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Dissociation of High-Affinity Cocaine Analog Binding and Dopamine Uptake Inhibition at the Dopamine Transporter

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ABSTRACT

Cocaine initiates its euphoric effects by binding to the dopamine transporter (DAT), blocking uptake of synaptic dopamine. It has been hypothesized that the DAT transmembrane aspartic acid residue D79 forms an ionic interaction with charged nitrogen atoms in both dopamine and cocaine. We examined the consequences of novel and previously studied mutations of the D79 residue on DAT uptake of [^{3}H]dopamine, DAT binding of the cocaine analog [^{3}H]WIN 35,428, and drug inhibition of each process, all under identical conditions. The rat D79E DAT mutation decreased dopamine uptake V_{max} by 7-fold and decreased dopamine turnover by 4-fold. Wild-type DAT displayed near-perfect agreement in the uptake and binding inhibition potencies for substrates, but cocaine and other nonsubstrate inhibitor drugs were ~3-fold less potent in uptake than in binding assays. Apparent affinities for substrates were unaffected by the D79E mutation unless the catechol moiety was modified. Strikingly, potencies for nonsubstrate inhibitors in uptake and binding assays matched for D79E DAT, because of a 3-fold lowering of binding affinities relative to WT DAT. The present findings reveal a complex role for D79 in determining substrate specificity and high-affinity binding of DAT inhibitors. We propose that at least two discrete inhibitor-binding DAT conformations or populations exist and that the DAT conformation/population responsible for inhibitor high-affinity binding is less responsible for dopamine uptake. The findings may be extensible to other psychostimulants and antidepressants that display discrepancies between binding affinity and monoamine uptake inhibition potency and may be relevant to development of a long-sought "cocaine antagonist".

Binding of cocaine by the dopamine transporter protein delays termination of dopaminergic neurotransmission by blocking dopamine reuptake into the presynaptic cell. The resultant excess of dopamine in the synapse leads to an increase in postsynaptic receptor binding of the neurotransmitter; this potentiation of the transduced signal eventually effects a physical dependence on cocaine. Almost no definitive information exists on the conformation of neurotransmitter transporter proteins, because no crystallographic data are available. More attainable are data derived from site-directed and chimeric mutants, and such studies have provided much of what is known about DAT protein structure as it pertains to function. Molecular cloning of cDNAs encoding the plasma membrane monoamine transporters (Blakely et al., 1991; Hoffman et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Shimada et al., 1991) has suggested candidate amino acid residues for direct contact with the monoamine substrates. Scanning the Na^+/Cl^- dependent transporter family, it is quickly noted that an aspartic acid residue in the first putative transmembrane domain (TM) of the plasma membrane monoamine transporter is not shared by transporters for GABA, glycine, proline, betaine, or the other members of this group (Fig. 1).

In a previous study, replacement of the rat DAT TM1 aspartic acid residue (D79) with alanine, glycine, or glutamic acid decreased the dopamine uptake V_{max} value, increased the K_m value, and decreased binding of the cocaine analog [^{3}H]WIN 35,428 (Kitayama et al., 1992). The authors proposed that the D79 residue directly contacts the positively charged amino group of dopamine, an idea borrowed from a model involving agonist docking to a G protein-coupled receptor (GPCR), the β-adrenergic receptor (Strader et al., 1988). Indeed, ionic competition for D79 has been viewed as

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ABBREVIATIONS: DAT, dopamine transporter; SERT, serotonin transporter; TM, transmembrane domain; WIN 35,428, (−)-3β-(4-fluorophenyl)- tropan-2β-carboxylic acid methyl ester tartrate; WT, wild type; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; KRH, Krebs-Ringers-HEPES; DHBA, dihydroxybenzylamine.

the mechanism of cocaine inhibition of dopamine uptake; this postulate has served as the central tenet for DAT-ligand interactions (Carroll et al., 1992). More recently, neutral 8-oxa analogs of WIN 35,428 and cocaine have proven to be quite potent DAT inhibitors relative to the charged, nitrogen-based parent compounds (Madras et al., 1996; Kozikowski et al., 1999), which places in doubt the necessity of a D79-cocaine ion pair.

The postulate that the dopamine amino group forms an ionic bond with D79 is inconsistent, however, with amino acid sequence homology and substrate commonalities within the Na^+/Cl^- dependent transporter family. Transporters for GABA, glycine, proline, creatine, betaine, and taurine possess a glycine residue at the analogous position (Fig. 1) (Guastella et al., 1990, 1992; Fremeau et al., 1992; Yamauchi et al., 1992; Uchida et al., 1992; Guimbal and Kilimann, 1993), yet all of these substrates possess the very same positively charged amino group as dopamine. Given that dopamine, norepinephrine, epinephrine and serotonin share a phenolic moiety not shared by the other six substrates above, a more logical role for the aspartate side chain would be to form an intramolecular contact that supported a phenolic (or aromatic) binding pocket. The fact that this charged, hydro*philic* residue is one of only two such residues located in the hydro*phobic* lipid bilayer further implies its importance to DAT function; thus, a better understanding of the contribution of D79 to substrate and inhibitor binding is essential.

No direct contact points have been unequivocally established between a transporter in this family and either its cognate neurotransmitter or cocaine, although specific serotonin transporter residues have been identified as contributors to cocaine or mazindol binding sites (Chen et al., 1997; Barker et al., 1998). Toward the goal of establishing discrete dopamine-DAT points of contact, we have tested the premise of a dopamine-DAT D79 ionic bond serving as the governing point of contact by generating mutants that alter the charge, hydrogen bonding potential, or length of the D79 side chain. Use of a dopamine analog that is one carbon shorter at D79E DAT did not re-establish wild-type (WT) levels of apparent substrate uptake, and WT and D79E DAT displayed a very similar affinity for dopamine, findings that did not support the postulated D79-dopamine interaction. To directly and accurately compare ligand binding affinities and dopamine uptake inhibition potencies, binding and uptake inhibition assays were carried out under the same conditions. High-affinity binding of WIN 35,428 was lost at the D79E DAT mutant; paradoxically, potencies for this and other DAT blockers in inhibiting dopamine transport were considerably less affected by the mutation. This result led to the inference that two DAT populations or conformations recognize DAT inhibitors and to the unexpected conclusion that the DAT population/conformation responsible for high-affinity cocaine analog binding is not primarily responsible for dopamine uptake.

rDAT	73	V	I	G	F	A	V	**D**	L	A	N	V	W	R	85
hNET	69	V	V	G	F	A	V	**D**	L	A	N	V	W	R	81
rSERT	92	V	I	G	Y	A	V	**D**	L	G	N	I	W	R	104
fET	82	V	V	G	F	S	V	**D**	L	A	N	V	W	R	94
rGAT1	57	C	V	G	Y	A	I	**G**	L	G	N	V	W	R	69
rGLYT1	40	S	V	G	Y	A	V	**G**	L	G	N	V	W	R	52
rPROT	50	C	I	G	Y	C	V	**G**	L	G	N	V	W	R	62
hCREAT	65	C	V	G	F	A	V	**G**	L	G	N	V	W	R	77
cBET	49	V	A	G	E	I	I	**G**	L	G	N	V	W	R	61
hTAURT	54	V	A	G	G	F	V	**G**	L	G	N	V	W	R	66

Fig. 1. Protein sequence alignment of DAT relative to nine other members of this Na^+-dependent transporter family. The position of the DAT D79 residue is indicated in bold, flanked by neighboring residues in putative TM1. The numbers before and after the protein sequence refer to the position of the first and last residue shown, respectively, relative to the entire polypeptide. rDAT, rat dopamine transporter; hNET, human norepinephrine transporter; rSERT, rat serotonin transporter; fET, frog epinephrine transporter; rGAT, rat GABA transporter; rGLYT, rat glycine transporter; rPROT, rat proline transporter; hCREAT, human creatine transporter; cBET, canine betaine transporter; hTAURT, human taurine transporter.

