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A Human Serotonin Transporter Mutation Causes Constitutive Activation of Transport Activity

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ABSTRACT

A rarely occurring variant of human serotonin transporter (hSERT) was tested for its functional consequences in HeLa and COS-7 cells. The variant, in which Ile-425 is converted to Val, was significantly different from wild type with respect to its catalytic properties. In both cell types, rates of serotonin (5-HT) transport were higher for the I425V variant. Both an increase in V_{max} and a decrease in K_M caused this increase in rate. The increase in V_{max} was not accounted for by increases in transporter expression or in the distribution of transporter between the cell surface and intracellular pools. The decrease in K_M was accompanied by a decrease in the K_D for binding of the cocaine analog 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane. In both HeLa and COS-7 cells, the nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine increased the activity of wild-type hSERT to that of the variant but did not change the activity of the I425V variant. This stimulation was prevented by the presence of oxyhemoglobin, which quenches nitric oxide, and by an inhibitor of guanylyl cyclase.

Serotonin transporter (SERT) is responsible for accumulation of serotonin (5-HT) by neurons, platelets, and other cells. Inhibitors that prevent 5-HT reuptake into serotonergic neurons increase synaptic 5-HT and have been used to treat a variety of neuropsychiatric disorders, including affective disorder, anxiety disorders, obsessive-compulsive disorder (OCD), and autism (Murphy et al., 1998; Stahl, 1998; Jones and Blackburn, 2002). Because physiological modulation of SERT activity also is expected to influence synaptic 5-HT, endogenous mechanisms of SERT regulation have been the object of considerable study. Investigations in cellular model systems have revealed such mechanisms as increases in message levels induced by staurosporine and forskolin (Ramamoorthy et al., 1995), retrieval from the plasma membrane in response to activators of protein kinase C (Anderson and Horne, 1992; Miller and Hoffman, 1994; Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy and Blakely, 1999), and activation through stimulation of cGMP-dependent protein kinase (PKG) (Miller and Hoffman, 1994). Although these regulatory processes might be important in modulating serotonergic neurotransmission, they previously have not been shown to be physiologically relevant.

Human SERT (hSERT) is a 630-amino acid plasma membrane protein that is believed to traverse the membrane 12 times. Site-directed chemical labeling experiments have determined that residues predicted to lie in each of the external loop domains of SERT are accessible to reagents added from the external medium (Chen et al., 1998), and each of the predicted internal loops has been localized to the cytoplasmic surface of the protein (Androutsellis-Theotokis et al., 2001; Androutsellis-Theotokis and Rudnick, 2002). Previous results have suggested that the transmembrane domains of SERT are likely to contain residues associated with the binding site for 5-HT. External loop domains 1, 2, 3, and 6 have been shown not to determine substrate or inhibitor selectivity (Smicun et al., 1999). In contrast, residues in the first and third transmembrane domains (TM1 and TM3) have been proposed as possible substrate binding determinants (Adkins et al., 2001; Barker et al., 1998, 1999; Chen et al., 1997; Chen and Rudnick, 2000).

A recent search for sequence variants in the *SLC6A4* gene encoding SERT uncovered 15 variants in genomic DNA from a population of 450 persons in the DNA Polymorphism Discovery Resource (Collins et al., 1998). Of these, six were synonymous and the remaining nine resulted in an amino acid change. One of these nine, an isoleucine-to-valine con-

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ABBREVIATIONS: SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine, serotonin; OCD, obsessive-compulsive disorder; PKG, cyclic GMP-dependent protein kinase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SNAP, *S*-nitroso-*N*-acetylpenicillamine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate; β-CIT, 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane; TM, transmembrane domain.

version at position 425, was also discovered in a screen of patients with serotonin-related neuropsychiatric disorders (Ozaki et al., 2003). Persons in two unrelated families who were heterozygous for the I425V variant exhibited symptoms of OCD plus Asperger's syndrome or anorexia nervosa and other disorders (Ozaki et al., 2003). Isoleucine is absolutely conserved at this position in all mammalian SERTs, and the change from Ile to Val is therefore likely to represent a recent mutation rather than a persistent polymorphism. Here we show that the I425V mutation constitutively activates SERT and prevents modulation of its activity by PKG.

