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Serotonin Transporter Phosphorylation by cGMP-Dependent Protein Kinase Is Altered by a Mutation Associated with Obsessive–Compulsive Disorder

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Human serotonin transporter (hSERT) activity expressed in HeLa cells was stimulated by agents that release nitric oxide, stimulate soluble guanylyl cyclase, or activate cGMP-dependent protein kinase (PKG). This stimulation was blocked by a PKG inhibitor. A naturally occurring mutation, I425V, associated with obsessive–compulsive disorder and other neuropsychiatric disorders, activated hSERT and eliminated stimulation via the PKG pathway. Inhibitors of soluble guanylyl cyclase or PKG decreased activity of the I425V mutant, but not wild type, indicating that both wild-type and mutant transporters could exist in both high and low activity forms. Mutation of Thr-276 in the fifth transmembrane domain (TM5) to alanine or aspartate prevented activation of wild-type hSERT through the PKG pathway and also blocked the inhibition of I425V activity by inhibitors of the pathway. The accessibility of positions in TM5 near Thr-276 was modified in T276D, but not in I425V. These results are consistent with the hypothesis that PKG phosphorylates hSERT at Thr-276 and increases its activity by modifying the substrate permeation pathway formed, in part, by TM5. The effect of the I425V mutation may shift the balance of hSERT toward the phosphorylated form, possibly by interfering with the action of a phosphatase. However, association of hSERT with protein phosphatase 2A was not decreased in the I425V mutant.

Key words: **serotonin; transporter; obsessive–compulsive disorder; phosphorylation; cGMP; mutation**

Introduction

Human serotonin transporter (hSERT) is responsible for terminating the action of serotonin [5-hydroxytryptamine (5-HT)] released by neurons and for regulating the level of extracellular 5-HT near the synapse (Murphy et al., 2004). Drugs that inhibit hSERT are widely used to treat depression and other disorders, including obsessive–compulsive disorder (OCD) (Murphy et al., 1998; Stahl, 1998; Jones and Blackburn, 2002). Recent evidence suggests that in SERT, the cytoplasmic half of the fifth transmembrane domain (TM5) contributes to the pathway through which 5-HT diffuses between its binding site to the cytoplasm (Zhang and Rudnick, 2006).

SERT is regulated by multiple signal transduction pathways dependent on cAMP, cGMP, calmodulin, p38 mitogen-activated protein (MAP) kinase, and protein kinase C (PKC) (Ramamoorthy et al., 1993; Jayanthi et al., 1994; Miller and Hoffman, 1994; Qian et al., 1997; Zhu et al., 2004a, 2005; Samuvel et al., 2005). Zhu et al. (2004a,b) found that activation of SERT in RBL cells by adenosine receptor agonists was blocked not only by inhibitors of adenosine A_3 receptors and cGMP synthesis but also by an inhibitor of p38 MAP kinase. They also measured increased cell-surface SERT expression with A_3 agonists that were blocked by a cGMP-dependent protein kinase (PKG) inhibitor (Zhu et al., 2004b) but not by a p38 MAP kinase inhibitor (Zhu et al., 2004a). They inferred that A_3 receptor activation increased SERT surface expression through PKG and increased SERT catalytic activity through p38 MAP kinase.

Miller and Hoffman (1994) enumerated 13 potential SERT phosphorylation sites for proline kinase, PKC, PKG, cAMP-dependent protein kinase, casein kinase I, and glycogen synthase kinase 3, including a PKC and PKG site, Ser-277, in TM5 near intracellular loop 2 (IL2). This site is absolutely conserved within biogenic amine transporters. Recent evidence indicates that the adjacent threonine-276 is phosphorylated in response to PKG activation (Ramamoorthy et al., 2007).

A small subset of patients with OCD carry a mutated form of hSERT that is also associated with anorexia nervosa and Asperger syndrome (Ozaki et al., 2003; Delorme et al., 2005). This I425V mutant was shown to transport 5-HT faster than wild-type hSERT, although surface expression of I425V was indistinguishable from that of wild type (Kilic et al., 2003) (but see Prasad et al., 2005). *S*-Nitroso-*N*-acetylpenicillamine (SNAP), which breaks down spontaneously to form nitric oxide (NO), increased the activity of wild type, but not the I425V mutant, and that stimulation was blocked by inhibitors of

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soluble guanylyl cyclase and by oxidized hemoglobin, which quenches NO (Kilic et al., 2003).

These results suggest that activation of the cGMP pathway, possibly through PKG, is responsible for increasing hSERT transport activity and that the I425V mutation mimics activation by cGMP. It is not clear from the previous work, however, whether activation resulted from direct phosphorylation of hSERT or indirectly. Also unclear was the nature of hSERT activation by the I425V mutation. The work presented here addresses both of these issues and provides a rationale for PKG activation of SERT.

Materials and Methods

Materials. Early-passage HeLa cells (CCL-2) were obtained from American Type Culture Collection (Manassas, VA). Recombinant VTF7–3 vaccinia virus encoding T7 RNA polymerase was prepared as described previously (Blakely et al., 1991). SNAP, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP), 8-bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP), and 5-HT were purchased from Sigma (St. Louis, MO). 3-(5′-Hydroxymethyl-2′-furyl)-1-benzylindazole was from Calbiochem (San Diego, CA). 8-(4-Chlorophenylthio)guanosine 3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-pCPT-cGMPS) was from Alexis (San Diego, CA). [^{3}H]5-HT (27.1 Ci/mmol) was purchased from PerkinElmer (Boston, MA). The expression plasmid for human vasodilator-stimulated phosphoprotein (VASP) pCDNA3-VSV-VASP and 16C2 mouse monoclonal antibody against phospho-VASP (Smolenski et al., 1998) were generous gifts from Dr. S. Lohmann (Institute for Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Würzburg, Germany).

Mutagenesis. Wild-type hSERT and the I425V mutant were described previously (Kilic et al., 2003). hSERT T276A, T276D, and T276E were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). After introduction of the desired mutations into pBluescript II SK$^+$ containing wild-type hSERT, the mutated region was excised by digestion with *Age*I and *Bgl*II and subcloned individually back into the original pBluescript-hSERT (T276A, T276D, and T276E) or pBluescript-I425V construct (double mutants, T276A/I425V, and T276D/I425V) via *Age*I/*Bgl*II restriction sites. SERT cysteine mutants K272C through A285C were described previously (Zhang and Rudnick, 2005a). Each of these mutants is in the X5C background (C15A/C21A/C109A/C357I/C622A) in which we replaced the five endogenous cysteine residues known to react with MTS (methane thiosulfonate) reagents (Androutsellis-Theotokis and Rudnick, 2002). Additionally, these mutants also contained, where indicated, I425V or T276D mutations. All mutations were screened by restriction mapping and confirmed by DNA sequencing.

Expression of hSERT. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells plated in either 24- or 96-well culture plates (100,000 cells per well) were infected with recombinant VTF7–3 virus and transfected with plasmid bearing hSERT cDNA under control of T7 promoter. Transfected cells were incubated for 18–20 h at 37°C with 5% CO_2 and used for the transport assay. Protein concentration was determined with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

Biotinylation. Cell-surface proteins were labeled at 4°C with the membrane-impermeant biotinylation reagent sulfo-NHS-SS-biotin (Pierce), as described previously (Chen et al., 1998). Briefly, cells expressing wild-type hSERT or mutants were incubated with sulfo-NHS-SS-biotin in 20 mM HEPES, pH 8.6, 2 mM $CaCl_2$, and 150 mM NaCl for 20 min; the excess reagent was quenched with glycine; and the cells were dissolved in SDS-lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% SDS, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% protease inhibitor mixture (Sigma)]. Cell-surface proteins were isolated from the cell extracts with immobilized streptavidin, and the transporter was detected in the pool of surface proteins by SDS-PAGE and Western blotting using SERT-specific SR-12 antibody. A horseradish peroxidase-conjugated anti-rabbit IgG was used to visualize the signal by Super Signal West Femto (Pierce).

