Phosphorylation of Threonine Residue 276 Is Required for Acute Regulation of Serotonin Transporter by Cyclic GMP*

Sammanda Ramamoorthy1, Devadoss J. Samuvel1, Eric R. Buck1, Gary Rudnick§, and Lankupalle D. Jayanthi‡

From the ‡Department of Neurosciences, Division of Neuroscience Research, Medical University of South Carolina, Charleston, South Carolina 29425 and the §Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06519

Cellular protein kinases, phosphatases, and other serotonin transporter (SERT) interacting proteins participate in several signaling mechanisms regulating SERT activity. The molecular mechanisms of protein kinase G (PKG)-mediated SERT regulation and the site of transporter phosphorylation were investigated. Treatment of rat midbrain synaptosomes with 8-bromo-cGMP increased SERT activity, and the increase was selectively blocked by PKG inhibitors. The Vmax value for serotonin (5-HT) transport increased following cGMP treatment. However, surface biotinylation studies showed no change in SERT surface abundance following PKG activation. 32P metabolic labeling face biotinylation studies showed no change in SERT surface abundance following PKG activation. 32P metabolic labeling experiments showed increased SERT phosphorylation in the presence of cGMP that was abolished by selectively inhibiting PKG. Phosphoamino acid analysis revealed that cGMP-stimulated native SERT phosphorylation occurred only on threonine residues. When added to CHO-1 cells expressing SERT, 8-bromo-cGMP stimulated 5-HT transport and SERT phosphorylation. Mutation of SERT threonine 276 to alanine completely abolished cGMP-mediated stimulation of 5-HT transport and SERT phosphorylation. Although the T276A mutation had no significant effect on 5-HT transport or SERT protein expression, mutation to aspartate (T276D) increased the level of 5-HT transport. We used the following: (a) rat midbrain synaptosomes to identify the molecular events in native SERT regulation and (b) expressed SERT to identify the molecular events in native SERT regulation and to study the mechanisms of protein kinase G (PKG)-mediated SERT regulation.

The intracellular domains of SERT (15) contain potential phosphorylation sites for several protein kinases (16–18). This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Neurosciences, Division of Neuroscience Research, 173 Ashley Ave., Box 403, Medical University of South Carolina, Charleston, SC 29425. Tel.: 843-792-3689; Fax: 843-792-4423; E-mail: rama@musc.edu

2 The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); 8-Br-cGMP, 8-bromo-cGMP; 3′,5′-cyclic monophosphate; CHO-1, Chinese hamster ovary; NET, norepinephrine transporter; NO, nitric oxide; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PKG, protein kinase G; PP2Ac, protein phosphatase 2A catalytic subunit; SERT, serotonin transporter; ANOVA, analysis of variance; h, human; PKC, protein kinase C; PKA, cAMP-dependent protein kinase.
cGMP and PKG-dependent SERT Phosphorylation

ulation by cGMP and PKG, and (b) a heterologous cell model system to identify the molecular site(s) of SERT phosphorylation in cGMP- and PKG-dependent transporter regulation. Using the synaptosomal preparation, we demonstrate that 8-Br-cGMP stimulates 5-HT uptake via PKG activation without altering SERT surface density. PKG activation also leads to SERT phosphorylation on threonine residue(s). Using SERT mutants with various intracellular threonines replaced, we find that cGMP- and PKG-mediated SERT regulation in CHO-1 cells requires phosphorylation of Thr-276 located in the fifth transmembrane domain near the second intracellular loop of SERT. These findings demonstrate for the first time a mechanistic basis for the direct link between 5-HT transport and alterations in SERT phosphorylation in response to changes in PKG activity.

EXPERIMENTAL PROCEDURES

Materials—8-Br-cGMP, H-8, fluoxetine, 5-HT, and gentamicin were obtained from Sigma. Okadaic acid was purchased from LC Laboratories/Alexis Biochemicals (San Diego, CA). Bisindolylmaleimide I was purchased from Calbiochem. [3H]5-HT (5-hydroxy-[3H]tryptamine trifluoroacetate), [32P]orthophosphate, protein A beads, ECL reagent, and ECL enhanced film were obtained from Amersham Biosciences. NHS-SS-biotin and monomeric avidin beads were from Pierce. All cell culture media and other reagents were purchased from Invitrogen. FuGENE 6 was from Roche Diagnostics. Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-calnexin was from StressGen (Victoria, Canada). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Other reagents were of the highest grade possible from standard sources.

Synaptosome Preparations—All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all animal use protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (100–150 g) were decapitated, and the midbrain was immediately homogenized in 10 volumes (w/v) of cold 0.32 M sucrose. The crude synaptosomal pellet (P2 fraction) was further purified as described previously (9). The purified synaptosomes were suspended in regular Krebs-Ringer-HEPES (KRH) buffer saturated with 95% O2 and 5% CO2. Protein concentration was determined by DC protein assay (Bio-Rad) using bovine serum albumin as standard.