Materials and Methods

Materials. [^{3}H]WIN 35,428 (~85 Ci/mmol) and [^{3}H]dopamine (~29 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). Nonradioactive WIN 35,428, cocaine, methylphenidate, and amphetamine were obtained from Research Triangle Institute (Research Triangle Park, NC) via the National Institute on Drug Abuse Division of Basic Research. Nonradioactive dopamine and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO); mazindol, norepinephrine, and the tyramines were obtained from RBI/Sigma (Natick, MA); and dihydroxybenzylamine was obtained from Aldrich (Milwaukee, WI). Scintillation counting materials were from Fisher Scientific (Pittsburgh, PA), and GF/B paper was from Brandel (Gaithersburg, MD). Anti-DAT antibody (rat) was obtained from Chemicon (Temecula, CA), biotinylation reagents were obtained from Pierce Chemical Co. (Rockford, IL), and all other Western blotting reagents were from Bio-Rad (Hercules, CA). COS-7 and CHO-K1 cell lines were obtained from American Type Culture Collection (Manassas, VA).

Mutagenesis and Cell Transfections. All site-directed mutagenesis was conducted as described previously (Spivak et al., 1997). COS-7 cells were cultured in Dulbecco's minimal essential medium plus 10% fetal bovine serum, 10% penicillin/streptomycin, and 20 mM L-glutamine at 37°C in 5% CO_2 in 750-cm^2 flasks. Cells were distributed in 24-well plates such that the monolayer would be 95% confluent when the transfection commenced. For an individual well of the 24-well plate, 1 μg of plasmid and 1.5 ml of "LIPO 2000" LipofectAMINE suspension were preincubated 20 min before adding to the well in Opti-MEM medium. After 5 h, an equal volume of Dulbecco's minimal essential medium plus 10% fetal bovine serum, 10% penicillin/streptomycin, and 20 mM L-glutamine was added to the well, and cells were used for experiments approximately 24 h after initiating the transfection. Stably-transfected DAT-CHO cell lines were prepared by LipofectAMINE-mediated transfection and selection of stable transfectants in the presence of 500 μg/ml G-418 in Ham's F-12 medium; cell lines were maintained in the same medium containing 100 μg/ml G-418.

Cell Surface Labeling of Expressed DAT Proteins. Stably transfected CHO cells or transiently transfected COS-7 cells were grown to confluence on six-well (35 mm/well) polylysine-free plates. After washing the cell monolayer 3 × 10 min with ice-cold phosphate-buffered saline supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/Ca/Mg buffer) (Ramamoorthy et al., 1998), 1 ml of a 1.5 mg/ml solution of freshly-prepared sulfosuccinimidyl-2-(biotinamido-)ethyl-1,3-dithiopropionate in PBS/Ca/Mg buffer was added for 25 min at 4°C. Free biotinylation reagent was quenched by washing thrice with cold PBS/Ca/Mg containing 100 mM glycine. After two more washes with PBS/Ca/Mg alone, cells were lysed with 0.2 ml of 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA in 10 mM Tris-HCl, pH 7.4, for 30 min on ice. Lysates were cleared by centrifugation at 14,000*g* for 10 min at 4°C. The upper 150 μl of

supernatant was transferred to a new tube and combined with 50 μl of NeutrAvidin resin (50% slurry), incubating overnight at 4°C and mixing with end-over-end rotation. The resin was concentrated to a pellet with 5000*g* centrifugation for 4 min at 4°C. The pellet was washed first three times by resuspending the resin in the lysis buffer, and then two times more in 0.1% Triton X-100, 500 mM NaCl and 5 mM EDTA in 50 mM Tris-HCl, pH 7.5; each wash was centrifuged as before with all steps at 4°C. After a final wash with 50 mM Tris-HCl, pH 7.5, the supernatant was completely removed, and biotinylated proteins were released from the NeutrAvidin resin by addition of standard SDS gel loading buffer containing 2-mercaptoethanol. Biotinylated DAT was separated from other biotinylated proteins by SDS-polyacrylamide gel electrophoresis, and identified with Western blotting (standard conditions, using a rat anti-DAT primary antibody diluted 1:4000). To test that biotinylation was specific for cell surface proteins, the same blot was stripped of antibodies with 100 mM 2-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.7, for 1 min at 40°C and subsequently reprobed with a monoclonal antibody against actin, an intracellular marker protein.

[^{3}H]Dopamine Uptake. Assays were conducted with cell monolayers in six-well plates. The monolayer was washed 2 × 2 ml with KRH buffer (25 mM HEPES, pH 7.3, 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM Mg_2SO_4, 1.2 mM KH_2PO_4, and 5.6 mM glucose), and uptake was typically initiated by addition of 1 ml of 10 nM [^{3}H]dopamine and 50 μM ascorbic acid in KRH to duplicate or triplicate cell monolayers. A time course was conducted for each DAT mutant to determine when uptake velocity was in the linear phase, typically occurring at 5 to 10 min at 22°C. Uptake was quenched by washing the monolayer with 2 × 2 ml of KRH + ascorbic acid. Cell monolayers were solubilized in 0.5 ml of 1% SDS, and transferred to scintillation vials for determination of incorporated tritium. DAT mutants demonstrating the ability to transport dopamine in this assay were studied in uptake "saturation" experiments in which final concentrations of 0.1 to 16 μM dopamine were employed, and [^{3}H]dopamine was diluted with nonradioactive dopamine to obtain a specific activity of 0.1 Ci/mmol. Nonspecific uptake was assessed by inclusion of 30 μM cocaine. Uptake inhibition experiments used 2 to 20 μM [^{3}H]dopamine plus nonradioactive DAT uptake blockers at the following concentration ranges: cocaine, 0.01 to 100 μM; WIN 35,428, 3 to 10,000 nM; mazindol, 3 to 10,000 nM; methylphenidate, 3 to 10,000 nM; and dihydroxybenzylamine (DHBA), 0.3 to 100 mM. All nonsubstrate inhibitors were preincubated 10 min with the cell monolayer before adding [^{3}H]dopamine. K_m and V_{max} values for transport and K_i values for uptake inhibition were determined with Prism 2.0 (GraphPad Software, San Diego, CA).