Experimental Procedures

Materials. Human cervical epithelioid carcinoma (HeLa) and COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA). pBluescript II SK+ was from Stratagene (La Jolla, CA), and pcDNA3 was from Invitrogen (Carlsbad, CA). Oxyhemoglobin was prepared as described by Ignarro et al. 1987) and was a generous gift from Dr. William Sessa. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were from Sigma-Aldrich (St. Louis, MO).

Site-Directed Mutagenesis and Expression of SERT. hSERT I425V was generated using the QuikChange site-directed mutagenesis kit (Stratagene). We designed an oligonucleotide primer (5′AAGAAGATGACGGCAAAGAAAGTCGACGCTGGCATGT-3′) that introduced valine into hSERT at position 425. After the QuikChange kit protocol, the mutated region was excised by digestion with *Bsa*BI and *Bgl*II and ligated into pBluescript II SK+ containing wild-type hSERT cDNA digested with *Bsa*BI and *Bgl*II. The resulting hSERT I425V plasmid was transformed into XL1-Blue supercompetent *Escherichia coli* cells (Stratagene), and colonies were screened by digestion with diagnostic restriction enzymes. Once mutant colonies were identified, the plasmid DNA was isolated and sequenced through the subcloned region.

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells plated in 24-well culture plates (~100,000 cells per well) were transfected with recombinant VTF7-3 vaccinia virus encoding T7 RNA polymerase as described previously (Blakely et al., 1991). Transfected cells were incubated for 16 to 20 h at 37°C before they were used for transport or immunoprecipitation experiments. Protein concentration was determined with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

For expression in COS-7 cells, hSERT and hSERT I425V were subcloned into the *Kpn*I/*Xba*I site of the mammalian expression vector pcDNA3. This construct placed SERT expression under control of the cytomegalovirus and T7 promoters suitable for expression in both HeLa cells using the vaccinia-T7 system and in COS-7 cells. COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% streptomycin at 37°C in a humidified 5% CO_2 environment. For uptake experiments, 100,000 cells per well were plated in 24-well culture plates (Falcon Plastics, Oxnard, CA) and transfected with hSERT or I425V cDNA using 100 ng/well and a 5:1 ratio of Lipofectin to DNA. Medium containing DNA and Lipofectin was removed 18 h after transfection and replaced with complete DMEM. Cells were assayed for uptake 48 h later. For cell-surface biotinylation experiments, cells (400,000 per well) in six-well culture plates were transfected as above with hSERT or I425V cDNA using 2 mg of DNA per well.

Western Blotting. Cells in a 10-cm (diameter) dish were collected by scraping into phosphate-buffered saline (PBS) (Sambrook et al., 1989) containing 1 mM phenylmethylsulfonyl fluoride [freshly prepared in acetone/ethanol (1:1)] and 2 mM EDTA, washed with the same buffer, and resuspended in 400 μl of PBS containing 0.44% SDS, 2 μg/ml DNase I, 1 mM phenylmethylsulfonyl fluoride, and 2 μl of protease inhibitor mixture (consisting of 5 mg/ml final concentra-

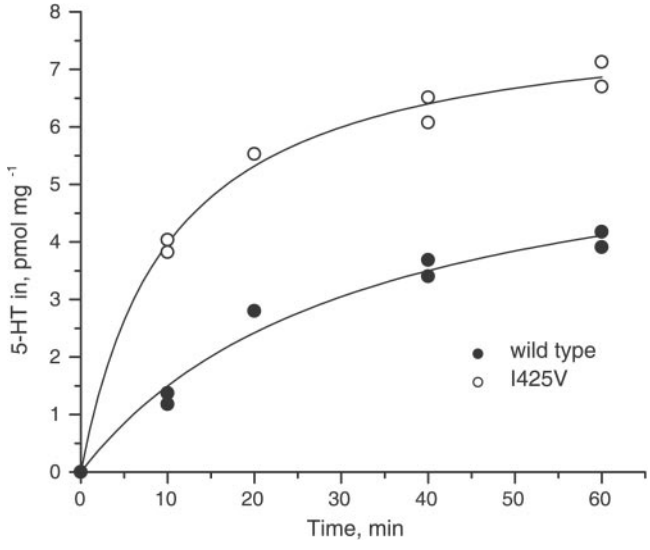

Fig. 1. Enhanced 5-HT transport by hSERT I425V relative to wild type. HeLa cells transiently expressing wild-type (●) and I425V mutant (○) transporters were assayed for [^{3}H]5-HT uptake at 10, 20, 40, and 60 min as described under *Materials and Methods*. Background accumulation of [^{3}H]5-HT was measured in the same experiment using untransfected cells and subtracted from each experimental value.