5-HT Uptake in Synaptosomes—10–20 µg of purified synaptosomes were incubated in 250 µl of assay buffer containing 0.1 mM ascorbic acid, 0.1 mM pargyline, and 20 mM [3H]5-HT for 3 min. Synaptosomes were precubated with the modulators at 37 °C for the indicated times followed by addition of [3H]5-HT to initiate 5-HT uptake as described previously (9). For saturation analysis, [3H]5-HT was mixed with unlabeled 5-HT and varied from 10 nM to 1 µM. Nonspecific [3H]5-HT uptake was calculated as the accumulation in the presence of 100 nM fluoxetine and was subtracted from total uptake. Uptake was terminated by addition of 3 ml of ice-cold stop buffer (phosphate-buffered saline containing 10 mM imipramine) followed by rapid filtration over 3% polyethyleneimine-coated GF-B filters using a Brandel cell harvester. Filters were washed rapidly with 5 ml of cold stop buffer, and radioactivity bound to the filter was counted by liquid scintillation spectrometry. All uptake assays were performed in triplicate, and mean values of specific uptake ± S.E. from at least three separate experiments were determined.

Surface Biotinylation—Cell surface SERT in purified synaptosomal preparations was determined as described previously (9). Briefly, 100 µg of purified synaptosomes were incubated with cGMP or vehicle for 15 min at 37 °C. The samples were washed quickly, and the pellets were treated with NHS-SS-Biotin (0.5 mg/1 mg of protein) for 20 min at 4 °C in cold Krebs-bicarbonate buffer. The samples were subsequently washed with the same buffer containing 100 mM glycine, and the pellet was resuspended in RIPA lysis buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, and 0.1% sodium deoxycholate) supplemented with protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 μM pepstatin, and 250 µM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM sodium fluoride, 50 mM sodium pyrophosphate, 5 mM activated orthovanadate, and 1 μM okadaic acid). The resuspended synaptosomes were trituated 10 times through a 25-gauge needle and centrifuged at 25,000 × g for 30 min. The biotinylated proteins were separated from clear supernatant fluid by incubation with monomeric avidin beads for 4 h at 4 °C, washing three times with RIPA buffer, and extraction with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 5% β-mercaptoethanol) for 30 min at room temperature. Aliquots from total extracts, unbound fractions, and bead eluate were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with SERT-specific SR-12 antibody as described previously (9). SERT-reactive proteins were visualized using ECL Plus reagent followed by exposure to Hyperfilm-ECL (GE Healthcare). Multiple exposures of immunoblots were taken to ensure the band development on the film was within the linear range. Band densities were quantified by scanning and analyzed using NIH ImageJ (version 1.32j) software. Subsequently, the blots were stripped and reprobed with anti-calnexin antibody to validate the surface biotinylation of plasma membrane proteins. SERT densities from total, nonbiotinylated (representing the intracellular pool), and biotinylated (representing the surface pool) fractions were normalized using levels of calnexin in the total extract.

SERT Phosphorylation—The protocol for assaying cGMP-dependent SERT phosphorylation was similar to that described previously (7–9). Synaptosomes (1 mg) were incubated with 4.0 mCi of [32P]orthophosphate (carrier-free) for 60 min prior to the addition of modulators (as indicated). Samples were then centrifuged, and the pellet was resuspended in RIPA buffer (containing protease and phosphatase inhibitors) by passing through a 25-gauge needle 10 times. The clear supernatant obtained after sedimenting the solubilized synaptosomes at 45,000 × g for 40 min at 4 °C was passed through a Sephadex G-50 spin column to remove unused [32P] and free [32P]-labeled ATP before immunoprecipitation with the SR-12 antibody. To test specificity, additional experiments were performed in parallel using SR-12 preimmune serum as described previously (9). The immunoprecipitates were captured by the addition of pro-
tein A-Sepharose. The beads were washed with RIPA buffer prior to extraction with 60 μl of Laemmli sample buffer for 30 min at 22 °C. Eluted proteins were subjected to SDS-PAGE (4–15%), and radiolabeled SERT was detected by autoradiography. Multiple exposures (1–4 days) were evaluated by digital quantitation using NIH ImageJ (version 1.32j) software to ensure that results were within the linear range of the film.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described previously (19). Briefly, the [32P]-labeled SERT was immunoprecipitated from cGMP-treated synaptosomes and subjected to SDS-PAGE as described above. The band corresponding to [32P]-labeled SERT was excised from the gel and incubated in 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1% SDS, 3 mM β-mercaptoethanol, and 100 μg of histone (as carrier) with continuous overnight shaking at 22 °C. Eluted protein was precipitated with 20% trichloroacetic acid and subjected to acid hydrolysis for 90 min in 5.7 N HCl at 110 °C, and the samples were subjected to high voltage electrophoresis as described previously (19). Standard phosphoamino acids were added to the radioactive samples during hydrolysis and located by ninhydrin spray. The [32P]-phosphoamino acids were added to the radioactive samples during hydrolysis and subjected to acid hydrolysis for 90 min in 5.7 N HCl at 110 °C, and the samples were subjected to high voltage electrophoresis as described previously (19). Standard phosphoamino acids were added to the radioactive samples during hydrolysis and located by ninhydrin spray. The [32P]-phosphoamino acids from SERT were located by autoradiography and aligned with standards as we have described previously (19).