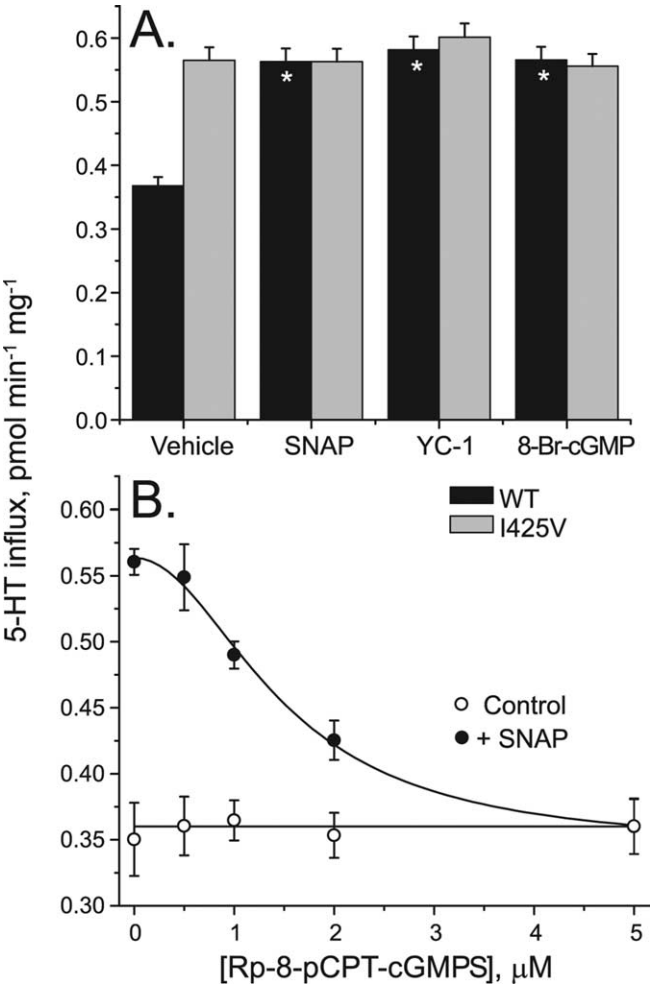

Figure 1. PKG activators stimulate wild-type hSERT but not I425V. ***A***, HeLa cells expressing wild-type hSERT (WT; black bars) or the I425V mutant (gray bars) were preincubated at room temperature with 100 μM SNAP for 15 min, 10 μM YC-1 for 30 min, or 100 μM 8-Br-cGMP for 30 min in PBS/CM, at which time transport was initiated by the addition of [^{3}H]5-HT to a final concentration of 20 nM. The incubation was continued in the presence of SNAP, YC-1, or 8-Br-cGMP for 10 min. [^{3}H]5-HT uptake was determined as described in Materials and Methods. Combined results of six separate experiments are shown. The asterisks indicate significant differences between transport activity after treatment with vehicle alone and the addition of the PKG pathway activator ($p < 0.01$, paired Student's *t* tests). ***B***, Rp-8-pCPT-cGMPS, a PKG inhibitor, blocks the effect of SNAP on wild-type hSERT activity. Cells expressing wild-type hSERT were treated with the indicated concentration of Rp-pCPT-cGMPS in the presence (filled circles) or absence (open circles) of 100 μM SNAP. After the preincubation at room temperature for 15 min, [^{3}H]5-HT was added to a final concentration of 20 nM. Transport was measured in a 10 min incubation as described in Materials and Methods. A representative experiment that was performed in triplicate and repeated in three separate assays with similar results is shown. Error bars indicate SD of the means from six experiments (***A***) or from triplicates within a representative experiment (***B***).

5-HT transport assay. [^{3}H]5-HT transport activity was assayed in monolayer cultures at room temperature. Transfected HeLa cells in 24-well plates were washed once with 500 μl of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) and incubated in PBS/CM for the indicated time at room temperature with or without various modulators. 5-HT uptake assays were initiated by the addition of [^{3}H]5-HT (20 nM final concentration), and the incubation was continued for 10 min. Saturation kinetic analyses for 5-HT K_M and V_{max} were performed in 96-well plates with a concentration range of 0.02–5 μM 5-HT generated by adding unlabeled 5-HT to a constant amount of [^{3}H]5-HT. The assays

Table 1. Effect of the p38 MAP kinase inhibitor SB203580 on hSERT activity before and after stimulation with 8-Br-cGMP

	Vehicle	8-Br-cGMP	SB203580	8-Br-cGMP + SB203580
Wild type	100 ± 4	129 ± 3	102 ± 2	126 ± 3
T276D	131 ± 3	127 ± 2	130 ± 1	130 ± 1
I425V	130 ± 4	130 ± 3	125 ± 3	126 ± 5

Cells expressing wild-type hSERT, T276D, or I425V mutant were treated with SB203580 (10 μM in 0.1% DMSO) for 10 min, 8-Br-cGMP (100 μM) for 30 min, or preincubated with SB203580 (10 μM) for 10 min before adding 100 μM 8-Br-cGMP for 30 min before transport assay. Transport was measured in a 10 min incubation with [^{3}H]5-HT (20 nM), as described in Materials and Methods. Data are means ± SD from six measurements in two separate experiments as the percentage of transport activity relative to wild type treated with vehicle (0.1% DMSO).

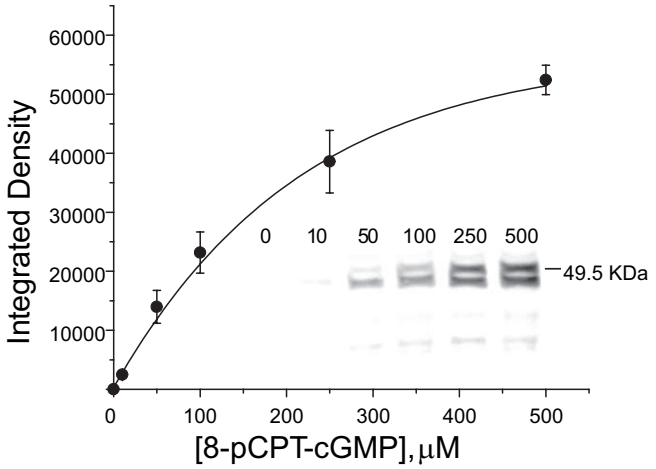

Figure 2. Western blot analysis of VASP phosphorylation in HeLa cells. HeLa cells transfected with pCDNA3-VSV-VASP were treated with the indicated concentration of 8-pCPT-cGMP in PBS/CM at room temperature for 30 min. Protein extracts from the cells were separated by SDS-PAGE and immunoblotted with monoclonal anti-phospho-VASP antibody 16C2 as described in Materials and Methods. The data in the graph represent densitometry results combined from three experiments, one of which is shown in the immunoblot representation (inset). The line adjacent to the immunoblot indicates the position of 49.5 kDa protein standard, and the numbers on the top of the immunoblot show the concentration of 8-pCPT-cGMP used for each sample. Error bars indicate SD of the means from three experiments.

were terminated by three rapid washes by aspiration with ice-cold PBS. The cells were then solubilized in 500 μl of 1% SDS or 30 μl of 0.1 M NaOH for 30 min. The extent of [^{3}H]5-HT accumulated was determined by liquid scintillation spectrometry in a PerkinElmer (Waltham, MA) Microbeta plate counter. All uptake measurements were corrected by subtracting blank values measured in the presence of 1 mM cocaine.