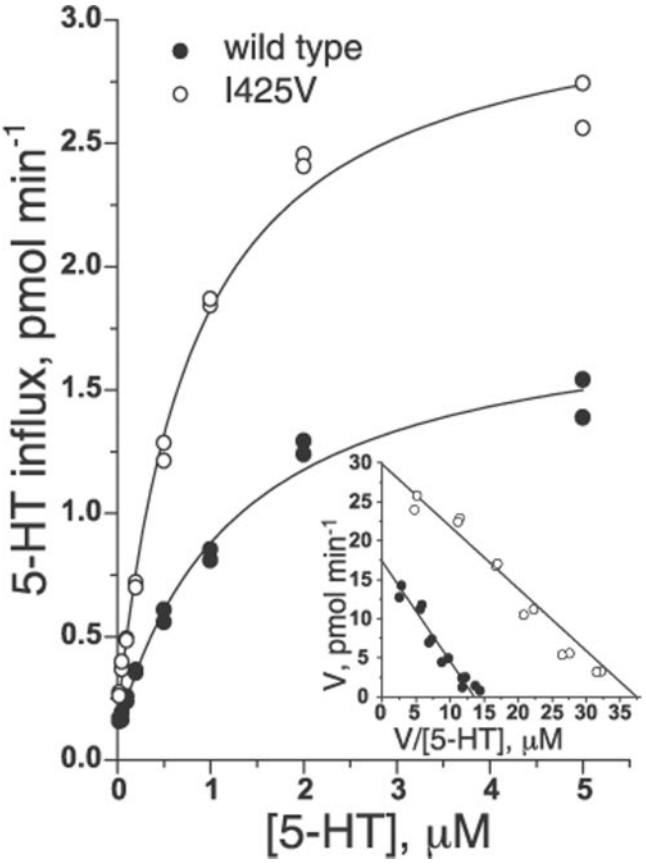

Fig. 2. Substrate dependence of transport in hSERT and mutant I425V. Initial rates of 5-HT influx were measured over the indicated range of 5-HT concentrations using 20 nM [^{3}H]5-HT with added unlabeled 5-HT to the final concentration. K_M and V_{max} values were determined by fitting the rate versus concentration data. The inset shows an Eadie-Hofstee plot of the data with lines drawn from the derived kinetic constants. See Table 1 for a summary of the calculated data.

tions of leupeptin, pepstatin A, chymostatin, bestatin, antipain, and aprotinin). The suspension was sonicated and mixed with 200 μl of Laemmli 3× sample buffer containing 0.7 M β-mercaptoethanol and was separated by 9% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The gel was transferred to a nitrocellulose membrane by the procedure of Towbin et al. (1979), and SERT was detected (Harlow and Lane, 1988) using anti-human polyclonal antibody 48 (diluted 1:2500), first described by Bauman et al. (2000) (a kind gift from Dr. Randy Blakely, Vanderbilt University, Nashville, TN). The signal was visualized by using an enhanced chemiluminescence Western Blotting detection system (Pierce). Immunoblots were quantitated by using an IS-1000 system (Alpha Innotech, San Leandro, CA) .

Cell Surface Biotinylation. Cell surface expression of the transporters was determined using the membrane-impermeant biotinylation reagent NHS-SS-biotin (Pierce) by a modification of the procedure of Gottardi et al. (1995) as described previously (Kilic and Rudnick, 2000). Briefly, cells were labeled with NHS-SS-biotin, the excess reagent was quenched, and the cells were solubilized. Cell surface proteins were isolated from the cell extract with immobilized streptavidin, and transporter was detected in the pool of surface proteins by gel electrophoresis and Western blotting using anti-hSERT antibody (Bauman et al., 2000). Immunoblots were quantitated using an Alpha Innotech IS-1000. Experiments were performed in triplicate and repeated in two to three separate assays.