Site-directed Mutagenesis—Threonine residues in hSERT intracellular domains were mutated to alanine or aspartic acid residues using the QuickChange site-directed mutagenesis kit (Stratagene). XhoI/AgeI or XbaI/AgeI fragments containing the mutated site(s) were then ligated into correspondingly cut DNA on both strands.

Cell Culture and Transient Transfections—CHO-1 cells were cultured as monolayers in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum, 2 mM glutamine in 25- or 75-cm² flasks in an atmosphere of 5% CO₂ and 95% humidity at 37 °C. For transient transfection experiments, trypsin-released cells were seeded in 6-well (200,000 cells/well) or 24-well (50,000 cells/well) plates. Cells were transfected with hSERT plasmids or the empty vector using FuGENE 6 transfection reagent according to the manufacturer’s protocols. Where indicated, cells were treated with different modulators as described after 24 h of transfection.

5-HT Uptake in Transfected CHO-1 Cells—5-HT uptake was performed as described previously (7, 20). Cells were preincubated with the modulators at 37 °C for the indicated times followed by addition of [³H]5-HT to initiate 5-HT uptake. Uptake was terminated after 10 min of incubation at 37 °C by rapid washings with KRH assay buffer containing 100 mM fluoxetine. Cells were lysed, and the accumulated radioactivity was measured by liquid scintillation. Specific 5-HT uptake measurements were performed as described above.

Cell surface Biotinylation and Immunoblotting—Cell-surface biotinylation on transfected cells was performed as described previously (7, 20) following treatments with different modulators as indicated elsewhere. Biotinylated and nonbiotinylated proteins were separated by monomeric avidin bead binding and subjected to SDS-PAGE as described above. Biotinylated and nonbiotinylated SERT proteins were identified by immunoblotting with SERT-specific antibody.

RESULTS

CAMP and PKG-dependent SERT Phosphorylation

SERT Phosphorylation in Transfected Cells—SERT phosphorylation in transfected CHO-1 cells was measured as described previously (7, 20). Briefly, cells were metabolically labeled with [³²P]orthophosphate and subjected to treatments with different modulators as indicated elsewhere. RIPA extracts of the cells were immunoprecipitated with SR-12 antibody along with parallel control experiments using SR-12 preimmune serum as described previously (9). Immunoprecipitates were captured by protein A-Sepharose beads and subjected to SDS-PAGE followed by autoradiography to visualize phosphorylated SERT as described above.

Immunoprecipitations and Immunoblotting—SERT immunoprecipitations and immunoblotting were performed on detergent extracts of synaptosomes and transfected cells as described previously (9, 14). Protein samples were separated using a 4–15% linear gradient SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with specific antibodies as indicated in figure legends. Immunoreactive bands were visualized by chemiluminescence (ECL Plus).

Statistical Analyses—Unless indicated otherwise, experiments were conducted in triplicate, and the mean values ± S.E. from three independent experiments are given. Values are expressed as mean ± S.E. Analysis by one-way analysis of variance was used followed by post hoc testing (Tukey-Kramer and Bonferroni). Student’s t test was performed for paired observations. A value of p < 0.05 was considered statistically significant.

CAMP Stimulates 5-HT Uptake in Midbrain Synaptosomes via PKG Activation—Fig. 1 shows that the membrane-permeant CAMP analog, 8-Br-cAMP, increases 5-HT uptake by midbrain synaptosomes in a time- and dose-dependent fashion. The time course of stimulation by 250 μM 8-Br-cAMP peaked at 10–15 min and then decreased (Fig. 1A). 5-HT uptake increased maximally (25%) at 250 μM 8-Br-cAMP and decreased above that concentration (Fig. 1B). 8-Br-cGMP-stimulated 5-HT uptake was blocked almost completely when synaptosomes were incubated with PKG-specific inhibitors, KT5823 and (R)ₙ-8-pCPT-cGMPS, for 30 min prior to exposure to 8-Br-cGMP (Fig. 2). Neither the PKC inhibitor bisindolylmaleimide I nor the PKA inhibitor KT5720 blocked 8-Br-cGMP-stimulated 5-HT uptake (Fig. 2). Thus, PKG (and not PKC or PKA) seems to be responsible for the stimulation of 5-HT uptake by 8-Br-cGMP.