Western blot analysis for VASP phosphorylation. HeLa cells plated in 12-well plates were infected with recombinant VTF7–3 virus and transfected with plasmid pCDNA3-VSV-VASP. Twenty hours after transfection, the cells were washed once with 1 ml of PBS/CM and incubated with the indicated concentrations of 8-pCPT-cGMP in PBS/CM at room temperature for 30 min. Then, the cells were washed with 1 ml of PBS/CM and solubilized in 300 μl of an SDS-containing stop solution (66.6 mM Tris-HCl buffer, pH 6.7, containing 2% SDS, 5% glycerol, 10% β-mercaptoethanol, and a trace of bromphenol blue). The resulting samples were boiled and separated by 9% SDS-PAGE. The proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membrane was incubated overnight at 4°C with monoclonal anti-phospho-VASP antibody 16C2 (diluted 1:2500) and subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody. The signal was visualized by chemiluminescence using Super Signal West Pico (Pierce). The images were captured, and phospho-VASP bands were quantified using a UVP (Upland, CA) Laboratory Imaging and Analysis System.

Binding assay. Binding of the high-affinity cocaine analog 2β-carbomethoxy-3β-(4-iodophenyl)tropane (RTI-55, β-CIT) was measured in crude membrane preparations from transfected HeLa cells as described previously (Zhang and Rudnick, 2005a). For membrane binding assays, frozen membranes from cells expressing rat SERT mutants were thawed on ice, applied to Multiscreen-FB 96-well filtration plates (Millipore, Bedford, MA), and washed five times by filtration with 100 μl of binding buffer (10 mM HEPES buffer, pH 8.0, containing 150 mM NaCl). 2-(Aminoethyl)methanethiosulfonate hydrobromide (MTSEA) was subsequently added to the membranes and incubated for 15 min at room temperature, and the membranes were then washed five times to remove unbound MTSEA. β-CIT binding was then initiated by the addition of 100 μl of the standard binding buffer containing 150 mM NaCl and 0.1 nM [^{125}I]β-CIT (RTI-55; PerkinElmer). Binding was allowed to proceed for 1.5 h at room temperature with gentle rocking. The reaction was stopped by washing three times with 100 μl of ice-cold binding buffer. The filters were removed from the plate and counted with a PerkinElmer Microbeta plate counter in 150 μl of Optifluor (PerkinElmer).

The EC_{50} concentration of MTSEA, sufficient to half-maximally inactivate each mutant, was used to calculate rate constants with the assumption of bimolecular kinetics and a first-order time course of activity loss (Rudnick, 2002). In the 15 min inactivation reaction, half-maximal inactivation gives a $t_{1/2}$ of 15 min and a pseudo first-order rate constant of 0.046 min^{-1}. From this value and the concentrations of MTSEA required for half-maximal inactivation, the rate constants shown in Figure 7 were calculated. We interpret a higher inactivation rate as a greater accessibility to MTSEA.

Immunoprecipitation and immunoblotting of PP2Ac. Protein phosphatase 2A (PP2A) was immunoprecipitated from detergent extracts of transiently transfected HeLa cells as described previously by Bauman et al. (2000). Cells expressing wild-type hSERT or the I425V mutant were treated with 100 μM 8-Br-cGMP for 30 min, treated with 10 μM Rp-8-pCPT-cGMPS for 30 min, or preincubated with Rp-8-pCPT-cGMPS for 30 min before adding 8-Br-cGMP. The cells were then washed three times with PBS/CM buffer and lysed by addition of 400 μl/well ice-cold modified RIPA (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) buffer containing 0.5% protease inhibitor mixture (Sigma) for 1 h at 4°C. After centrifugation at 14,000 rpm for 30 min, the SERT-specific SR-12 antibody was added to the resulting supernatants to immunoprecipitate hSERT overnight at 4°C with end-over-end mixing, followed by a 1 h incubation with protein A-Sepharose beads at 20°C. The immunoadsorbents were washed five times with ice-cold RIPA buffer before the addition of SDS-PAGE sample buffer. Samples were separated by SDS-PAGE (10%), electroblotted to a nitrocellulose membrane, probed with anti-PP2Ac antibody (BD Transduction Laboratories, San Diego, CA) overnight at 4°C, and subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse antibody. The signal was detected by chemiluminescence using Super Signal West Femto.

Data analysis. Nonlinear regression fits of experimental and calculated data were performed with Origin (OriginLab, Northampton, MA), which uses the Marquardt-Levenberg nonlinear least-squares curve-fitting algorithm. The statistical analysis given was from multiple experiments. Data with error bars represent the mean SD for multiple experiments. Statistical analysis comparing vehicle and modulator-modified uptake was performed using Student's paired *t* tests.

Results

Previous results indicated that an Ile to Val mutation at position 425 of hSERT led to an increase in transport activity and insensitivity to stimulation by NO donors (Kilic et al., 2003). To extend this observation, we tested the ability of other activators of the PKG pathway to increase hSERT activity. Figure 1*A* shows the activity of wild-type hSERT and the I425V mutant after treatment with SNAP, an NO donor, YC-1 [3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole], a stimulator of soluble guanylyl cy-

clase, and 8-Br-cGMP. Each of these agents markedly stimulated the activity of wild-type hSERT, but not I425V, and the activity of wild-type hSERT stimulated by each of these agents was similar to that of the I425V mutant. Moreover, Figure 1*B* shows that the stimulatory effect of SNAP was completely reversed by the presence of the PKG inhibitor Rp-8-pCPT-cGMPS. Incubation with SB203580, a specific inhibitor of p38 MAP kinase, failed to affect SERT activity or, in contrast to results of Zhu et al. (2004a), subsequent stimulation by 8-Br-cGMP (Table 1). The observation that hSERT activity could be stimulated not only by SNAP, which decomposes to NO, but also by YC-1, which stimulates guanylyl cyclase, and 8-Br-cGMP suggests that all these agents are acting through PKG.

Further evidence consistent with this conclusion is shown in Figure 2. To demonstrate that the HeLa cells used for these experiments expressed PKG, we transfected the cells with cDNA encoding VASP, a 46 kDa substrate for PKG (Halbrugge et al., 1990). Using an antibody specific for phosphorylated VASP (Smolenski et al., 1998), we observed PKG activity in these cells stimulated by addition of the cGMP analog 8-pCPT-cGMP. Immunoblot analysis is shown as an inset, and combined analysis of multiple experiments is shown in the body of Figure 2. Phosphorylation of VASP was detectable at 8-pCPT-cGMP concentrations as low as 50 μM and increased up to 500 μM, the highest concentration tested. The effects of 8-pCPT-cGMP on VASP phosphorylation were blocked >50% by 10 μM Rp-8-pCPT-cGMPS and completely blocked at 100 μM (data not shown). Using antibodies directed against PKG I and II isoforms, we observed an immunoreactive 65 kDa band corresponding to a degradation product that has been described for PKG I (Smolenski et al., 2000) but no significant PKG II immunoreactivity (data not shown). However, the message for both isoforms was detected using reverse transcription-PCR (data not shown).

