable of partially improving DA uptake by Asp-345 mutants. Finally, the inability of D345N to mediate the amphetamine-induced DA efflux convincingly points to a defect in the return of the DA-transporter complex. This is seen most clearly in experiments with Zn$^{2+}$/H11001 (30–100/M), where the DA preloading level and the uptake $V_{\text{max}}$ were similar between WT and D345N, but the initial DA efflux rate mediated by the mutant remained extremely slow. It should be noted that Zn$^{2+}$ itself does not induce DA efflux and does not alter the initial rate of the amphetamine-induced DA efflux. Thus, the lack of DA efflux at D345N in the presence of Zn$^{2+}$/H11001 cannot be accounted for by a different effect of Zn$^{2+}$ on DA efflux.

A notable property of Asp-345 mutants is that they lack binding activity for various radiolabeled inhibitors, including cocaine analogs CFT and CIT, and the non-cocaine-like inhibitors mazindol and GBR12935 (14, current study), but still allow these inhibitors to potently inhibit DA uptake. In a previous study with D345N (14), we demonstrated the following. 1) The lack of binding is not due to a rapid dissociation of the ligand-transporter complex escaping the capture by filtration separation approach or isotopic artifacts because the irreversible inhibitor $^{125}$I-labeled 3-(p-chlorophenyl)tropane-2-carboxylic acid failed to covalently label the mutated transporter. 2) The relative potent inhibition of DA uptake is not due to binding of the inhibitors to a conformational state exclusively occurring during the transport cycle because the presence of substrates did not improve CFT binding. The current study provides an explanation for these phenomena. We found that the D345N mutation tremendously reduced the binding capacity of surface transporters for CFT and GBR12935 without affecting the binding affinity and uptake inhibitory potency for the two compounds. These findings are consistent with a substantial loss of a conformational state where DA binds to initiate uptake, and inhibitors bind to initiate uptake inhibition. In this scenario, despite the fact that the number of DATs residing in a conformational state favoring the binding of inhibitors becomes too small to allow appreciable labeling by a trace amount of the radiolabeled inhibitor, the inhibitor still binds to a small portion of the DAT remaining in this state with considerable affinity, and the uptake signal is sensitive enough to reflect this inhibition. This conformational state that is lost is likely to be the outward-facing state, because analysis of DA transport suggests that mutation of Asp-345 impedes the conversion of the mutant toward the outward-facing state. In further support, although both Na$^{+}$/H11001 and Zn$^{2+}$/H11001 are thought to stimulate CFT binding via inducing a conformational change, only Zn$^{2+}$, which is found to constrain the transporter in an outward-facing like state, was able to show a significantly greater stimulation of CFT binding to this mutant.

If binding of cocaine analogs fully underlies their inhibition of DA uptake, the ionic dependence for binding activity and uptake inhibition should be similar. However, at D345N, Na$^{+}$ did not stimulate the binding of cocaine analogs but still enhanced the potency for cocaine to inhibit DA uptake. It is possible that occupancy of the outward-facing state may not be the sole action through which cocaine analogs inhibit DA uptake by D345N. For instance, a Na$^{+}$-dependent conformational change induced by cocaine could influence multiple steps of DA transport or impact the function of oligomeric transporter complexes (34–36), thus providing uptake inhibition with Na$^{+}$.
dependence nonidentical to that for inhibitor binding. To seek direct evidence for the mutation-induced alteration in conformational changes associated with cocaine binding, we examined the ability of cocaine to modify the reaction of endogenous cistines of D345N with the sulhydryl reagent MTSET. MTSET is membrane-impermeant and preferentially reacts with cistines that are water-accessible from the extracellular side of the membrane. It has been demonstrated that reaction of MTSET with extracellular Cys-90 and/or Cys-306 of the DAT stimulates CFT binding, whereas that with intracellular Cys-135 and/or Cys-342 inhibits it (29, 30). In membrane preparations where cistines on both extracellular and intracellular portions of the human DAT are exposed to MTSET, cocaine potentiates the stimulatory effect of MTSET on CFT binding by protecting Cys-135 and Cys-342 from reaction as well as by increasing the reaction of Cys-90 (30). However, in intact cells expressing WT (29, current study) or a mutant X-90C (data not shown), in which Cys-90 is retained but four reactive cistines Cys-135, Cys-306, Cys-319, and Cys-342 are replaced, whereas MTSET reacts with Cys-90 to stimulate CFT binding, cocaine does not potentiate this stimulation. This difference suggests that the cocaine-induced conformational change varies with DAT preparations, increasing the accessibility of Cys-90 in membrane preparations but not in intact cells. Intriguingly, in cells expressing D345N, cocaine regained its ability to potentiate the MTSET stimulation of CFT binding. This result strongly argues that cocaine does bind to D345N and stabilize a conformational state different from that for WT. The conclusion is reinforced by the ion sensitivity of cocaine actions. Thus K+ substitution for Na+, although it did not alter MTSET reaction as judged by CFT binding and DA uptake, 1) prevented cocaine from potentiating the MTSET stimulation of CFT binding to D345N, and 2) caused cocaine to enhance the inhibitory effect of MTSET on DA uptake by WT but not that by D345N. The results also inspire speculation regarding the mechanism for the DAT preparation-dependent effect of cocaine on MTSET modification of Cys-90 and/or Cys-306. It could be contemplated that the co-presence of intracellular content and Asp-345 constrains the plasma membrane DAT in a conformational state where cocaine binding cannot alter the accessibility of Cys-90 and/or Cys-306. Thus, a loss of either intracellular content or Asp-345, by isolation of the membrane from cells or by mutation of Asp-345, would promote a different conformation of DAT amenable to cocaine-induced enhancement of the accessibility of Cys-90/306.

In conclusion, aspartate 345 is critical for substrate transport in both forward and reverse directions. Replacement of it traps the transporter in a conformational state that cannot readily assume the outward-facing conformation that is essential for detection of the radiolabeled inhibitor binding. However, cocaine and other DAT inhibitors may still bind directly to a small portion of the mutated transporter that resides in the outward-facing state, thereby blocking the initial step of DA uptake. This may be sufficient to explain their relatively potent inhibitory effect on DA uptake. Furthermore, following its binding to D345N, cocaine likely induces a Na+-dependent conformational change unlike that in WT, perhaps providing an example of uptake inhibitory activity resulting from, but not directly associated with, inhibitor binding.

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Aspartate 345 of the Dopamine Transporter Is Critical for Conformational Changes in Substrate Translocation and Cocaine Binding
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