For subsequent experiments in this study, preparations were treated with 250 μM 8-Br-cGMP for 15 min, conditions that caused maximal stimulation of 5-HT uptake. Under these conditions, the Kₚ and Vₘₐₓ values for SERT were determined in vehicle- and 8-Br-cGMP-treated midbrain synaptosomes. 8-Br-cGMP treatment increased the maximal velocity (Vₘₐₓ) by ~25% (from 2138.2 ± 35.9 to 2725.0 ± 23.5 fmol/mg protein/min). There was no significant change in the Kₚ value following cGMP treatment (Kₚ values, control 40.39 ± 0.2 nm and 8-Br-cGMP 36.77 ± 3.3 nm).

Lack of Surface SERT Redistribution in cGMP-induced SERT Activation—One potential mechanism by which 8-Br-cGMP could increase Vₘₐₓ for 5-HT transport is by altering trafficking
cGMP and PKG-dependent SERT Phosphorylation

**FIGURE 1.** Time and dose dependence of 8-Br-cGMP on 5-HT uptake in synaptosomes. Purified rat midbrain synaptosomes were preincubated with vehicle or 250 μM 8-Br-cGMP for the times indicated (A) or treated with the indicated concentrations of 8-Br-cGMP for 10 min (B) before 5-HT uptake assays. 5-HT uptake was measured after a 3-min incubation at 37 °C, using [3H]5-HT (20 nM) as described under “Experimental Procedures.” Nonspecific assays. 5-HT uptake was measured after a 3-min incubation at 37 °C, using indicated concentrations of 8-Br-cGMP for 10 min (Fig. 1). The 32P-labeled band was not found when immunoprecipitation was performed with preimmune serum (Fig. 4).

**FIGURE 2.** Effect of PKA, PKC, and PKG inhibitors on 8-Br-cGMP-stimulated 5-HT uptake in synaptosomes. Purified rat midbrain synaptosomes were preincubated with kinase inhibitors for 30 min at 37 °C prior to the addition of 8-Br-cGMP. Synaptosomes were pretreated with vehicle, 10 μM KT5823, 100 μM (R)-8-pcPT-cGMP, 500 nM bisindolylmaleimide I (BIM I), or 100 μM KT5720 and incubated with or without 8-Br-cGMP (250 μM) for 15 min before measuring 5-HT uptake as described under “Experimental Procedures.” Values are expressed as mean ± S.E. *, p < 0.02 versus control; #, <0.02 versus 8-Br-cGMP alone by one-way ANOVA with Tukey-Kramer post-hoc analysis.

events such that more functional SERT is available on the cell-surface for 5-HT uptake. Therefore, we measured cell-surface SERT by surface biotinylation and immunoblotting (Fig. 3). Fig. 3 shows that acute treatment with 8-Br-cGMP had no effect on either SERT surface density or total SERT protein levels. These results suggest that 8-Br-cGMP activation of PKG in synaptosomes activates 5-HT uptake by increasing the intrinsic activity of SERT.

cGMP Induces PKG-dependent SERT Phosphorylation—If PKG is necessary for the regulation of SERT activity by 8-Br-cGMP, it is possible that PKG directly phosphorylates SERT, increasing its intrinsic activity. To address whether SERT is phosphorylated by PKG in synaptosomal preparations, we tested the effect of pretreatment with 8-Br-cGMP on the extent of SERT phosphorylation in synaptosomes metabolically labeled with 32P-O4. Fig. 4 shows that pretreatment of such synaptosomes with 8-Br-cGMP led to a marked increase in SERT phosphorylation. 8-Br-cGMP-induced SERT phosphorylation was effectively blocked by the PKG inhibitors KT5823 and (R)-8-pcPT-cGMPs but not by KT5720 or bisindolylmaleimide I, inhibitors of PKA or PKC, respectively (Fig. 4). The increase in 32P-SERT mirrored the effects of 8-Br-cGMP on SERT activity (Fig. 1). The 32P-labeled band was not found when immunoprecipitation was performed with preimmune serum (Fig. 4).

cGMP Increases Phosphorylation of SERT Threonine Residue(s)—Having shown that PKG activation leads to SERT phosphorylation, we next sought to determine the phosphoamino acid composition of PKG-phosphorylated SERT. Under basal, unstimulated conditions, 32P-labeled SERT was detected (Fig. 4). However, because of the low level of SERT phosphorylation, we could not determine the phosphoamino acid composition of unstimulated phospho-SERT. Fig. 5 shows that after 15 min of incubation with 8-Br-cGMP, the increased phosphorylation of SERT was exclusively in phosphothreonine. These data suggest that 8-Br-cGMP induces SERT phosphorylation on threonines but not on serines or tyrosines.