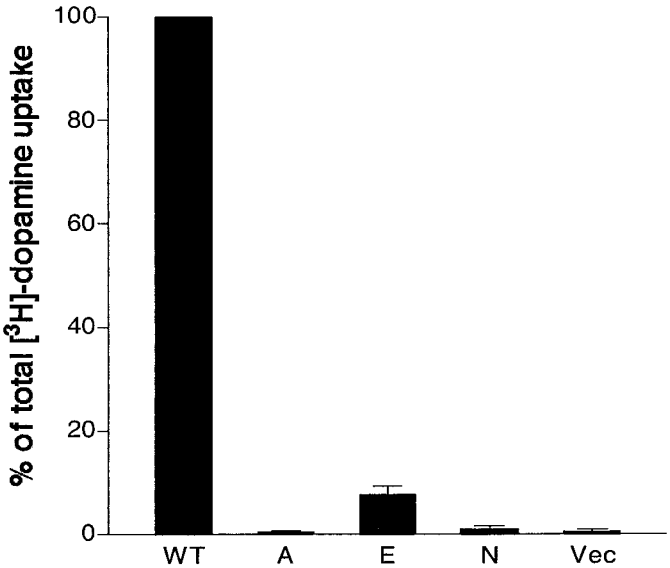

Fig. 2. Determination of [^{3}H]dopamine uptake ability by COS-7 cells expressing WT or D79 mutant DAT proteins. The extent of total [^{3}H]dopamine uptake as a percentage of WT DAT was assessed for cells transiently transfected with plasmids encoding D79A (A), D79E (E) or D79N (N) DAT, or the plasmid vector lacking DAT sequence (Vec). The data represent an average of three separate experiments.

Ligand Binding Assays. [^{3}H]WIN 35,428, structurally similar to cocaine but much more stable in vitro, was the radioligand employed for all experiments. With the exception of the membrane binding experiment referred to under *Results*, all binding assays were conducted exactly as described above for the dopamine uptake assay except that [^{3}H]dopamine was replaced with 1 nM [^{3}H]WIN 35,428, and radioligand and nonradioactive competitor were incubated with cells for 15 min (the same incubation period allowed for an uptake blocker in the uptake assay). Nonradioactive competitor concentrations were as indicated above for uptake inhibition. Nonspecific binding was assessed by addition of 10 μM mazindol except when mazindol was the drug tested, in which case 50 μM cocaine was used to assess nonspecific binding. For the membrane binding experiment, membranes of CHO cells stably transfected with WT or D79E DAT cDNAs were prepared by suspending 2.5×10^8 cells in 40 ml of 10 mM HEPES, pH 8.0, 50 mM $MgCl_2$ buffer. The suspension was processed in a Polytron homogenizer (Kinematica AG, Basel, Switzerland), and the homogenate was centrifugated for 20 min at 48,000*g* (all steps at 4°C). After protein quantitation, membranes were resuspended in KRH buffer and combined with 2 to 20 nM [^{3}H]WIN 35,428 and increasing concentrations of nonradioactive WIN 35,428 for 60 min at 22°C. Membranes were aspirated with a Brandel harvester onto GF/B glass fiber filter paper, washed three times with 5 ml of ice-cold KRH, and the filters were measured for radioactivity via liquid scintillation counting. For all binding assays, data were analyzed with the Prism 2.0 software to obtain K_d, K_i, and B_{max} values.

Results

Site-directed mutagenesis of the rat DAT D79 residue was employed to define features of the TM1 aspartic acid side chain important for both dopamine uptake and recognition of drugs that inhibit uptake. Substitution with glutamic acid (D79E), asparagine (D79N), or alanine (D79A) addressed the importance of a negative charge, hydrogen-bonding potential and side-chain length at this position. Transiently transfected COS-7 cells were screened for the ability to take up 10 nM [^{3}H]dopamine (Fig. 2). No specific uptake was detected for cells bearing the D79A or D79N DAT mutants. Levels of [^{3}H]dopamine incorporation by cells bearing the D79A or D79N DAT mutants did not differ appreciably from that for cells transfected with the pIRES vector alone. D79E-transfected cells, in contrast, clearly exhibited [^{3}H]dopamine uptake, albeit at a level well below that of cells bearing "wild-type" (wild-type + N-terminal epitope tag) DAT.[1] The inability of D79A DAT to transport dopamine could not be accounted for by poor surface expression (Fig. 3a), suggesting that a negative charge, hydrogen bonding potential, a longer side chain, or a combination of these is required at position 79 of DAT for dopamine uptake. The total D79N DAT expression level was equal to or better than the other DAT proteins, but its surface expression was relatively low—sufficiently lower than that of D79E DAT to account for the functional differ-

[1] The N-terminally epitope-tagged DAT was pharmacologically indistinguishable from wild-type DAT (data not shown). The introduced epitope was not employed in the experiments reported within, because antisera directed against an authentic DAT N-terminal epitope proved superior for the biotinylation studies.

ence between these mutants. It was therefore not possible to infer that a negatively charged side chain at position 79 is critical for dopamine transport (the failure of D79A DAT may be caused by the shorter side chain or the lack of H-bonding potential). Extracellular biotinylation of the exclusively intracellular β-actin protein was not detected with an anti-actin monoclonal antibody at intact cells, and was observed only for cell lysates (data not shown), meaning that the immunoreactivity of DAT proteins could not be explained by intracellular biotinylation due to cell permeation with biotin.

To more thoroughly characterize D79E DAT relative to WT DAT, stably transfected CHO-K1 cells were prepared. Biotinylation experiments again established that WT and D79E DAT proteins were present at the cell surface, but total, intracellular, and cell surface expression of D79E DAT were approximately half that of WT DAT (Fig. 3b). The kinetics of [^{3}H]dopamine uptake were determined for each stable cell line (Fig. 4). The WT DAT turnover rate in CHO cells was in agreement with that reported for hDAT in oocytes (Sonders et al., 1997) and rDAT in LLC-PK cells (Gu et al., 1994), and 7-fold higher than that for D79E DAT CHO cells (Table 1). Half of the V_{max} decrease at D79E DAT cells could be attributed to fewer transporter proteins at the cell surface, the latter evidenced by the biotinylation data (Fig. 3b) and supported by the B_{max} values for [^{3}H]WIN 35,428 binding (Table 1). Thus, the D79E mutation rendered a decrease of approximately 4-fold in DAT dopamine turnover rate (Table 1). A decrease in transport capacity was also observed for the analogous TM1 SERT mutant D98E (Barker et al., 1999).