Transport and Binding Measurements. Transport of [^{3}H]5-HT was measured by adding 250 ml of PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ containing 20 nM [^{3}H]5-HT (PerkinElmer Life Sciences, Boston, MA) to each well and incubating for 10 min at 22°C. Reactions were terminated by aspiration of the substrate and rapid washing three times with ice-cold PBS. Cells were lysed with 250 ml of 1% SDS, and the well contents were transferred to scintillation vials for counting. The protein concentration was determined from parallel wells by using the Micro BCA protein assay reagent kit (Pierce). All uptake measurements were corrected by subtracting the blank values measured in the presence of 100 mM cocaine. Binding of the high affinity cocaine analog 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane (β-CIT), was measured as described previously (Kilic and Rudnick, 2000). Results are from triplicate samples and were repeated in two to three separate experiments.

Immunocytochemistry. Normal or transfected COS-7 cells were plated on coverslips at 50% confluence (in six-well culture plates) and grown for 2 days. Cells were then rinsed with PBS, fixed for 10 min in methanol, and stored in PBS at 4°C. After rehydration for 5 min in PBS, the cells were permeabilized for 15 min in PBS plus 0.3% Triton X-100 and 0.1% bovine serum albumin (permeabilization buffer) and blocked for 30 min in goat serum dilution buffer [16% goat serum (Sigma), 0.3% Triton X-100, 20 mM sodium phosphate, pH 7.4, and 0.45 M NaCl]. The cells were then incubated for 1 h in goat serum dilution buffer with polyclonal rabbit anti-hSERT antibody 50 (1:200 dilution) (Bauman et al., 2000). After three 5-min washes with permeabilization buffer, fluorescent (fluorescein isothiocyanate) goat anti-rabbit IgG (VectorLaboratories, Burlingame, CA) were added to the cells at 1:100 dilution and incubated for 1 h. At the end of the incubation, the cells were again washed three times with permeabilization buffer and once with 5 mM sodium phosphate, pH 7.5, for 5 min. Coverslips then were mounted onto slides with Vectashield (Vector Laboratories). Immunofluorescence was ob-

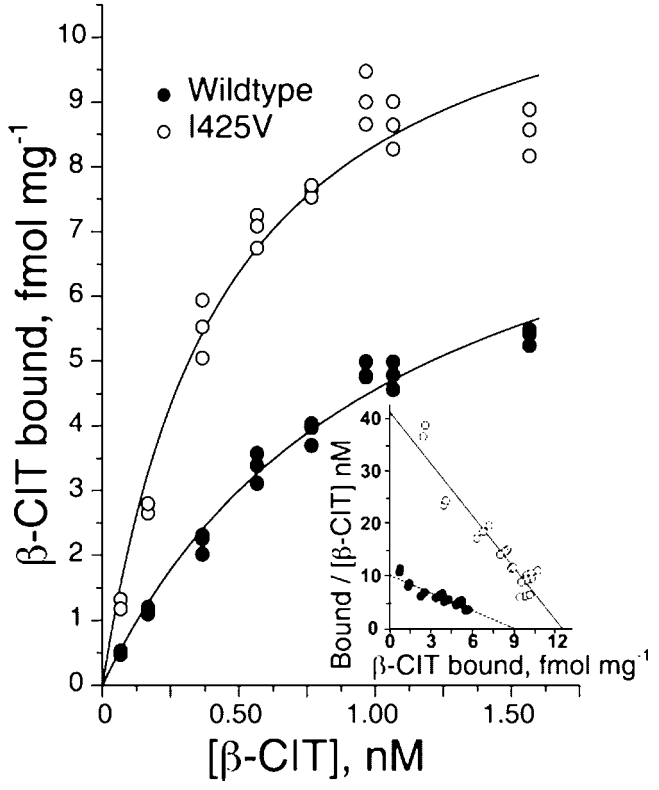

Fig. 3. Inhibitor binding to hSERT and mutant I425V. Equilibrium binding of β-CIT was measured after 1 h of incubation with 0.03 nM [^{125}I]β-CIT containing unlabeled β-CIT to the final indicated concentration. K_D and B_{max} values were determined by fitting the binding versus concentration data. The inset shows a Scatchard plot of the data with lines drawn from the derived binding constants. See Table 1 for a summary of the calculated data.