Although the I425V mutant did not respond to agents that stimulate PKG, we found that extended incubation with inhibitors of soluble guanylyl cyclase and PKG decreased transport activity of this mutant. The results demonstrate that neither 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Fig. 3*A*) nor Rp-8-pCPT-cGMPS (Fig. 3*B*) affected the activity of wild-type hSERT under these conditions, but both agents led to decreased activity of the I425V mutant to transport levels similar to that of wild-type hSERT. The loss of activity occurred over a period of hours, in contrast to the activation of wild type, which occurred in minutes. These results suggest that both wild-type hSERT and I425V can exist in both high and low activity states and that the effect of the Ile to Val mutation at position 425 is to alter the balance between the two states in favor of the high activity state. Furthermore, they suggest that the I425V mutation might impair the removal of a phosphate added to SERT by PKG.

The results in Figures 1–3 are consistent with direct phosphorylation of hSERT by PKG. In recent work by Ramamoorthy et al. (2007), Thr-276 has been identified as a site of PKG-mediated phosphorylation of SERT. We therefore tested the stimulatory effect of SNAP and 8-Br-cGMP on the hSERT mutants T276A and T276D expressed in HeLa cells and found that it was not stimulated by either 8-Br-cGMP (Fig. 4) or SNAP (data not shown), consistent with the proposal that Thr-276 is the target for PKG phosphorylation of SERT. T276E behaved like T276D (data not shown). The activity of T276D and T276E was approximately equal to that of wild-type hSERT in cells stimulated with 8-Br-cGMP (Fig. 4) but was not stimulated under similar conditions. However, neither aspartate nor glutamate is equivalent to phosphoryl-threonine, and we do not believe that T276D or T276E is activated in the same way as I425V or wild type in the presence of cGMP for reasons described below.

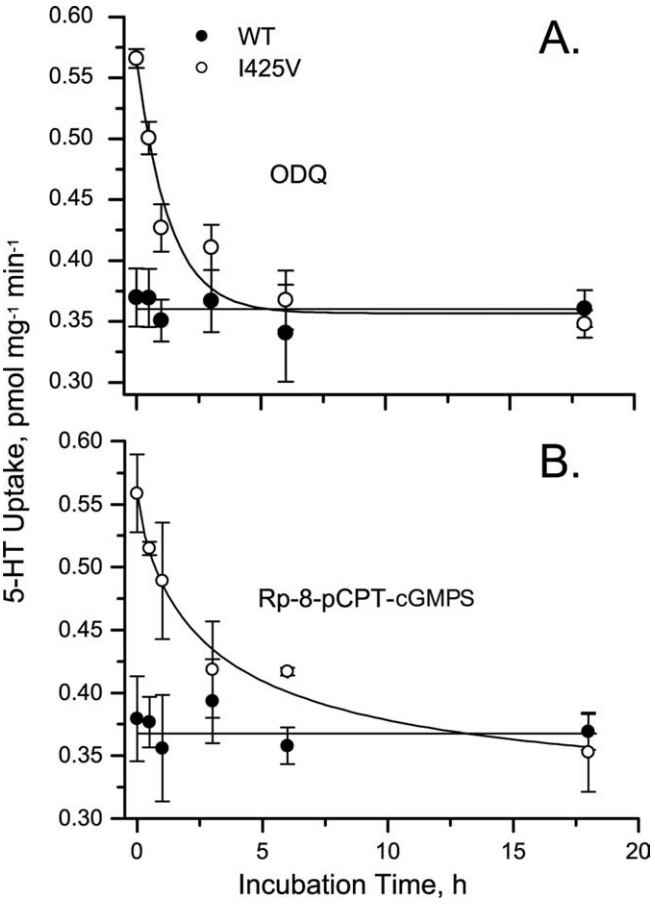

Figure 3. ODQ and Rp-8-pCPT-cGMPS decrease transport activity of I425V but not wild-type hSERT. HeLa cells were transfected with cDNA encoding either wild-type hSERT (WT; filled circles) or the I425V mutant (open circles). At various times after transfection, 1 μM ODQ (***A***) or 5 μM Rp-8-pCPT-cGMPS (***B***) was added and incubated for the indicated time before transport assay. At 18 h after transfection, all the cells were washed once and [^{3}H]5-HT was added to a final concentration of 20 nM. [^{3}H]5-HT uptake was determined as described in Materials and Methods. A representative experiment that was performed in triplicate and repeated in three separate assays with similar results is shown. Error bars indicate SD of the means from triplicates within a representative experiment.

The increased activity of T276D was apparently caused by a decrease in K_M. The kinetic analysis illustrated in Figure 5 shows that the increase in activity was observed only at low concentrations of 5-HT and that a decrease in the V_{max} of T276D led to lower transport rates relative to wild type at higher 5-HT concentrations. Figure 5*B* shows the rate versus concentration profiles for wild type, T276A, and T276D, and Figure 5*A* shows T276D activity as a function of 5-HT, plotted as a percentage of wild type. At limiting 5-HT concentrations, T276D transports at 130% the rate of wild-type hSERT, and at high concentrations, the relative rate falls to less than half of that, ~60% of the wild-type rate. Similar results were observed with T276E: 122% of wild-type activity at low concentrations and 70% of the wild-type V_{max}.

Changes in K_M can arise from alterations in kinetic parameters similar to those that increase V_{max}, but they can also be attributable to changes in substrate affinity. Figure 5*C* shows that 5-HT had a higher affinity for T276D than for wild type, as demonstrated by its ability to displace the cocaine analog β-CIT in equilibrium binding measurements. These results argue against the T276D mutation mimicking the phosphorylated state of

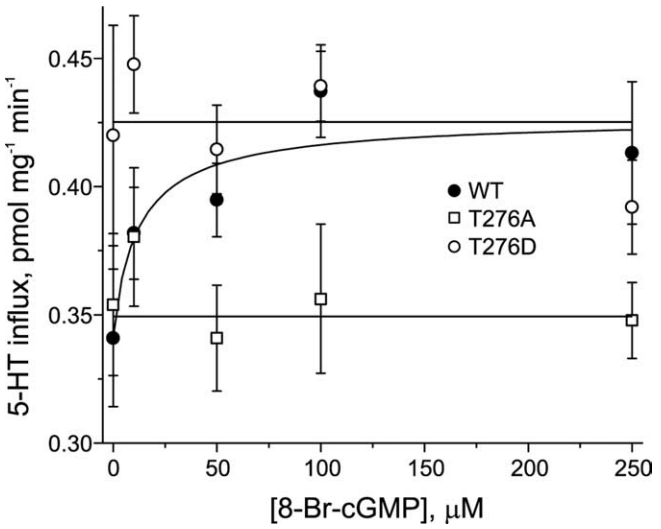

Figure 4. Effect of 8-Br-cGMP on transport activity of wild-type hSERT, T276A, or T276D. HeLa cells expressing wild-type hSERT (WT; filled circles), T276A (open squares), or T276D (open circles) were preincubated with the indicated concentration of 8-Br-cGMP for 30 min, at which time transport was initiated by addition of [^{3}H]5-HT to a final concentration of 20 nM. Transport was measured in a 10 min incubation in the presence of 8-Br-cGMP as described in Materials and Methods. The results represent data combined from two experiments, each with triplicate measurements for each sample. The curve representing the wild-type points is the best-fit rectangular hyperbola for saturating effect of 8-Br-cGMP. Error bars indicate SD of the means from two experiments.