On the other hand, this mutation had little effect on the K_m value for dopamine uptake (Fig. 4 and Table 2). Because this value cannot be equated with an affinity constant, dopamine affinity was assessed by its ability to displace [^{3}H]WIN 35,428 binding at WT and D79E DAT. (Binding affinity K_i values for all other substrates and inhibitors in the study were also assessed in this way, and this K_i value will hereafter be referred to as the "binding affinity" of the ligand for WT or D79E DAT.) By this measure, dopamine displayed an almost identical binding affinity for WT and D79E DAT proteins (Fig. 5 and Table 2). This result may be inconsistent with the idea that the D79 residue is the governing element of the DAT dopamine-binding pocket because the added steric bulk of the glutamate side chain would leave less room for dopamine (assuming that dopamine does not adopt an energetically unfavorable conformation to accommodate the mutation). To further address this steric issue, DHBA was employed as a potential DAT substrate. This compound is identical to dopamine except for the absence of one of two methylene groups between the amino and dihydroxyphenyl groups. If the governing DAT-dopamine point of contact were

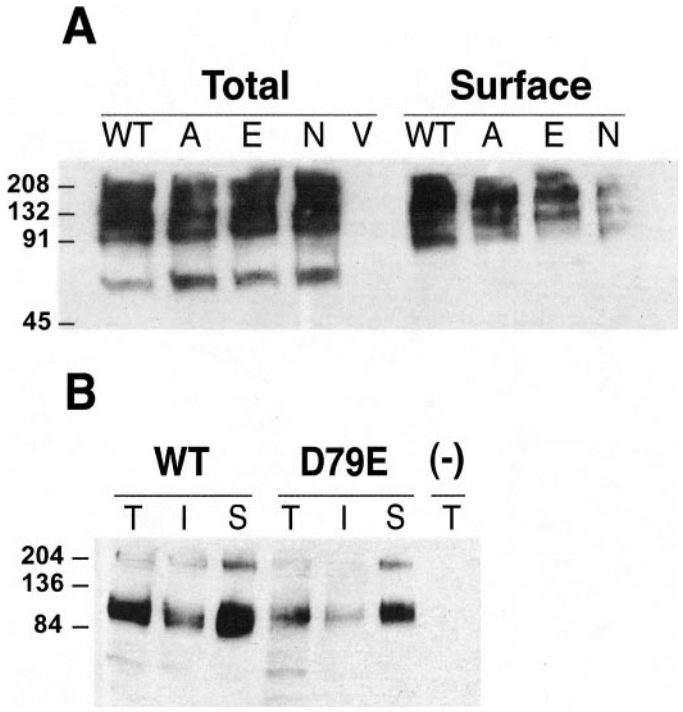

Fig. 3. Assessment of cell surface expression of WT and mutant DAT proteins. A, COS-7 cells transiently expressing WT, D79A, D79E, or D79N DAT were subjected to a biotinylation agent and processed as described under *Materials and Methods*. DAT protein was visualized after Western transfer with the aid of a DAT-selective antibody directed against an endogenous amino acid sequence of the N terminus. No DAT immunoreactivity was observed for the total cell lysate of cells transfected with the plasmid vector alone (V). A triplet of immunoreactive surface bands was observed, presumably corresponding to mature (fully glycosylated) multimer (>200 kDa), mature monomer (~120 kDa), and an immature monomer (~95 kDa). Total lanes additionally contain a band corresponding to a ~70-kDa polypeptide, presumably the unglycosylated monomer. Quantitation of bands was achieved using scanning densitometry, using exposures shorter than those shown in the figure to ensure that the scan was in the linear range of the film. In the figure, the total and surface band densities (expressed as a percentage of WT) were 94 and 76% for D79A DAT, 107 and 61% for D79E DAT, and 114 and 16% for D79N DAT, respectively. B, CHO-K1 cells stably-transfected with WT or D79E DAT were similarly biotinylated, and DAT immunoreactivity of lysates determined. Total cell lysate (T), the intracellular fraction of the lysate (I), and the biotinylated and affinity-purified cell surface fraction (S) were applied to the polyacrylamide gel. Total cell lysates of untransfected CHO cells (–) displayed no DAT immunoreactivity. The major band indicated an apparent molecular mass of 94 kDa, presumably the mature monomer. A much weaker band of ~200 kDa is evident, consistent with a DAT dimer. The apparent molecular masses of both COS- and CHO-expressed mature monomers are in agreement with published values (Patel, 1997). In the figure, the total, intracellular, and surface band densities (expressed as a percentage of WT) for D79E DAT were 51, 36, and 48% respectively. A representative result of three to six experiments is shown.

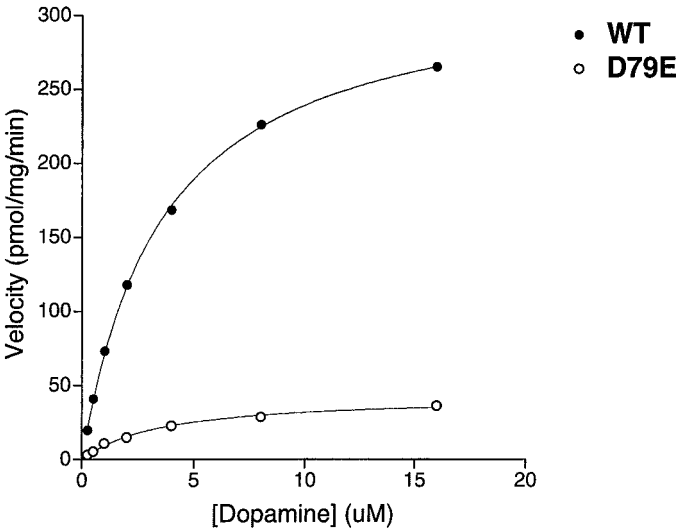

Fig. 4. WT and D79E DAT dopamine uptake saturation kinetics. CHO cells stably transfected with WT (●) or D79E (○) DAT were incubated with increasing concentrations of a fixed ratio of [^{3}H]dopamine: nonradioactive dopamine. A representative result of three separate experiments is shown.

TABLE 1

Kinetics of dopamine uptake by CHO cells stably expressing WT or D79E DAT

V_{max} values were derived from experiments incubating the stably transfected cells with dopamine at 0.1 Ci/mmol for 5 min at 22°C. B_{max} values were derived from [^{3}H]WIN 35,428 saturation binding. Values are presented as mean ± S.E. for three independent experiments.

	WT DAT	D79E DAT
V_{max} (pmol/mg/min)	348 ± 22	48 ± 4[a]
B_{max} (pmol/mg)	10.2 ± 0.4	6.1 ± 0.6
V_{max}/B_{max}	34.1/min	7.9/min

[a] $P < 0.05$ versus WT DAT (Student's t test).

TABLE 2

Effect of DAT substrates on CHO cells stably expressing WT or D79E DAT

K_m and K_i values were derived from experiments incubating the stably transfected cells with nonradioactive substrates in the presence of [^{3}H]dopamine or [^{3}H]WIN 35,428 at 22°C in KRH buffer. Values are presented as mean ± S.E. for three to six independent experiments.

	K_m or K_i	
	WT DAT	D79E DAT
	μM	
[^{3}H]DA Uptake		
Dopamine	1.7 ± 0.3	2.3 ± 0.3
DHBA	5760 ± 540[a]	782 ± 22[a,b]
Norepinephrine	5.4 ± 0.5[a]	5.2 ± 0.3[a]
m-Tyramine	1.4 ± 0.3	1.2 ± 0.3[a]
p-Tyramine	1.7 ± 0.1	3.3 ± 0.4[b]
(+)-Amphetamine	0.5 ± 0.1	2.4 ± 0.3[b]
(−)-Amphetamine	2.8 ± 0.5	4.2 ± 0.5[a]
[^{3}H]WIN Inhibition		
Dopamine	2.1 ± 0.2	2.5 ± 0.4
DHBA	801 ± 22[a,c]	791 ± 27[a]
Norepinephrine	2.3 ± 0.5[c]	5.5 ± 0.7[a,b]
m-Tyramine	1.0 ± 0.2[a]	6.6 ± 1.2[a,b,c]
p-Tyramine	0.8 ± 0.1[a,c]	4.3 ± 0.5[b]
(+)-Amphetamine	0.4 ± 0.1[a]	3.7 ± 0.8[b]
(−)-Amphetamine	2.0 ± 0.2	8.1 ± 0.5[a,b,c]