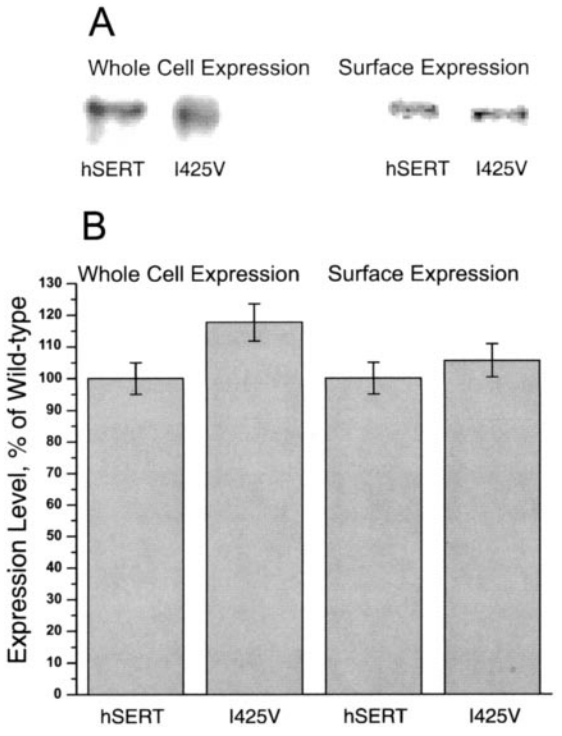

Fig. 4. Whole-cell and surface expression of hSERT and mutant I425V. Standard curves for quantitation of hSERT and I425V mutant expression were prepared as described previously (Kilic and Rudnick, 2000). For whole-cell expression, HeLa cells expressing hSERT or I425V were lysed, their proteins were separated by polyacrylamide gel electrophoresis and visualized by Western blot analysis, and the integrated density values were determined by densitometry. For cell surface expression, cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads and then separated and visualized as above. The results of Western blot analysis are shown above a summary of combined data from three densitometric scans. The small shift in position between the wild-type and I425V mutant bands was infrequently observed and is unlikely to indicate a difference in electrophoretic mobility.

served and photographed with a Zeiss Axiophot epifluorescence photomicroscope (Zeiss, Welwyn Garden City, UK).

Data Analysis. Nonlinear regression fits of experimental and calculated data were performed with Origin (OriginLab Corp, Northampton, MA), which uses the Marquardt-Levenberg nonlinear least-squares curve-fitting algorithm. Each figure shows a representative experiment that was performed at least twice. The statistical analysis given in text was from multiple experiments, with data analyzed using analysis of variance with Tukey's post hoc tests and unpaired *t* tests as appropriate using StatView software (SAS Institute, Cary, NC). Data with error bars represent the mean ± S.D. for triplicate samples.

Results

Kinetic Characterization of hSERT I425V Mutant. To determine whether the I425V mutation had altered functional activity, we generated the same mutation in a pBlueScript plasmid carrying the hSERT cDNA. The mutated cDNA was used to transfect HeLa cells infected with the vTF7-3 strain of vaccinia virus (Blakely et al., 1991). In this system, bacteriophage T7 RNA polymerase, encoded by the virus, transcribes hSERT cDNA, which follows the T7 promoter sequence of the plasmid. As shown in Fig. 1, the rate of 5-HT accumulation by cells expressing the I425V mutant was greater than that of cells expressing wild-type hSERT. The difference was most pronounced at the earliest time points.

To determine the reason for this increased 5-HT influx, we measured the transport rate over a range of 5-HT concentrations for wild type and I425V. The results in Fig. 2 demonstrate that the increased transport by I425V was maintained over a wide range of substrate concentrations and that high substrate concentrations did not eliminate the difference between wild type and mutant. These data were analyzed by fitting to hyperbolic saturation kinetics, yielding a difference in K_M and V_{max} values of 0.62- and 1.72-fold, respectively. The mutant transporter demonstrated a higher maximal rate and saturated at a lower 5-HT concentration than the wild type. This is shown graphically in the Eadie-Hofstee plot shown as an inset to Fig. 2. The line representing mutant protein intersected the *y*-axis at a higher value (V_{max}) than that of wild type, and had a less-negative slope ($-K_M$).