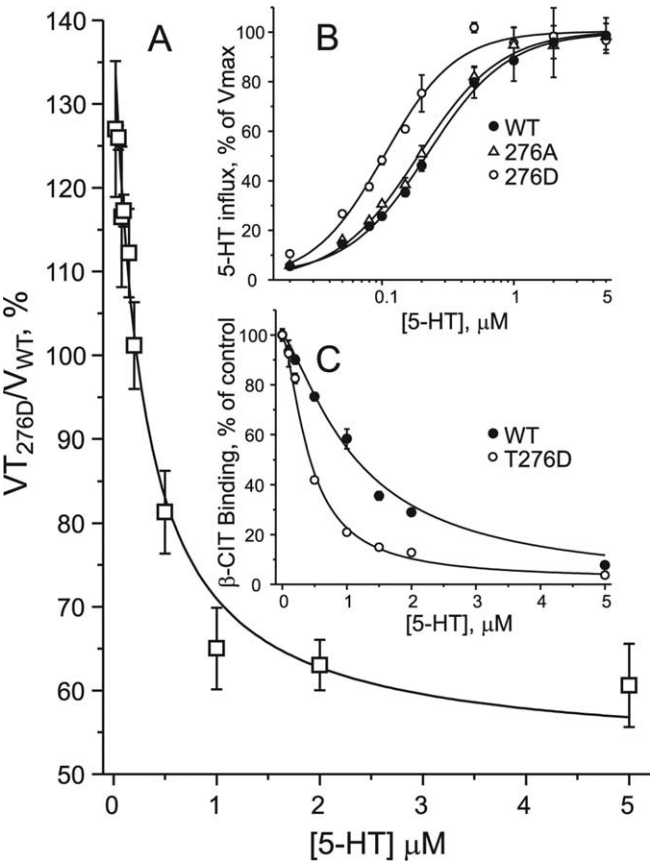

Figure 5. Saturation analysis for hSERT wild type (WT) and mutants T276A and T276D. The rate of 5-HT influx as a function of 5-HT concentration was measured over a range from 0.02 to 5 μM for wild type, T276A, and T276D expressed in HeLa cells. ***A***, The ratio (V_{T276D}/V_{WT}) of wild type-to-T276D influx is shown as a function of 5-HT concentration. ***B***, Rates relative to V_{max}. K_M and V_{max} values were calculated by fitting the rate versus 5-HT concentration using Origin software (OriginLab). From analysis of these results, K_M values of 0.24 $\pm$ 0.01, 0.19 $\pm$ 0.01, and 0.11 $\pm$ 0.01 μM and V_{max} values of 6.55 $\pm$ 0.23, 5.54 $\pm$ 0.11, and 4.03 $\pm$ 0.11 pmol min^{-1} mg^{-1} protein were obtained for wild type, T276A, and T276D, respectively. The results represent data combined from four experiments. T276D values differed significantly ($p < 0.01$) from those of wild-type hSERT. ***C***, Displacement of β-CIT from cells expressing SERT wild type or T276D. Half-maximal displacement occurred at 1.15 $\pm$ 0.17 μM for wild type and 0.42 $\pm$ 0.03 for T276D. Error bars indicate SD of the means from three experiments.

SERT, in which the primary effect is an increase in V_{max} (Ramamoorthy et al., 2007).

Cell-surface expression of T276A and T276D was measured by biotinylation as described previously (Chen et al., 1998). The level of expression of these mutants was not statistically different from that of wild-type hSERT (95 $\pm$ 4 and 106 $\pm$ 14%, respectively, for total expression; 104 $\pm$ 4 and 95 $\pm$ 6%, respectively, for surface expression, relative to wild type). Activation of 5-HT transport by 8-Br-cGMP or the I425V mutation led to an increase in V_{max} with little change in K_M (Table 2).

The decrease in I425V activity resulting from incubation with ODQ (Fig. 3) was also found to be dependent on the presence of threonine at position 276. Double mutants containing both I425V and either alanine or aspartate at 276 did not lose activity when treated with ODQ, in contrast with I425V alone, which decreased over the incubation time (Fig. 6). ODQ also had no effect on T276D (data not shown). Replacement of Ile-425 with valine did not change the activity of T276D, as it did in the wild-type background, although it did increase the activity of T276A (Fig. 6, Table 2). This increase, observed at low concentrations of 5-HT, results from a decrease in K_M, which is accompanied by a decrease in V_{max} similar to the kinetic changes in T276D (Table 2) and unlikely to be related to phosphorylation at Thr-276. It is significant, however, that the increase in wild-type hSERT activity resulting from PKG activation and the decrease in I425V activity resulting from PKG inhibition both required a threonine at position 276.

Because Thr-276 is in TM5 of SERT, which contributes to the substrate permeation pathway (Zhang and Rudnick, 2006), we evaluated the possibility that modifications at this position alter the conformation of TM5 and thereby influence catalytic activity. We measured the influence of I425V and T276D mutations on the reactivity of cysteine residues inserted in place of residues Lys-272 through Ala-285. Previous investigation revealed that in rat SERT this region is in an α-helical conformation, consistent with its alignment with TM5 in LeuT (Beuming et al., 2006), and that rates of MTSEA reaction with these cysteine mutants varied by factors of >100-fold from one position to another.

Figure 7 presents these results as second-order rate constants for MTSEA reactivity as measured by inactivation of SERT binding to the high-affinity cocaine analog β-CIT (Zhang and Rudnick, 2005a). Figure 7*A* shows that the I425V mutation had essentially no effect on the magnitude of these rates or the pattern of relative rates within this region. In contrast, mutation of Thr-276 to Asp led to marked changes in the rate of inactivation at several positions. Figure 7*C* shows the ratio of the rates in the 276D and X5C backgrounds. It is evident that several positions, notably 274, 277, and 283, reacted 7- to 10-fold faster in T276D. Other positions showed smaller rate increases, but G278C and T284C rates were unchanged and V280C reacted slower in the context of T276D. These data point to a conformational change effected by the T276D mutation. Conformational changes in TM5 may also occur when Thr-276 is phosphorylated.

The increased activity of I425V occurred under basal condi-

Table 2. Kinetic characteristics and surface expression levels of wild-type (WT) and hSERT mutants at Thr-276 and Ile-425

	K_M (μM)	V_{max} (pmol/min/mg)	Surface expression (% of wild type)
WT	0.24 ± 0.04	6.55 ± 0.23	100 ± 5
WT + 8-Br-cGMP	0.20 ± 0.03	8.28 ± 0.13*	ND
I425V	0.26 ± 0.02	8.16 ± 0.59*	104 ± 1
T276A	0.19 ± 0.01	5.54 ± 0.11	104 ± 4
T276A-I425V	0.12 ± 0.01*	4.03 ± 0.26*	103 ± 3
T276D	0.11 ± 0.01*	4.03 ± 0.11*	95 ± 7
T276D-I425V	0.08 ± 0.01*	4.24 ± 0.21*	95 ± 6
T276E	0.13 ± 0.02*	4.48 ± 0.19*	87 ± 8

WT and hSERT mutants were tested to measure transport rate over a range (0.02–5 μM) of 5-HT concentrations. K_M and V_{max} were calculated by fitting the rate versus concentration data using Origin software (OriginLab). The results are from multiple experiments. Asterisks indicate K_M and V_{max} values significantly different ($p < 0.05$) from that of wild type. ND, Not determined.