[a] $P < 0.05$ versus dopamine for the given assay and DAT construct (via Student's t test).
[b] $P < 0.05$ versus WT DAT for that assay (via Student's t test).
[c] $P < 0.05$ for WIN inhibition versus dopamine uptake at the same DAT construct (via Student's t test).

at D79 and the role of the D79 residue were simply to form an interaction with the amino group of dopamine, the extra methylene group in the glutamic acid side chain would exactly compensate for the shorter DHBA ligand. Binding and uptake of DHBA by D79E DAT should thus be as efficient as dopamine binding and uptake at WT DAT and superior to that of dopamine at D79E DAT. This was not the case; DHBA binding affinities were identical at WT and D79E DAT, and DHBA remained a quite inferior inhibitor of [^{3}H]dopamine uptake (Fig. 5 and Table 2). The potency of DHBA in inhibiting [^{3}H]dopamine uptake was 7-fold higher at D79E DAT compared with WT DAT. Still, this K_i value for DHBA at D79E DAT was 460-fold higher than the dopamine K_m value at WT DAT and 377-fold higher than the K_i value for dopamine binding at WT DAT. The DHBA K_i value for [^{3}H]dopamine uptake inhibition at D79E DAT agrees well with that for inhibition of [^{3}H]WIN 35,428 binding; curiously, the [^{3}H]dopamine uptake inhibition potency of DHBA at WT DAT was 7-fold less than its apparent affinity for inhibiting [^{3}H]WIN 35,428 binding. Ligand binding assays were conducted under conditions that matched those for assaying uptake inhibition.

Other dopamine analogs assessed the effect of adding substituents to the methylene groups or of altering the catechol hydroxyl groups (Table 2). Norepinephrine (addition of a β-OH group to dopamine) was equally effective at WT and D79E DAT in inhibiting dopamine uptake but was 2-fold less effective at displacing [^{3}H]WIN 35,428 at D79E DAT than at WT DAT. The dopamine uptake inhibition potencies of *m*- or *p*-tyramine (each lacking one of the catechol OH groups) were unchanged or 2-fold lower as a result of the D79E mutation, respectively, yet the mutation decreased binding affinities for both tyramines by more than 5-fold. Of the DAT substrates tested, the D79E mutation most dramatically affected the dopamine uptake inhibition potency (5-fold loss) and binding affinity (9-fold loss) of the more psychoactive isomer of amphetamine (lacking both catechol OH groups and adding an α-methyl group), *S*(+)-amphetamine. For *R*(−)-amphet-

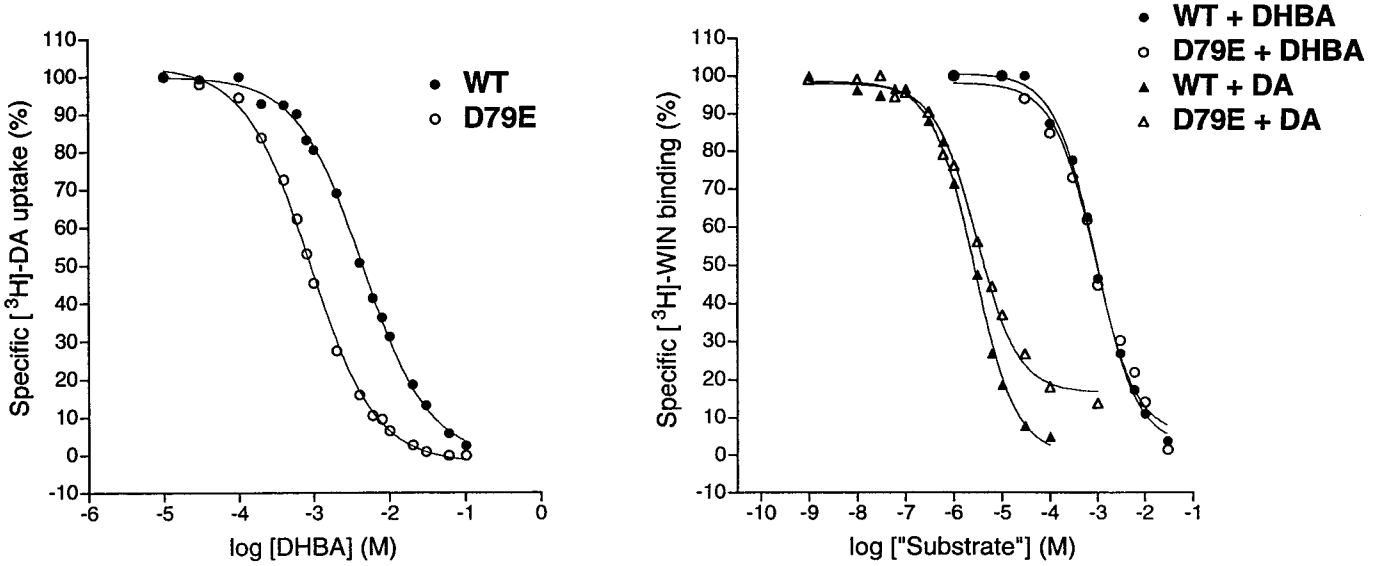

Fig. 5. Dopamine or DHBA inhibition of [^{3}H]dopamine uptake (left) or [^{3}H]WIN 35,428 binding (right) under identical conditions at CHO cells stably transfected with WT or D79E DAT. The data are representative of at least three independent experiments. Because intact naive CHO cells possess a low-affinity site ($K_d > 1$ μM) for [^{3}H]WIN 35,428, it was not possible to completely displace radioligand specific binding unless very high concentrations of inhibitor were employed.

amine, however, dopamine uptake inhibition potency was not significantly affected by the mutation.

The dopamine uptake inhibition potencies of the classic uptake inhibitors cocaine, WIN 35,428, mazindol, and methylphenidate were compared at WT and D79E DAT cells. Of the four inhibitors, only the dopamine uptake inhibition potency of cocaine was altered (2-fold decrease) in a statistically significant fashion by the mutation (Fig. 6 and Table 3). Binding affinities for each inhibitor were also determined, using intact cell monolayers and under conditions essentially identical to those for dopamine uptake assays. All inhibitors were shown to reach equilibrium with WT or D79E DAT within 15 min; K_i values did not differ from those obtained after 60- or 120-min binding assays (data not shown). For all four inhibitors, the potency of inhibition of [^{3}H]WIN 35,428 binding matched the potency for inhibition of [^{3}H]dopamine uptake at D79E DAT, as would be expected if occupancy of the [^{3}H]WIN 35,428 binding site prevented substrate translocation. In contrast to the D79E mutant, inhibitory potencies derived from uptake assays and binding assays did not match for intact cells stably expressing WT DAT: WIN 35,428, mazindol, methylphenidate, and cocaine were 3- to 4-fold more potent as inhibitors of [^{3}H]WIN 35,428 binding than as inhibitors of [^{3}H]dopamine uptake (Fig. 6 and Table 3). These results suggest the existence of a conformational state or binding site in WT DAT with high affinity for WIN 35,428 that is not detectable in D79E DAT.