Differences in Ligand Binding. The effect of the I425V mutation on substrate transport kinetics could be caused by changes in the absolute number of transporters, turnover rate, ligand binding affinity, or a combination of these factors. As a first step in evaluating possible changes, we measured equilibrium binding of the high-affinity cocaine analog β-CIT to membranes prepared from cells expressing I425V and wild-type hSERT. The results, shown in Fig. 3, demonstrate that binding was enhanced in the mutant relative to the wild type. A fit of these data demonstrated that there was a modest increase (20–40%) in the maximal number of binding sites (B_{max}) and a much larger decrease (3-fold) in the dissociation constant, indicating higher affinity binding to the mutant. These changes are reflected in the Scatchard plot shown as an inset to Fig. 3. The line representing the mutant intersected the *x*-axis at a higher value (B_{max}) than wild type and had a steeper negative slope ($-1/K_M$) than wild type. The small increase in the number of binding sites did not account for the large increase in V_{max} shown in Fig. 2.

Cell Surface Localization. An additional possible explanation for the increased V_{max} is that the distribution of transporters between cell surface and intracellular locations might have been different for the mutant and wild-type proteins. To test this possibility, we treated cells expressing hSERT and the I425V mutant with the membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin to label proteins on the cell surface. The cells were solubilized and biotinylated proteins were extracted using streptavidin-agarose. An antibody against hSERT was used to determine the relative abundance of mutant and wild-type proteins by quantitative Western blot analysis. No significant differences were detected between the surface expression of the mutant and wild-type proteins (Fig. 4), with only a minor difference in whole-cell expression. Relative to wild type, the level of whole-cell expression for I425V was 116%. The lack of increased surface expression in the mutant suggested that most of the increase in V_{max} of I425V represents an increase in the average rate of transport per molecule of cell surface transporter.

Expression in COS-7 Cells. To examine the possibility that the differences observed between wild-type and I425V hSERT might be artifacts caused by the preparation of plasmid DNA or the expression system, we subcloned the wild-type and mutant cDNAs into another vector, pcDNA3. Because this vector contains both T7 and CMV promoters, as well as the simian virus 40 promoter and origin of replica-

TABLE 1

Kinetic and binding characteristics of hSERT wild-type and I425V mutant in HeLa and COS-7 cells

Cells were grown to confluence on 24-well plates. Three transport experiments performed with confluent cells on 48-well plates each gave similar results; average values are shown. Transport and binding measurements were performed as described under *Materials and Methods*. K_M and V_{max} values and their associated uncertainties were obtained by nonlinear regression analysis.

	Rate	K_m	V_m	K_D,	B_{max}
	pmol/min/mg	*μM*	*pmol/min/mg*	*nM*	*fmol/mg*
HeLa BS					
Wild type	0.131 ± 0.010	1.28 ± 0.19	1.73 ± 0.06	1.07 ± 0.13	9.47 ± 0.62
Mutant	0.267 ± 0.005***	0.80 ± 0.09*	2.98 ± 0.05***	0.44 ± 0.06**	12.0 ± 0.6**
pcDNA3					
Wild type	0.136 ± 0.006				
Mutant	0.271 ± 0.019***				
No DNA	0.022 ± 0.001				
COS-7					
Wild type	0.416 ± 0.007	1.04 ± 0.51	1.37 ± 0.56	0.58 ± 0.11	2.6 ± 0.33
Mutant	0.851 ± 0.151**	0.88 ± 0.14NS	2.98 ± 0.17**	0.32 ± 0.04*	3.7 ± 0.24 **
No DNA	0.064 ± 0.002				

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS, not significant.

tion, it serves as a good expression vector in both the vaccinia-T7 system and in COS-7 cells. The pcDNA3 plasmids were tested in both HeLa cells and in COS-7 cells, and results obtained are summarized in Table 1. The difference in transport rate observed with the pBlueScript plasmids (Fig. 1) was also found with the pcDNA3 plasmids when expressed both in HeLa and COS-7 cells (Table 1). Moreover, the increases in V_{max} and B_{max} and the decrease in K_M and K_D observed for transport and binding in HeLa cells were also found in COS-7 cells (Table 1).

Immunocytochemistry. Because the kinetics and equilibrium binding behavior of the I425V mutant differed from that of wild-type hSERT, we examined the possibility that its distribution within the cell might also differ. Using an antibody that recognizes the C-terminal region of hSERT, we permeabilized and stained COS-7 cells expressing both wild-type and I425V. The results showed no consistent difference in the distribution between intracellular and cell surface locations (data not shown).