cGMP-induced SERT Regulation Requires Phosphorylation of SERT on Threonine 276—To identify the site of threonine phosphorylation in SERT, and to verify its functional importance in 5-HT transport, we used heterologous expression of hSERT mutants in CHO-1 cells. We individually mutated to alanine each of the 18 threonines predicted by homology with the structure of LeuT (21, 22) to have cytoplasmic exposure. We then expressed the mutants in CHO-1 cells and screened for the ability of 8-Br-cGMP to stimulate 5-HT uptake. SERT contains nine threonines in the N terminus (Thr-3, -4, -33, -59, -66, -67, -68, -69, and -81), one near the second intracellular loop (Thr-276), two in the fourth intracellular loop (Thr-349 and Thr-448), one in the fifth intracellular loop (Thr-519), and five in the C terminus (Thr-600, -603, -613, -616, and -618). Consistent with the stimulatory effect of 8-Br-cGMP on 5-HT uptake in synaptosomes (Fig. 1), exposure of CHO-1 cells expressing wild-type hSERT to 8-Br-cGMP (250 μM) for 15 min...
stimulated 5-HT uptake, and the effect was blocked when the PKG inhibitor (R)-8-pcPT-cGMPs was applied prior to 8-Br-cGMP treatment (Fig. 6). Of all the single threonine mutants, only T276A failed to respond to 8-Br-cGMP with an increase in 5-HT uptake (Figs. 6 and 7A). In cells expressing all other single mutants, 5-HT uptake responded to 8-Br-cGMP comparably to wild type. Following 8-Br-cGMP treatment, we found enhanced SERT incorporation of $^{32}$P in cells expressing wild-type hSERT. However, 8-Br-cGMP failed to enhance $^{32}$P incorporation into SERT in cells expressing the T276A mutant (Fig. 7, C and D). In the absence of any modulator, we detected some SERT phosphorylation in cells expressing wild-type hSERT or the T276A mutant (Fig. 7C). This basal SERT phosphorylation is due in part to p38 MAPK (7–9) and was not affected by PKG inhibitors (Figs. 4 and 7C). Total and surface expression of the SERT T276A mutant was similar to that of wild-type hSERT (Fig. 7B). In all of the other 17 threonine mutants, 8-Br-cGMP increased SERT phosphorylation, although the amount of the increase varied, probably because of differences in expression levels (data not shown). Consistent with the effect of 8-Br-cGMP in synaptosomes, 8-Br-cGMP had no effect on SERT surface expression in CHO-1 cells (Fig. 7B). This finding suggests that the phosphorylation of Thr-276 is critical for stimulation of SERT activity by 8-Br-cGMP activation of PKG.

Converting Thr-276 to Aspartate Mimics cGMP Induction of 5-HT Uptake—Phosphorylation of SERT at the target Thr-276 adds a negative charge that might influence SERT activity. Having shown that SERT phosphorylation at Thr-276 is critical for 8-Br-cGMP and PKG-mediated SERT modulation, we next tested whether substitution of Thr-276 with aspartic acid, (which in its ionized form carries a negative charge) would mimic these effects on 5-HT uptake. Substitution of Thr-276 to aspartic acid (T276D) per se did not affect total or surface expression of SERT (Fig. 8, B and C). However, T276D had 25% higher 5-HT uptake activity, similar to the 8-Br-cGMP stimulation of 5-HT uptake observed in wild-type hSERT (Fig. 8A). Furthermore, cells expressing T276D hSERT failed to respond to 8-Br-cGMP treatment at the concentration shown to increase 5-HT uptake in cells expressing wild-type hSERT (Fig. 8A). These data further support the idea that 8-Br-cGMP activates PKG to phosphorylate SERT on Thr-276 leading to

**FIGURE 3. Effect of 8-Br-cGMP on cell surface SERT density in synaptosomes.** A, SERT immunoblot. Synaptosomes were treated with 250 $\mu$M 8-Br-cGMP or vehicle for 30 min and then biotinylated with sulfo-NHS-SS-biotin as described under “Experimental Procedures.” Aliquots (50 $\mu$L) of total nonbiotinylated and entire biotinylated fractions were subjected to SDS-PAGE followed by immunoblotting with affinity-purified SR-12 SERT antibody. A representative SERT immunoblot of four separate experiments is shown. B, quantitative analysis of SERT band densities. Biotinylation of SERT proteins were quantified using NIH image, and the densities of SERT band from four separate experiments are presented as mean ± S.E. No significant changes were observed in surface or intracellular SERT following 8-Br-cGMP treatment compared with vehicle treatment. (Student’s $t$ test).