Interestingly, specific binding of [^{3}H]WIN 35,428 at D79E DAT was undetectable using either suspended cells (the monolayer was scraped from the plate) or a cell membrane preparation in the KRH uptake buffer, whereas [^{3}H]WIN 35,428 binding affinity at WT DAT was unaffected by these

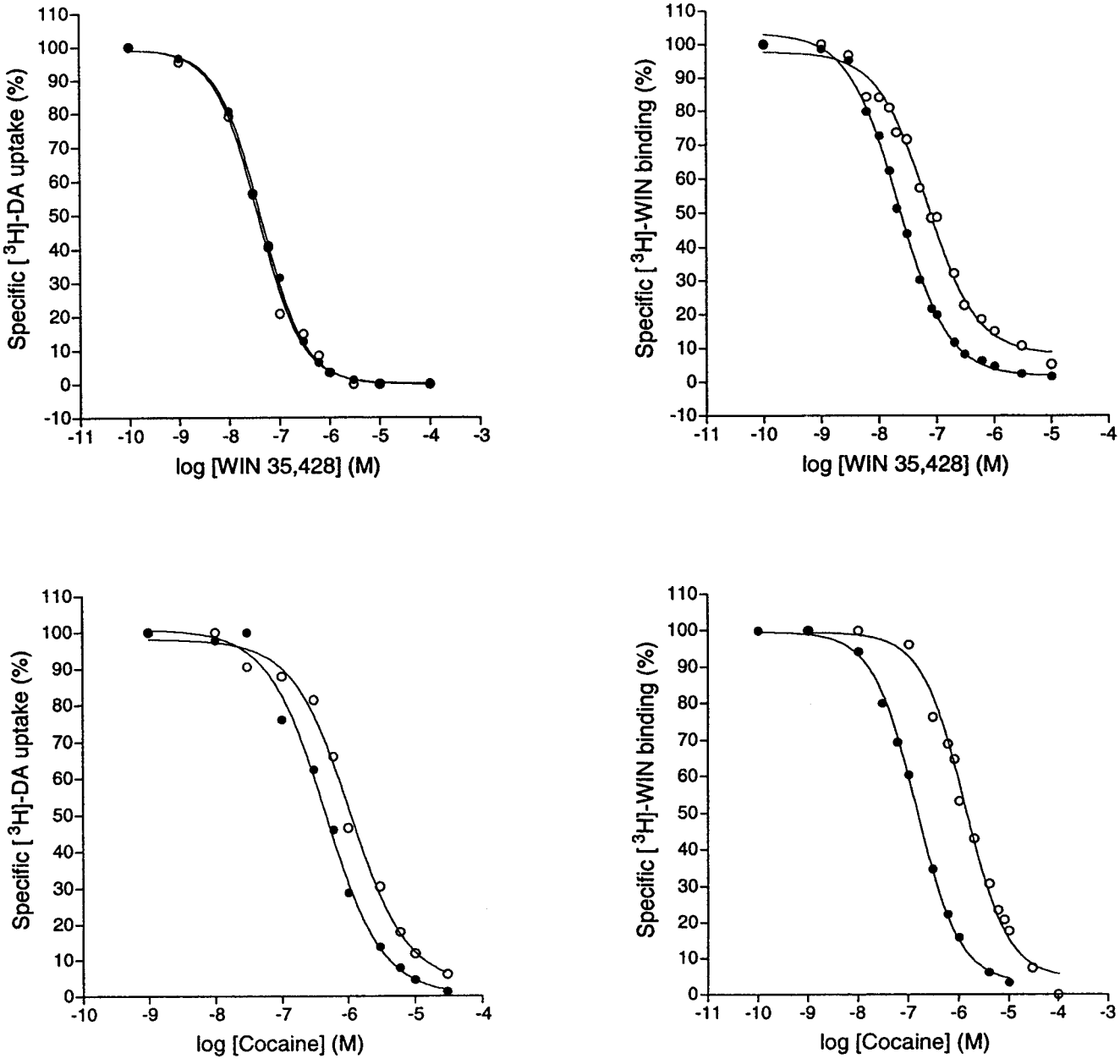

Fig. 6. WIN 35,428 (top) or cocaine (bottom) inhibition of [^{3}H]dopamine uptake (left) or [^{3}H]WIN 35,428 binding (right) under identical conditions at CHO cells stably transfected with WT DAT (●) or D79E DAT (○). The data are representative of at least three independent experiments.

conditions (K_d = 20 ± 1, 19 ± 1, and 18 ± 4 nM for attached, suspended, and membrane-prepared WT DAT CHO cells, respectively). No appreciable specific uptake of [^{3}H]dopamine was observed for suspended cells possessing either transporter protein (data not shown).

The results with WT DAT cells suggested that a high-affinity binding site for uptake inhibitors may have been masked in the dopamine uptake assay. To search for two inhibitor sites at WT DAT, 24-point concentration curves were employed for cocaine inhibition of [^{3}H]dopamine uptake. A reproducible second WT DAT site was indeed suggested from visual inspection of the curve (but was not sufficiently defined for GraphPad analysis), and this site seemed to be in the vicinity of the 128 nM K_i value previously measured for high-affinity binding. To a lesser extent, D79E DAT also occasionally displayed this high-affinity cocaine site (Fig. 7). Similarly, DHBA typically displayed a mild deviation from the monophasic uptake inhibition curve for WT DAT that was consistent with its measured higher affinity (800 μM) binding site (data not shown).

Discussion

The initial focus of the study was to test the premise that the DAT D79 residue serves as the governing point of contact for dopamine via an ionic interaction. Our results do not rule out such an interaction, but the fact that the DAT D79E mutant was most sensitive to modifications of the substrate catechol moiety may suggest that this portion of dopamine is more likely to be recognized by a binding site defined in part by D79. In the course of the work, a second and more surprising implication was that the WT DAT site, conformation, or population responsible for higher affinity binding of [^{3}H]WIN 35,428 was not principally responsible for the observed inhibition of dopamine uptake.

Based on models for dopamine interaction with its G protein-coupled receptor, related GPCR findings, and DAT mutagenesis, the negatively charged carboxylate moiety of the D79 DAT residue has been postulated to contact the positively charged amino group of dopamine (Kitayama et al., 1992). In agreement with Kitayama et al. (1992), we observed that of several substitutions at the D79 position, the glutamic acid mutation was least deleterious to dopamine transport via DAT. However, we did not detect an appreciable difference in the dopamine K_m values at WT and D79E DAT (Table 2), contrasting with the previously observed 4-fold increase in K_m value at the mutant. Dopamine affinity was assessed by inhibition of [^{3}H]WIN 35,428 binding and was again found not to differ between WT and D79E DAT in a statistically-significant fashion (Table 2).