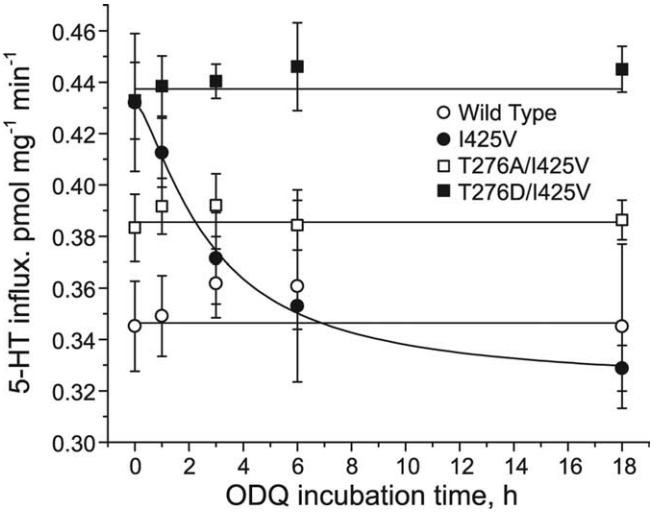

Figure 6. ODQ does not decrease transport activity of Thr-276 mutants in the I425V background. Wild-type hSERT (open circles) and mutants I425V (filled circles), T276A/I425V (open squares), and T276D/I425V (filled squares) were expressed in HeLa cells, and the effect of 1 μM ODQ treatment was tested as described in Figure 3. The results represent data combined from two experiments, each with triplicate measurements for each sample. Error bars indicate SD of the means from two experiments.

tions and did not require stimulation of PKG. This observation might be explained if the mutation decreased dephosphorylation of Thr-276, leading to hyperphosphorylation at that position even at unstimulated levels of cGMP. Because PP2A has been found in association with SERT (Bauman et al., 2000), we measured the effect of the I425V mutation on this association in the presence and absence of 8-Br-cGMP and a PKG inhibitor. The results, shown in Figure 8, demonstrate that 8-Br-cGMP increased association of PP2A with wild-type hSERT, although inhibition of PKG did not decrease association. In contrast, PP2A association with hSERT I425V was increased relative to wild type and was not stimulated by addition of 8-Br-cGMP. However, inhibition of PKG decreased PP2A association with I425V to the level observed in the unstimulated wild type. PP2A association with hSERT paralleled the effects of PKG stimulation and inhibition on activity as shown in Figures 1 and 3.

Discussion

The results presented here extend our understanding of the mechanism by which PKG stimulates SERT and how that stimulation is influenced by the I425V mutation, which is associated with multiple psychiatric disorders including OCD and Asperger syndrome (Ozaki et al., 2003; Delorme et al., 2005). The present results are consistent with recent evidence that PKG increases hSERT activity by phosphorylation of Thr-276 in TM5 (Ramamoorthy et al., 2007) and the proposal that I425V mutation (Kilic et al., 2003) interferes with this regulation by inhibiting dephosphorylation of that site. Our results also support a mechanism in which phosphorylation of Thr-276 alters the conformation of TM5, leading to more efficient permeation of 5-HT from its binding site to the cytoplasm. This is the first example of a specific phosphorylation site linked to SERT regulation.

The current results provide further evidence that PKG is responsible for modulating hSERT activity and also for the dysregulation associated with the I425V mutation. It is clear that the HeLa cells used in this study retain a functional PKG. Furthermore, each of the agents that elevate or mimic cGMP increased hSERT activity to approximately the same level, which was similar to that of the I425V mutant. Moreover, the stimulation was prevented in the presence of a PKG inhibitor that had no effect on basal activity, and none of the agents that stimulated activity had a measurable effect on the I425V mutant. Additionally, mutation of Thr-276 blocked the influence of the cGMP pathway on SERT activity. We consider unlikely the alternative possibility that the 8-Br-cGMP effects are mediated by cAMP through inhibition of phosphodiesterase 3 activity (Butt et al., 1992) because stimulation of activity and phosphorylation were shown to be blocked by specific inhibitors of PKG but not PKA (Ramamoorthy et al., 2007). Together, these results support the hypothesis that stimulation of PKG leads to activation of hSERT by direct phosphorylation of the transporter.

Previous work determined that a consequence of the I425V mutation was to increase V_{max} and to slightly decrease K_M (Kilic et al., 2003; Prasad et al., 2005), although in the present study, only the V_{max} change was observed (Table 2), just as with 8-Br-cGMP stimulation of wild type (Table 2) (Ramamoorthy et al., 2007). The present results with T276D and T276E indicate a decrease in both kinetic parameters, leading to activation at low 5-HT concentrations and inhibition as substrate approaches saturation (Fig. 5). Although we expected aspartate or glutamate to mimic a phosphorylated threonine, it seems that there are sufficient structural differences between phosphothreonine and either aspartate or glutamate that the effect on catalysis is not identical. We reached similar conclusions in a previous study (Zhang and Rudnick, 2005b). Moreover, the K_M decrease that increased rate at low 5-HT concentrations in T276D was found in binding experiments to represent a change in substrate affinity and not an effect on transport kinetics.

Our present results indicate that Thr-276 is required both for 8-Br-cGMP to stimulate hSERT activity (Fig. 4) and for the guanylyl cyclase inhibitor ODQ to decrease the activity of the I425V mutant (Fig. 6). The most direct explanation for these observations is that hSERT was phosphorylated by PKG on Thr-276 and that this reaction increased the rate of transport and also increased SERT association with PP2A (Fig. 8). Ramamoorthy et al. (2007) also found that PKG-mediated incorporation of ^{32}P into SERT required Thr-276.

The location of Thr-276, near the cytoplasmic end of TM5, suggests a possible mechanism for regulation of SERT by phosphorylation. Evidence suggests that TM5 participates in forming

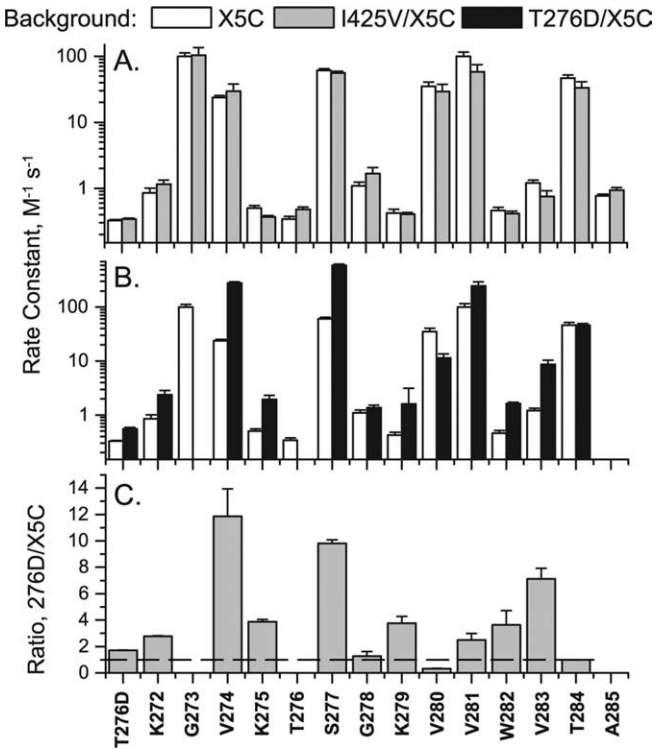

Figure 7. Reactivity of cysteines at positions 272–285 in the IL2–TM5 region toward MTSEA. ***A***, ***B***, Membranes prepared from cells expressing the indicated cysteine mutants in the X5C (***A***, ***B***), I425V/X5C (***A***), and T276D/X5C (***B***) backgrounds were treated with a range of MTSEA concentrations for 15 min at room temperature, washed, and assayed for residual β-CIT binding as described in Materials and Methods. Pseudo first-order rate constants for inactivation were estimated for each mutant from fits of MTSEA-dependent inactivation. The concentration corresponding to half-maximal inactivation was used to calculate the rate constant, assuming first-order kinetics for MTSEA. The results represent combined data from three experiments for each mutant. ***C***, The ratio of rate constants with and without the T276D mutation is shown for each cysteine mutant. Error bars indicate SD of the means from three experiments.