Effect of Nitric Oxide Donors. SERT endogenously expressed in rat basophilic leukemia cells is known to be subject to up-regulation by a nitric oxide-dependent pathway (Miller and Hoffman, 1994). Figure 5 shows that hSERT expressed in COS-7 cells is also activated by this pathway. Addition of the nitric oxide donor SNAP increased the activity of heterologously expressed hSERT to approximately twice the control value in the absence of SNAP. In different cultures or passages of HeLa and COS-7 cells, the extent of this stimulation varied from 50 to 100%. However, the stimulated wild type activity was, in each case, near the activity of the I425V mutant. Maximal activity was obtained with SNAP concentrations of 100 μM. We sometimes observed inhibition at higher concentrations. In contrast, the I425V mutant was not affected by SNAP. Figure 6 shows that the stimulation by SNAP was eliminated by addition of 8 μM oxyhemoglobin, a scavenger of nitric oxide (Fig. 6A), or by 1 μM ODQ, a selective inhibitor of nitric oxide-sensitive guanylyl cyclase (Fig. 6B). Neither of these agents had a marked effect on the activities of wild-type hSERT or the I425V mutant, but each of them blocked the stimulation of hSERT activity by SNAP.

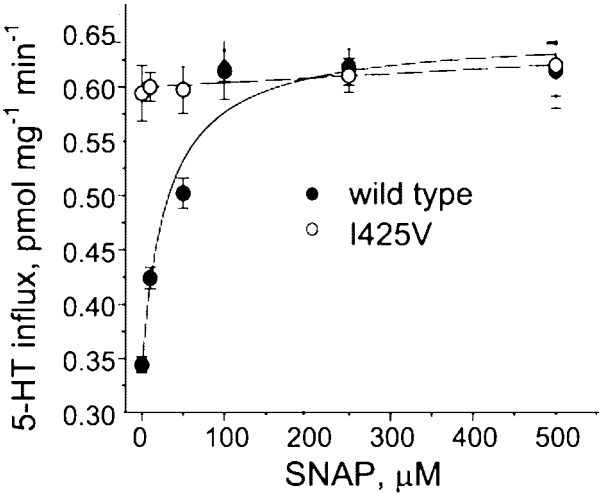

Fig. 5. Effect of SNAP on transport activity of hSERT and mutant I425V. COS-7 cells expressing hSERT (●) or mutant I425V (○) were incubated with the indicated concentration of SNAP for 10 min, at which time transport was initiated by addition of [^{3}H]5-HT to a final concentration of 20 nM. Transport was measured in a 10-min incubation in the continued presence of SNAP. Similar results were obtained using HeLa cells expressing hSERT and I425V.

Discussion

The results presented here demonstrate that the I425V mutation of hSERT, which was found to be associated with multiple behavioral disorders in humans, increased the transport activity of this protein. The increase was found to result from a defect in regulation of hSERT. Wild-type hSERT activity was stimulated by addition of SNAP (which decomposes in solution to liberate nitric oxide) as described previously for rat SERT in RBL cells (Miller and Hoffman, 1994), but I425V was unaffected by SNAP addition (Fig. 5). Because the enhanced activity of wild type hSERT in the presence of SNAP was roughly equal to that of I425V, we propose that the mutation may constitutively activate SERT I425V in the same way that nitric oxide stimulates wild-type hSERT.

The increased activity of the I425V mutant was not merely a result of increased expression or more efficient delivery to the cell surface. Direct measurements of cell surface expression demonstrated similar levels relative to wild-type transporter. Moreover, the K_M for 5-HT decreased in this mutant, consistent with a change in its fundamental properties rather than a difference in expression level. An additional indication that the I425V mutation alters fundamental properties of the transporter is the increased affinity for β-CIT observed in equilibrium binding experiments. B_{max} values for β-CIT binding to I425V were 20 to 40% higher than to wild-type hSERT (Table 1). This is at least partly a reflection of the increased overall level of expression for the mutant. Taken together, these results point to a direct effect on binding and kinetics of hSERT because of the mutation.