Experiments with DHBA, identical to dopamine except shorter by a methylene group, more directly addressed the role of the D79 residue in substrate recognition. The 7-fold increase in dopamine uptake inhibition potency observed for DHBA resulting from the D79E mutation was hardly an indication that the putative D79-substrate ion pair had been re-established, because it did not significantly approach the increase of more than 3300-fold needed to meet the level of dopamine transport by WT DAT. Moreover, DHBA affinities at WT and D79E DAT were identical (Table 2). This 7-fold increase in dopamine uptake inhibition potency for the shorter DHBA molecule may reflect some alleviation of substrate steric hindrance created by the D79E substitution, without necessarily involving an interaction between substrate amino group and D79 carboxylate. It is possible that the residue contributes to the tertiary structure of the transporter or even formation of the dopamine binding pocket without actually contacting dopamine. During the initial phase of our experiments, a study involving the analogous TM1 aspartic acid residue of SERT was reported (Barker et al., 1999). In concert with the present DAT results, only the SERT glutamic acid substitution at this position (D98E) displayed detectable substrate uptake, and K_m values for serotonin uptake were essentially the same for wild-type and D98E SERT. The mutation effected a compensatory 12-fold affinity increase for a "shorter" serotonin analog employed to accommodate the longer glutamic acid side chain. By itself, the result might suggest that an ionic bond forms between D98 and serotonin; however, serotonin was equipotent at wild-type and D98E SERT. It is conceivable that serotonin contacts the D98E side chain, but it should be noted that a less dramatic mutation-induced gain-of-function was required to approximate the potency of a "full-length" serotonin analog at wild-type SERT compared with the potency difference of more than 3000-fold between dopamine and DHBA at WT DAT. Still, it must be acknowledged that of all the DAT ligands tested in our study, only DHBA displayed an increase in dopamine uptake inhibition potency as a result of the D79E mutation.

The possibility that D79 is involved in recognition of some feature of the substrate catechol ring moiety was also tested. The dopamine uptake inhibition potencies for the tyramines and (−)-amphetamine at WT versus D79E DAT indicated, however, that loss of one or both catechol hydroxyl groups was essentially tolerated by D79E DAT. Binding affinities for these three substrates, on the other hand, were reduced severalfold by the mutation. The result suggests that one DAT conformation or population is more sensitive to alterations in the substrate catechol moiety than is a second DAT conformation/population. As explained in the Introduction, the aromatic moiety of dopamine would be the most logical substrate pharmacophore to show dependence on the D79 DAT residue. It should be noted that after correcting for

TABLE 3

Effect of classic DAT inhibitors on CHO cells stably expressing WT or D79E DAT

K_d (WIN 35,428 only) and K_i values were derived from experiments incubating the stably-transfected cells with nonradioactive substrates in the presence of [^{3}H]dopamine or [^{3}H]WIN 35,428 at 22° C in KRH buffer. Values are presented as mean ± S.E. for three to six independent experiments. Note that values are nanomolar, whereas those in Table 2 are micromolar.

	K_d or K_i	
	WT DAT	D79E DAT
	nM	
[^{3}H]DA uptake		
WIN 35,428	74 ± 17	71 ± 12
Cocaine	555 ± 29	1126 ± 32[a]
Mazindol	31 ± 3	47 ± 4
Methylphenidate	152 ± 20	161 ± 9
[^{3}H]WIN inhibition		
WIN 35,428	20 ± 1[b]	68 ± 4[a]
Cocaine	128 ± 5[b]	1066 ± 84[a]
Mazindol	13 ± 1[b]	41 ± 3[a]
Methylphenidate	57 ± 3[b]	167 ± 2[a]

[a] $P < 0.05$ versus WT DAT for that assay (via Student's t test).
[b] $P < 0.05$ for WIN inhibition versus dopamine uptake at the same DAT construct (via Student's t test).

differences in surface protein levels, D79E DAT displayed a 4-fold lower turnover number (V_{max}/B_{max}) than WT DAT (Table 1). Thus, the role of the D79 side chain in dopamine transport would seem to be more complex than merely contributing to the dopamine binding site.

The D79E mutation had no effect on the dopamine uptake inhibition potency of WIN 35,428, mazindol, or methylphenidate, and cocaine potency was decreased by only half. The binding affinity for each inhibitor was assessed under conditions identical to those employed in the dopamine uptake inhibition assay, and binding affinity constants matched those for uptake inhibition for D79E DAT. Curiously, this correlation was not seen for WT DAT; binding affinities were higher than dopamine uptake inhibition potencies for all inhibitors tested (Fig. 6 and Table 3). Moreover, D79E DAT did not display significant [^{3}H]WIN 35,428 affinity unless cells bearing this protein were attached to the culture plate as a monolayer. The requirement for an attached cell monolayer for [^{3}H]WIN 35,428 binding at D79E DAT is currently not understood. It is possible that the "off rate" of WIN 35,428 binding at this mutant protein is too rapid for detection with glass-fiber vacuum filtration. Another possibility is that scraping the CHO cells disrupts a DAT association with either another protein, e.g., phosphatase 2A, syntaxin-1A, PICK1, or α-synuclein (Bauman et al., 2000; Deken et al., 2000; Lee et al., 2001; Torres et al., 2001), or itself, as an oligomer (Kilic and Rudnick, 2000; Hastrup et al., 2001), and that such an association is required for 20 nM [^{3}H]WIN 35,428 binding.

Our results suggest that for each uptake inhibitor tested there exists a discrete site, conformation, or population for WT DAT that exhibits "higher" or "lower" binding affinity for the drug. The D79E mutation largely eliminates the high-affinity component (Fig. 8). Considering that the uptake inhibition potencies for three of four inhibitors 1) were insensitive to the mutation and 2) matched the binding affinities at D79E DAT, it appears that the lower affinity component for each drug at WT DAT was principally responsible for blocking translocation of dopamine into the cell. It is understandable why the [^{3}H]WIN 35,428 binding curve for WT DAT did not display 2 obvious phases. In binding displacement assays, use of a low concentration of radioligand favors detection of the site with highest affinity, at the expense of lower affinity sites. Thus, the apparently monophasic WT DAT curve corresponding to the 20 nM K_d value may be a weighted average between the lower affinity component and a component with an affinity greater than 20 nM.

On the other hand, such explanations do not account for the disparity between uptake inhibition potency and binding affinity observed for all inhibitors tested at WT DAT. Because the gap between these two values was greatest for cocaine, this drug was chosen for 24-point uptake inhibition experiments that sought to detect a high-affinity component. A second phase of the WT DAT curve corresponding to the high-affinity value was frequently present using this assay and occasionally present, to a lesser extent, in the D79E DAT curve (Fig. 7). Although visually apparent, this phase was not sufficiently prominent to be detected with the regression analysis. The WT DAT population that recognizes inhibitors with higher affinity may be a poorer transporter of dopamine than the population containing the lower affinity inhibitor binding site (Fig. 8). This explanation accounts for the underrepresentation of the "high-affinity" DAT population in the [^{3}H]dopamine uptake inhibition assay but not in the [^{3}H]WIN 35,428 binding assay. Interestingly, syntaxin-1A modulates GABA uptake via direct contact with the GABA transporter (Deken et al., 2000); it is possible that association of DAT with a cytoskeletal protein (or another DAT molecule) modulates uptake and at the same time creates the higher affinity binding site for classic inhibitors. Alternatively, it cannot be ruled out that the "high-affinity" DAT population is a minority for WT DAT and nearly undetectable in the case of D79E DAT.