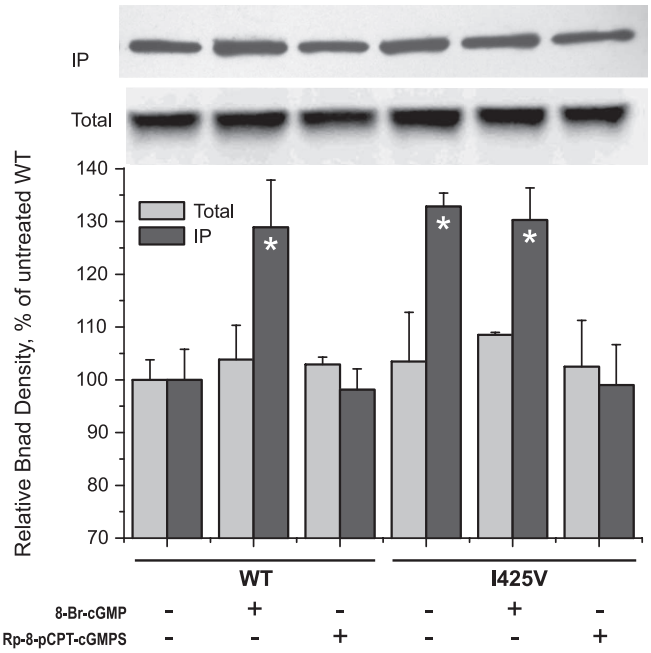

Figure 8. Effect of 8-Br-cGMP, PKG inhibition, and the I425V mutation on association of PP2a with hSERT. Cells expressing hSERT wild type (WT) or the I425V mutant were treated with 8-Br-cGMP (100 μM) for 30 min, treated with Rp-8-pCPT-cGMPS (10 μM) for 30 min, or preincubated with Rp-8-pCPT-cGMPS for 30 min before adding 8-Br-cGMP. The cells were then solubilized, and hSERT was immunoprecipitated using anti-SERT SR-12 antibody. The immunoprecipitates were separated by SDS-PAGE (10%) and transferred to nitrocellulose, and PP2A was detected with an anti-PP2Ac antibody as described in Materials and Methods. The top panel shows representative blots of PP2Ac detected in cell lysates (Total) and immunoprecipitates (IP), and the bottom panel shows aggregate data from three experiments of PP2Ac band density relative to wild-type hSERT without treatment. The asterisks indicate significant differences in SERT-associated PP2A after treatment with vehicle alone and the addition of inhibitor or activator ($p < 0.05$, paired Student's *t* tests). Error bars indicate SD of the means from three experiments.

the pathway by which 5-HT diffuses from its binding site to the cytoplasm (Zhang and Rudnick, 2006). Opening and closing this pathway may entail conformational changes on the part of TM5. Recent evidence supports a coordinated conformational change that opens the cytoplasmic pathway to which TM5 contributes and closes the extracellular permeation pathway (Jacobs et al., 2007). We propose that phosphorylation of Thr-276 alters the structure of TM5 and that this structural change allows more rapid transport of 5-HT from its binding site to the cytoplasm. That modification of Thr-276 alters TM5 conformation was demonstrated by the altered reactivity of TM5 cysteine mutants with T276D (Fig. 7). There is also evidence that structural changes in TM5 increase transport. Many TM5 mutations in SERT led to increased catalytic activity (Zhang and Rudnick, 2005a), arguably through alterations in the structure of this region that increase substrate permeation through SERT. Thr-276, therefore, is ideally situated in a position where its phosphorylation could alter the conformation of TM5 in a way that increases the catalytic activity of SERT.

Because PKG is expected, like PKA, to phosphorylate substrates in an extended conformation (Knighton et al., 1991), it is likely that significant changes in secondary structure would precede PKG phosphorylation of Thr-276, which is in an α-helical domain of TM5 (Zhang and Rudnick, 2005a; Beuming et al., 2006). We observed an increase in the accessibility of cysteine residues in positions 272–279, near the cytoplasmic end of TM5, in the presence of K^+, which participates in the transport reaction (Zhang and Rudnick, 2006). The increased accessibility of this region surrounding Thr-276 may represent a conformational change similar to the one required for phosphorylation by PKG.

The adjacent serine at 277 in TM5 was initially invoked by Miller and Hoffman (1994) as a potential site for PKG-mediated SERT phosphorylation that is conserved among monoamine transporters. However, PKG stimulated incorporation of ^{32}P into threonine, but not serine or tyrosine, residues (Ramamoorthy et al., 2007). The possibility remains that mutation at Thr-276 altered the conformation of hSERT so as to make another intracellular threonine more reactive toward PKG, but there is no other evidence to support this more complicated hypothesis. Moreover, all other predicted intracellular threonine residues in hSERT were individually mutated with no loss of stimulation or phosphorylation by 8-Br-cGMP (Ramamoorthy et al., 2007). The region surrounding Thr-276 is absolutely conserved within the biogenic amine transporters. Although a recent report suggests that norepinephrine and dopamine transporters were unresponsive to 8-Br-cGMP (Zhu et al., 2004b), the corresponding Thr-258 in norepinephrine transporter was identified as a site of PKC-mediated phosphorylation (Jayanthi et al., 2006).

Our results are not in agreement with the implications of recent results from Blakely and coworkers (Zhu et al., 2004a,b), in which activation of PKG was proposed to increase SERT surface expression and activation of p38 MAP kinase was proposed to activate SERT catalytic activity. Prasad et al. (2005) also observed

increased activity in I425V but found that surface expression rather than increased catalytic activity was responsible. In our system, we previously observed catalytic activation in the form of a lower K_M with the higher-activity I425V mutant (Kilic et al., 2003). Our present results are more consistent with a change only in V_{max} (Table 2). Moreover, we observed no increase in cell-surface expression with either of those mutants or with T276A, which is refractory to stimulation by 8-Br-cGMP [Kilic et al. (2003), their Fig. 4] (Table 2). Finally, we observed no effect of the p38 MAP kinase inhibitor SB203580 on basal or 8-Br-cGMP-stimulated activity (Table 1). We do not understand the origin of this apparent discrepancy, although the results were obtained in different expression systems and may be influenced by cell-specific factors. One possibility is that altered catalytic activity might influence surface expression, a phenomenon previously observed in the regulation of SERT by PKC (Ramamoorthy and Blakely, 1999).

The present results provide a plausible explanation for the effect of the I425V mutation on transport. Our ability to stimulate the activity of the wild-type transporter with PKG suggests that Thr-276 is predominantly in the unphosphorylated form under resting conditions. Any basal activity of PKG is presumably opposed by protein phosphatases, such as PP2A, which has been shown to be associated with SERT (Bauman et al., 2000) and whose association with hSERT increased when PKG was stimulated (Fig. 8). After activation of PKG, Thr-276 was phosphorylated (Ramamoorthy et al., 2007) with concomitant activation of transport activity. If the I425V mutation inhibits the ability of PP2A to dephosphorylate Thr-276, then basal PKG activity may be sufficient to maximally phosphorylate the transporter, leading to activation. As we observed (Fig. 6), inhibiting PKG activity or cGMP synthesis led to a slow decrease in I425V, but not wild-type, activity. We therefore propose that the I425V mutation makes phospho-SERT a poorer substrate for a phosphatase, such as PP2A.

We have eliminated two potential ways that the I425V mutation could inhibit dephosphorylation of Thr-276. The data in Figure 8 indicate that inhibition is not mediated by decreased PP2A association, which is actually increased in I425V. The data in Figure 7 show that the mutation has no effect on accessibility of TM5 in the region adjacent to the phosphorylation site, thus rendering unlikely the possibility that the I425V mutation alters the conformation around Thr-276 and thereby disrupts interaction with cytoplasmic PP2A. The expected increase in phosphorylation at Thr-276 in the I425V mutant was not observed as a change in TM5 reactivity, probably because Thr-276 was dephosphorylated during the preparation of membranes for reactivity measurements. In any case, it is not obvious how a mutation near the extracellular end of TM8 could affect the ability of a cytoplasmic phosphatase to act on Thr-276, near the cytoplasmic end of TM5. Almost 24 Å separate the corresponding residues in the crystal structure of the bacterial SERT homolog LeuT (Yamashita et al., 2005).