The mechanism by which the I425V mutation constitutively activates SERT is presently unknown. Earlier results from studies of cysteine scanning mutagenesis suggested that TM3 contained residues associated with the binding site for 5-HT and cocaine (Chen et al., 1997). TM1 has also been postulated to contain residues that determine substrate and

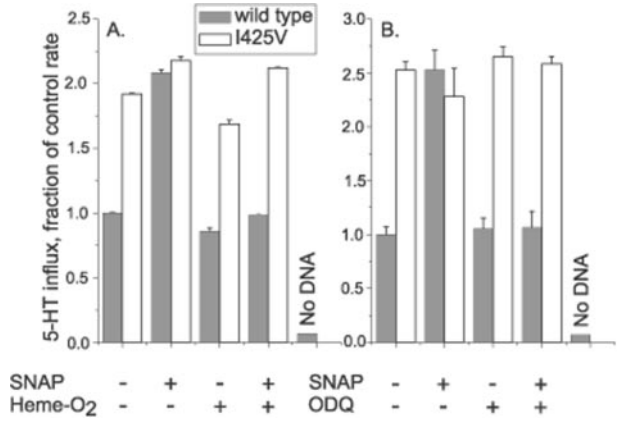

Fig. 6. Oxyhemoglobin and ODQ block the effect of SNAP on hSERT. HeLa cells expressing hSERT or I425V were preincubated, where indicated, with 100 μM SNAP and 8 μM oxyhemoglobin or 1 μM ODQ. After a 10-min preincubation, transport was initiated by addition of [^{3}H]5-HT to a final concentration of 20 nM. Transport was measured in a 10-min incubation in the continued presence of SNAP, oxyhemoglobin, and ODQ.

inhibitor selectivity (Barker et al., 1998, 1999) as has TM12 (Barker and Blakely, 1996). From its location in TM8, Ile-425 may contribute to the 5-HT binding site or translocation pathway, and replacement of the endogenous isoleucine with valine may result in a change that mimics the effect of nitric oxide observed in the wild type. It is also possible that the mutation affects the level of phosphorylation of SERT by PKG or its interaction with another phosphoprotein. Given the location of Ile-425 in a transmembrane domain, it is not immediately obvious how a modification there could affect its interaction with a soluble kinase or phosphatase, although interaction with another integral membrane protein might be influenced by the mutation.

Rat SERT was previously shown to be stimulated by adenosine A_3 receptor agonists in RBL cells (Miller and Hoffman, 1994). Inhibitors of nitric-oxide synthase or of cGMP-dependent protein kinase blocked the stimulation, and the effect was mimicked by addition of SNAP in the absence of A_3 receptor agonists. It was proposed that A_3 receptor activation stimulated rat SERT indirectly by activating nitric oxide synthesis, leading to cGMP production and cGMP-dependent protein kinase phosphorylation of SERT (Miller and Hoffman, 1994). The observation that ODQ blocked the SNAP effect in our experiments (Fig. 6) suggests that even in this heterologous expression system, nitric oxide effects on SERT are mediated through cGMP. In contrast with our data and those from RBL cells, evidence from studies with synaptosomes suggested inhibition of 5-HT uptake by SNAP and sodium nitroprusside (Pogun et al., 1994; Asano et al., 1997). Therefore, the same stimulus, possibly even the same phosphorylation of SERT, might have opposite effects, depending on the cell type. Other mechanisms that have been described for SERT regulation involve increased synthesis (Cool et al., 1991) and altered cell surface expression (Qian et al., 1997; Ramamoorthy and Blakely, 1999). It is possible that other mechanisms of regulation in synaptosomes, such as internalization of SERT, were responsible for decreased activity in response to SNAP, rather than the increase in activity observed here and in RBL cells (Miller and Hoffman, 1994).

This is the first example of a naturally occurring mutation in the SERT coding sequence that affects the transporter's functional activity. The association of the I425V mutation with symptoms of OCD, Asperger's syndrome, and anorexia nervosa suggests that altered regulation of hSERT can lead to profound behavioral changes. It is particularly interesting that persons who carry this mutation are refractory to treatment with selective serotonin reuptake inhibitors (Ozaki et al., 2003). Most patients suffering from these disorders do not carry the I425V mutation. It is possible that the benefits of selective serotonin reuptake inhibitors in these patients depend on their ability to regulate SERT activity by the pathway disrupted in I425V.

Acknowledgments

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