The lack of correlation between cocaine potencies for bind-

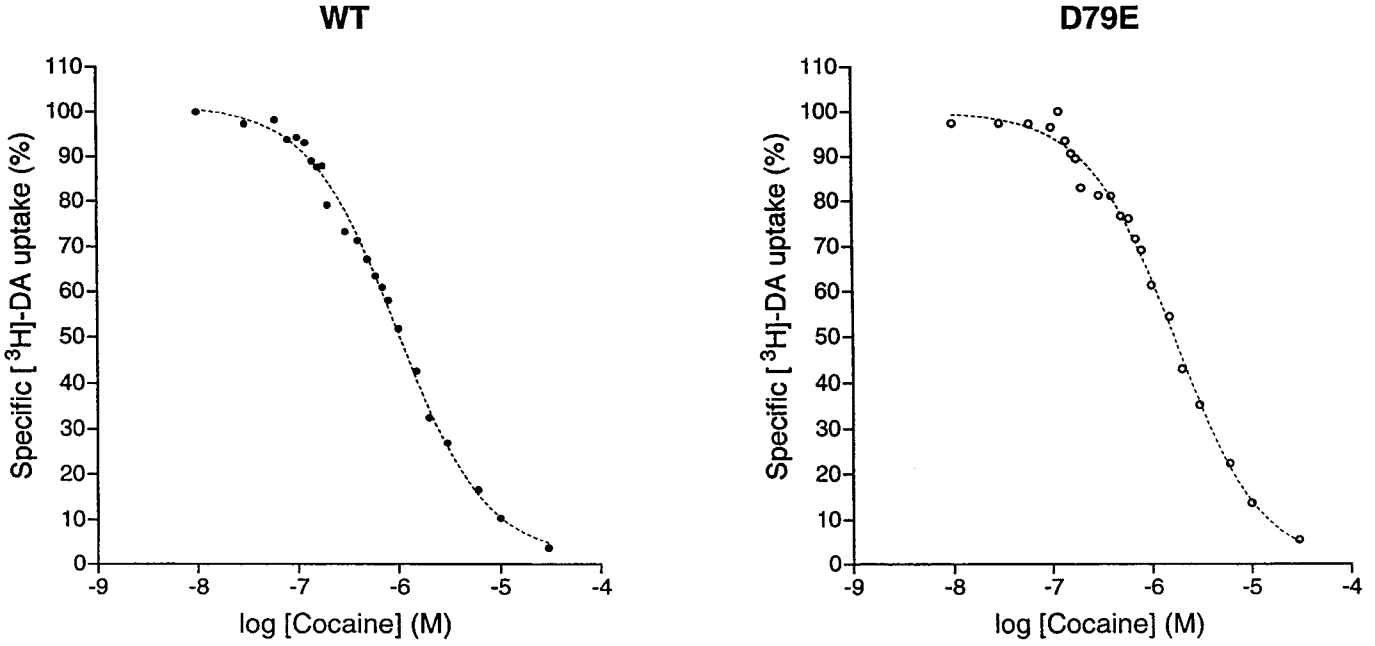

Fig. 7. Cocaine 24-point curves for inhibition of [^{3}H]dopamine uptake by CHO cells stably transfected with WT (●) or D79E (○) DAT. The dashed line indicates a one-site regression fit. The data are representative of three independent experiments.

ing and uptake inhibition at WT DAT has been noted previously. Using COS cells transiently-transfected with DAT, Pristupa et al. (1994) reported K_i values of 240 and 743 nM for cocaine binding and uptake inhibition, respectively, numbers very close to our own. Using DAT-transfected human embryonic kidney 293 cells, Eshleman et al. (1999) observed a 2- to 3-fold greater cocaine potency for uptake inhibition than for binding. Literature values for dopamine uptake inhibition potency of cocaine range from 149 nM (Eshleman et al., 1999) to 137,000 nM (Reith and Coffey, 1994); this variance seems to be dependent on cell/tissue type and experimental conditions. Again, DAT interactions with DAT binding proteins or DAT post-translational modifications are among possibilities that may be relevant to the differences in cocaine potency. We have observed cocaine dopamine uptake inhibition potency to fluctuate within a 3-fold range as a function of cell state when employing CHO cells stably transfected with WT DAT (C. K. Surratt, in preparation).

Our findings may be of great relevance to ongoing efforts in the development of anti-cocaine medications. Despite decades of investigation, the search for a therapy against dependence on cocaine has been largely unsuccessful. The principal focus in the development of potential anti-cocaine medications has been to inhibit high-affinity cocaine or WIN 35,428 binding while attempting to spare dopamine uptake. Our finding that the potency of high-affinity inhibitor binding to WT DAT diverges from that for inhibition of dopamine uptake suggests that development of a "cocaine antagonist" therapeutic should not be focused on blocking high-affinity cocaine binding. The D79E DAT mutant may thus serve as an important diagnostic tool in the development of a clinically useful cocaine antagonist. Future experiments will attempt to further define the dopamine uptake inhibition DAT site for various classic inhibitors, and will include mapping the cocaine and WIN 35,428 binding sites of D79E DAT using the substituted cysteine accessibility method (Ferrer and Javitch, 1998). Localizing drug binding sites relevant to dopamine uptake inhibition should markedly enhance the prospects for computer-aided rational design of anti-cocaine medications.

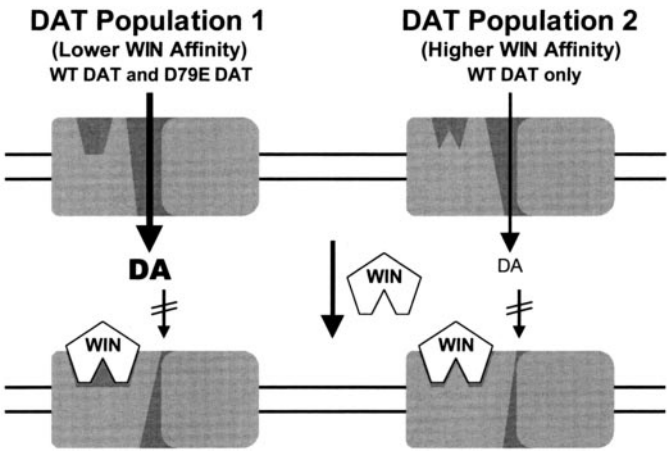

Fig. 8. A scenario consistent with the observed dissociation of high-affinity WIN 35,428 binding and dopamine uptake inhibition at DAT. Two DAT populations (rectangular structures) are represented at the cell surface, within the plasma membrane (parallel horizontal lines). The large majority of [^{3}H]dopamine uptake is proposed to be mediated by DAT population 1; population 2 is thus under-represented with respect to total uptake of the dopamine radiotracer. Population 1 displays a lower affinity (~70 nM) for [^{3}H]WIN 35,428 (WIN, inscribed in quasi-pentagonal structure) than does population 2 (~ 20 nM); Population 1 is thus under-represented with respect to total binding of the WIN 35,428 radiotracer. Higher levels (e.g., > 500 nM) of WIN 35,428 (or other DAT inhibitors employed in the study) thoroughly eliminate dopamine uptake by both DAT populations, but the uptake inhibition curves reflect actions principally at population 1 (the primary [^{3}H]dopamine conduit). The D79E mutation eliminates DAT population 2, eliminating the higher affinity binding of DAT inhibitors but affording little or no effect on their dopamine uptake inhibition potencies.

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