**FIGURE 4. Effect of 8-Br-cGMP on native SERT phosphorylation in synaptosomes.** A, synaptosomes were metabolically labeled with [$^{32}$P]orthophosphate for 60 min at 37 °C and treated with vehicle and the indicated regulatory agents. Kinase inhibitors (KT5823, 10 $\mu$M; (R)-8-pcPT-cGMPs, 100 $\mu$M; bisindolylmaleimide I (BIM1), 500 nM; and KT5720, 100 $\mu$M) were added 30 min before addition of 8-Br-cGMP (250 $\mu$M) and a further incubation of 15 min at 37 °C. RIPA extraction, immunoprecipitation (IP), SDS-PAGE and autoradiography were performed as described under “Experimental Procedures.” The $^{32}$P-labeled SERT was specifically immunoprecipitated by SERT-immune sera. Parallel experiments were performed using preimmune sera, to validate specificity of SERT specific antibody (Ab) SR-12. Note that there was no $^{32}$P-labeled SERT band when preimmune serum was used. The autoradiogram shown is a representative of three experiments. B, the bar graph shows the relative intensity of $^{32}$P-labeled SERT. Data are presented as mean values ± S.E. Asterisk indicates significant differences compared with vehicle controls ($p < 0.05$; Student’s $t$ test).
increased 5-HT uptake, as suggested by the results of the phosphorylation experiments.

**DISCUSSION**

Although SERT is a phosphoprotein, the direct role of SERT phosphorylation in regulating SERT activity was unknown. In this study, we examined the molecular mechanisms responsible for the activation of SERT by cGMP using both endogenous SERT in midbrain synaptosomes and heterologously expressed hSERT in CHO-1 cells. Our functional and mutational studies indicate that stimulation of SERT by PKG required phosphorylation of Thr-276 providing the first insight into the molecular link between SERT phosphorylation and 5-HT transport.

Activation of adenosine receptors in RBL cells or in CHO-1 cells transfected with SERT and adenosine receptors stimulates 5-HT uptake through PKG activation (11, 13, 23). Nitric oxide (NO) stimulation of SERT expressed in HeLa and COS cells is also via the cGMP pathway (10). In platelets, activation of histamine receptors stimulates 5-HT uptake through the activation of PKG (12). Cyclic GMP analogs also stimulate 5-HT uptake in these model systems (11, 13, 23). However, the detailed molecular mechanisms, including the site of SERT phosphorylation, were not established. In agreement with these previous studies, we show here both in neuronal preparations and in transfected CHO-1 cells that activation of PKG by 8-Br-cGMP stimulates 5-HT uptake. However, in both synaptosomes and CHO-1 cells, the increase in $V_{\text{max}}$ was not because of increased SERT surface expression, suggesting that PKG raises the intrinsic rate at which SERT transports 5-HT.

A biphasic effect of 8-Br-cGMP was apparent from the dose and time response experiments. Stimulation of 5-HT uptake was maximal at intermediate concentrations of 8-Br-cGMP and decreased at higher doses and longer times of...
cells were metabolically labeled with [32P]orthophosphate for 90 min at 37 °C before addition of 8-Br-cGMP as described under "Experimental Procedures" and in the legend to Fig. 3. Quantitation of SERT band density. Surface SERT expression using biotinylation was performed as described under "Experimental Procedures" and in the legend to Fig. 4. The autoradiogram shown is a representative of three experiments.

Surface expression, and phosphorylation in CHO-1 cells. CHO-1 cells were transfected with either empty vector (pcDNA3), wild-type hSERT, or T276A-hSERT expression plasmids as indicated. 24 h after transfection, cells were assayed for 5-HT uptake, surface biotinylation, and SERT phosphorylation as described under "Experimental Procedures." A, 5-HT uptake assays. Cells were washed once with KRH buffer and treated with 8-Br-cGMP (250 μM) or vehicle for 15 min at 37 °C before measuring 5-HT uptake as described under the legend to Fig. 6. B, SERT surface expression, and quantitation of SERT band density. Surface SERT expression using biotinylation was performed as described under "Experimental Procedures" and in the legend to Fig. 3. C, SERT phosphorylation. Cells were metabolically labeled with [32P]orthophosphate for 90 min at 37 °C before addition of 8-Br-cGMP (250 μM) and the subsequent 15 min incubation before immunoprecipitations as described under "Experimental Procedures" and in the legend to Fig. 4. The autoradiogram shown is a representative of three experiments. D, quantitation of phospho-SERT band densities. The bar graph shows the relative intensity of 32P-labeled SERT averaged from three phosphorylation experiments. Values are expressed as mean ± S.E. Asterisk indicates significant differences compared with vehicle (p < 0.01 one-way ANOVA with Bonferroni post-hoc analysis).

FIGURE 7. Effect of Thr-276 point mutation on 8-Br-cGMP effects on 5-HT uptake, SERT surface expression, and phosphorylation in CHO-1 cells. CHO-1 cells were transiently expressing hSERT or T276A-hSERT and treated with adenosine receptor-transfected CHO-1 cells or 8-Br-cGMP (250 μM) or vehicle for 15 min at 37 °C before measuring 5-HT uptake as described under the legend to Fig. 6. B, SERT surface expression, and quantitation of SERT band density. Surface SERT expression using biotinylation was performed as described under "Experimental Procedures" and in the legend to Fig. 3. C, SERT phosphorylation. Cells were metabolically labeled with [32P]orthophosphate for 90 min at 37 °C before addition of 8-Br-cGMP (250 μM) and the subsequent 15 min incubation before immunoprecipitations as described under "Experimental Procedures" and in the legend to Fig. 4. The autoradiogram shown is a representative of three experiments. D, quantitation of phospho-SERT band densities. The bar graph shows the relative intensity of 32P-labeled SERT averaged from three phosphorylation experiments. Values are expressed as mean ± S.E. Asterisk indicates significant differences compared with vehicle (p < 0.01 one-way ANOVA with Bonferroni post-hoc analysis).