References

Androutsellis-Theotokis A, Rudnick G (2002) Accessibility and conformational coupling in serotonin transporter predicted internal domains. J Neurosci 22:8370–8378.

Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, Blakely RD (2000) Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. J Neurosci 20:7571–7578.

Beuming T, Shi L, Javitch JA, Weinstein H (2006) A comprehensive structure-based alignment of prokaryotic and eukaryotic neurotransmitter/Na+ symporters (NSS) aids in the use of the LeuT structure to probe NSS structure and function. Mol Pharmacol 70:1630–1642.

Blakely RD, Clark JA, Rudnick G, Amara SG (1991) Vaccinia-T7 RNA polymerase expression system: evaluation for the expression cloning of plasma membrane transporters. Anal Biochem 194:302–308.

Butt E, Nolte C, Schulz S, Beltman J, Beavo JA, Jastorff B, Walter U (1992) Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. Biochem Pharmacol 43:2591–2600.

Chen JG, Liu-Chen S, Rudnick G (1998) Determination of external loop topology in the serotonin transporter by site-directed chemical labeling. J Biol Chem 273:12675–12681.

Delorme R, Betancur C, Wagner M, Krebs MO, Gorwood P, Pearl P, Nygren G, Durand CM, Buhtz F, Pickering P, Melke J, Ruhrmann S, Anckarsater H, Chabane N, Kipman A, Reck C, Millet B, Roy I, Mouren-Simeoni MC, Maier W, et al. (2005) Support for the association between the rare functional variant I425V of the serotonin transporter gene and susceptibility to obsessive compulsive disorder. Mol Psychiatry 10:1059–1061.

Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U (1990) Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. J Biol Chem 265:3088–3093.

Jacobs MT, Zhang YW, Campbell SD, Rudnick G (2007) Ibogaine, a non-competitive inhibitor of serotonin transport, acts by stabilizing the cytoplasmic-facing form of the transporter. J Biol Chem 282:29441–29447.

Jayanthi LD, Ramamoorthy S, Mahesh VB, Leibach FH, Ganapathy V (1994) Calmodulin-dependent regulation of the catalytic function of the human serotonin transporter in placental choriocarcinoma cells. J Biol Chem 269:14424–14429.

Jayanthi LD, Annamalai B, Samuvel DJ, Gether U, Ramamoorthy S (2006) Phosphorylation of the norepinephrine transporter at threonine 258 and serine 259 is linked to protein kinase C-mediated transporter internalization. J Biol Chem 281:23326–23340.

Jones BJ, Blackburn TP (2002) The medical benefit of 5-HT research. Pharmacol Biochem Behav 71:555–568.

Kilic F, Murphy DL, Rudnick G (2003) A human serotonin transporter mutation causes constitutive activation of transport activity. Mol Pharmacol 64:440–446.

Knighton DR, Zheng JH, Ten Eyck LF, Xuong NH, Taylor SS, Sowadski JM (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science 253:414–420.

Miller KJ, Hoffman BJ (1994) Adenosine A_3 receptors regulate serotonin transport via nitric oxide and cGMP. J Biol Chem 269:27351–27356.

Murphy DL, Andrews AM, Wichems CH, Li Q, Tohda M, Greenberg B (1998) Brain serotonin neurotransmission: an overview and update with an emphasis on serotonin subsystem heterogeneity, multiple receptors, interactions with other neurotransmitter systems, and consequent implications for understanding the actions of serotonergic drugs. J Clin Psychiatry 59 [Suppl 15]:4–12.

Murphy DL, Lerner A, Rudnick G, Lesch KP (2004) Serotonin transporter: gene, genetic disorders, and pharmacogenetics. Mol Interv 4:109–123.

Ozaki N, Goldman D, Kaye WH, Plotnicov K, Greenberg BD, Lappalainen J, Rudnick G, Murphy DL (2003) Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype. Mol Psychiatry 8:933–936.

Prasad HC, Zhu CB, McCauley JL, Samuvel DJ, Ramamoorthy S, Shelton RC, Hewlett WA, Sutcliffe JS, Blakely RD (2005) Human serotonin transporter variants display altered sensitivity to protein kinase G and p38 mitogen-activated protein kinase. Proc Natl Acad Sci USA 102:11545–11550.

Qian Y, Galli A, Ramamoorthy S, Risso S, DeFelice LJ, Blakely RD (1997) Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression. J Neurosci 17:45–57.

Ramamoorthy S, Blakely RD (1999) Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. Science 285:763–766.

Ramamoorthy S, Cool D, Mahesh V, Leibach F, Melikian H, Blakely R, Ganapathy V (1993) Regulation of the human serotonin transporter cholera toxin-induced stimulation of serotonin uptake in human placental choriocarcinoma cells is accompanied by increased serotonin transporter

messenger RNA levels and serotonin transporter-specific ligand binding. J Biol Chem 268:21626–21631.

Ramamoorthy S, Samuvel DJ, Buck ER, Rudnick G, Jayanthi LD (2007) Phosphorylation of threonine residue 276 is required for acute regulation of serotonin transporter by cyclic GMP. J Biol Chem 282:11639–11647.

Rudnick G (2002) Chemical modification strategies for structure-function studies. In: Transmembrane transporters (Quick MW, ed), pp 125–141. Hoboken, NJ: Wiley.

Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S (2005) A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. J Neurosci 25:29–41.

Smolenski A, Bachmann C, Reinhard K, Honig-Liedl P, Jarchau T, Hoschuetzky H, Walter U (1998) Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. J Biol Chem 273:20029–20035.

Smolenski A, Poller W, Walter U, Lohmann SM (2000) Regulation of human endothelial cell focal adhesion sites and migration by cGMP-dependent protein kinase I. J Biol Chem 275:25723–25732.

Stahl SM (1998) Mechanism of action of serotonin selective reuptake inhibitors - Serotonin receptors and pathways mediate therapeutic effects and side effects [review]. J Affect Dis 51:215–235.

Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E (2005) Crystal structure of a bacterial homologue of Na+/Cl-dependent neurotransmitter transporters. Nature 437:215–223.

Zhang YW, Rudnick G (2005a) Cysteine scanning mutagenesis of serotonin transporter intracellular loop 2 suggests an alpha-helical conformation. J Biol Chem 280:30807–30813.

Zhang YW, Rudnick G (2005b) Serotonin transporter mutations associated with obsessive-compulsive disorder and phosphorylation alter binding affinity for inhibitors. Neuropharmacology 49:791–797.

Zhang YW, Rudnick G (2006) The cytoplasmic substrate permeation pathway of serotonin transporter. J Biol Chem 281:36213–36220.

Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD (2004a) Adenosine receptor, protein kinase G and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. Mol Pharmacol 65:1462–1474.

Zhu CB, Hewlett WA, Francis SH, Corbin JD, Blakely RD (2004b) Stimulation of serotonin transport by the cyclic GMP phosphodiesterase-5 inhibitor sildenafil. Eur J Pharmacol 504:1–6.

Zhu CB, Carneiro AM, Dostmann WR, Hewlett WA, Blakely RD (2005) p38 MAPK activation elevates serotonin transport activity via a trafficking-independent, protein phosphatase 2A-dependent process. J Biol Chem 280:15649–15658.