Our phosphorylation studies suggested that PKG triggers SERT phosphorylation on threonine residue(s) in synaptosomal preparations. Replacement of Thr-276, located near the cytoplasmic end of the fifth transmembrane domain (22), prevented stimulation of SERT activity, but mutation of each of the 17 other predicted intracellular threonine residues in SERT failed to block stimulation of 5-HT transport by 8-Br-cGMP. Moreover, substitution of Thr-276 with alanine also eliminated the ability of 8-Br-cGMP to stimulate SERT phosphorylation but did not perturb SERT basal phosphorylation. Substitution of Thr-276 with aspartic acid (T276D) to mimic the phosphorylated form of Thr-276 resulted in higher 5-HT uptake than in wild type or T276A, consistent with Thr-276 as the site of 8-Br-cGMP effects. Moreover, T276D did not respond to 8-Br-cGMP. These results further support the involvement of Thr-276 phosphorylation in PKG-mediated SERT regulation.

Our results do not rule out the possibility that, under certain experimental conditions, PKG-mediated SERT regulation could proceed through alternative pathways or that in different cell types SERT regulation or trafficking in response to PKG may differ from what we have observed in synaptosomes and CHO-1 cells. Such changes could reflect cell-specific expression of SERT-associated proteins or signaling pathways. For example, exposure of 8-Br-cGMP to HeLa cells transiently expressing hSERT resulted in increased 5-HT uptake and surface binding of the high affinity cocaine analog 125I-labeled RTI-55 to SERT (24). In addition, activation of adenosine receptor increased 5-HT uptake and SERT surface expression via activation of PKG and p38 MAPK in RBL-2H3 cells and SERT and adenosine receptor-transfected CHO-1 cells (13). However, we were unable to reproduce 8-Br-cGMP-mediated increase in surface SERT, although we did observe the reported increase in 5-HT uptake following 8-Br-cGMP treatment. We3 and Prasad and co-workers (24) found that the effects of 8-Br-cGMP on 5-HT uptake and surface expression were highly sensitive to the level of hSERT expression in heterologous cell models.

Nevertheless, we found that activation of PKG increased 5-HT uptake with no change in surface SERT expression in the physiologically relevant native preparation (synaptosomes) and in heterologously transfected CHO-1 cells. The discrepancies in 8-Br-cGMP-mediated increase in surface SERT between our studies and others remain to be understood. However, our results are consistent with those of others to the extent that all show increase in surface SERT, although we did observe the reported increase in 5-HT uptake following 8-Br-cGMP treatment. We3 and Prasad and co-workers (24) found that the effects of 8-Br-cGMP on 5-HT uptake and surface expression were highly sensitive to the level of hSERT expression in heterologous cell models.

Nevertheless, we found that activation of PKG increased 5-HT uptake with no change in surface SERT expression in the physiologically relevant native preparation (synaptosomes) and in heterologously transfected CHO-1 cells. The discrepancies in 8-Br-cGMP-mediated increase in surface SERT between our studies and others remain to be understood. However, our results are consistent with those of others to the extent that all show increase in 5-HT uptake following 8-Br-cGMP treatment. We3 and Prasad and co-workers (24) found that the effects of 8-Br-cGMP on 5-HT uptake and surface expression were highly sensitive to the level of hSERT expression in heterologous cell models.

Nevertheless, we found that activation of PKG increased 5-HT uptake with no change in surface SERT expression in the physiologically relevant native preparation (synaptosomes) and in heterologously transfected CHO-1 cells. The discrepancies in 8-Br-cGMP-mediated increase in surface SERT between our studies and others remain to be understood. However, our results are consistent with those of others to the extent that all show increase in 5-HT uptake following 8-Br-cGMP treatment. We3 and Prasad and co-workers (24) found that the effects of 8-Br-cGMP on 5-HT uptake and surface expression were highly sensitive to the level of hSERT expression in heterologous cell models.

Because some of our conclusions are based on results with heterogeneous expression of mutant SERTs, there is a possibility that phosphorylation of Thr-276 is a downstream effect because of PKG phosphorylation of another protein or that another threonine residue in SERT is phosphorylated by PKG.

3 D. J. Samuvel, L. D. Jayanthi, and S. Ramamoorthy, unpublished observations.
cGMP and PKG-dependent SERT Phosphorylation

**FIGURE 8.** Effect of Thr-276 replacement with aspartic acid (T276D) on SERT functional expression and 8-Br-cGMP effects. CHO-1 cells were transfected with wild-type hSERT or T276D-hSERT expression plasmids as indicated. 24 h after transfection, cells were assayed for 5-HT uptake (A) and SERT surface expression (B and C) as described under “Experimental Procedures” and in the Fig. 7 legend. *, p < 0.05 indicates significant difference compared with vehicle-treated wild type; #, p < 0.05 compared with vehicle treated wild type by one-way ANOVA with Bonferroni post-hoc analysis.

and mutations at Thr-276 block the phosphorylation or its subsequent effect on activity. We did not observe ablation of PKG-mediated SERT stimulation or phosphorylation with any threonine mutants tested except for those at Thr-276. It is conceivable, but unlikely, that phosphorylation could occur at another threonine residue that is not predicted to be exposed to the cytoplasm, and that mutation of Thr-276 somehow blocks that process.

By analogy with the corresponding region of LeuT (21, 22) and from cysteine scanning experiments with rSERT (25), Thr-276 is in an α-helical region of the fifth transmembrane domain. The family of protein kinases containing PKG is believed to phosphorylate their peptide substrates in an extended (not helical) conformation (26). This consideration suggests that if the current assessment of TM regions in SERT is accurate, either a kinase active on α-helices (27) phosphorylates Thr-276 or the conformation of this region undergoes a transition that allows it to be a substrate for PKG. Recent data suggest that this part of SERT is involved in conformational transitions that accompany 5-HT transport (28), and some of these transitions may allow the direct action of PKG on Thr-276. Although our working hypothesis is that PKG phosphorylates SERT directly, it is possible that PKG regulates SERT catalytic function through other downstream kinase(s) or by some other indirect mechanism. Future studies involving direct phosphorylation of SERT in vitro and sequencing of phosphorylated SERT and site-specific phospho-SERT antibodies are warranted for understanding the direct regulatory role of Thr-276 in modulating SERT activity by PKG.

Despite these reservations, the simplest interpretation of our data would conclude that Thr-276 is the target residue for PKG. In addition, we have demonstrated PKG-mediated SERT regulation and phosphorylation in neuronal preparations, thus revealing a potential physiological regulation of 5-HT transport in the central nervous system. The amino acid sequence encompassing Thr-276 is identical in dopamine transporter, NET and SERT. Recently we showed the requirement of Thr-258 and Ser-259 phosphorylation of hNET for PKC-mediated NET sequestration and raft-mediated redistribution (29). A recent report showed that in CHO-1-transfected cells, 8-Br-cGMP has no effect on NET and dopamine transporter activities, suggesting a selective action of PKG on SERT regulation among the biogenic amine transporters (23).

Protein phosphorylation and dephosphorylation are common rapid and reversible means of transducing signals from cell surface receptors into varied cellular responses. It should not be surprising that neurons are able to use phosphorylation to control neurotransmission by altering SERT activity. Activation and deactivation of SERT via phosphorylation and dephosphorylation can affect the availability of synaptic 5-HT, which regulates serotonergic signaling. cGMP signaling through PKG is widely distributed in the central nervous system and peripheral nervous system, including areas known to express SERT (30, 31). PKG might be activated by cGMP generated either by receptor activation or generation of NO (31). Presynaptic receptors for adenosine and histamine and nitric-oxide synthase are expressed in serotonergic neurons and might be involved in PKG-mediated SERT regulation (32–37). Because it is rapidly diffusible, NO could be formed in neurons (both pre- and post-synaptic) or glia (31). Thus, NO could be a messenger for regulating 5-HT neurotransmission through PKG-mediated SERT phosphorylation. Furthermore, presynaptic adenosine, histamine receptors, and NO regulate 5-HT release (32, 33, 38–40). Thus, it is possible that stimulation of PKG via presynaptic receptors or NO-mediated cGMP synthesis regulates both synaptic 5-HT release and uptake for normal serotonergic signaling in the central nervous system. Any perturbation in SERT homeostasis might lead to altered 5-HT signaling and 5-HT related behavior. Alternatively, structural mutations in SERT might disturb PKG-mediated SERT regulation and phosphorylation. Recent findings suggest that SERT coding mutations T44A, G56A, E215K, I425V, K605N, and P621S constitutively stimulated SERT activity and eliminated PKG-dependent SERT up-regulation by NO and 8-Br-cGMP as well as p38 MAPK-dependent SERT up-regulation by anisomycin (10, 24, 41). Intriguingly, hSERT coding mutation G56A, which is associated with autism, exhibits elevated 5-HT uptake and basal SERT phosphorylation that could not be further increased.
by 8-Br-cGMP treatment (24). The identification of as yet unknown complex SERT regulatory paradigms in vivo as well as the influence of naturally occurring SERT coding variants found in psychiatric diseases may further our understanding of the molecular link underlying SERT phosphorylation and regulation.

REFERENCES
1. Amara, S., and Kuhar, M. (1993) Annu. Rev. Neurosci. 16, 73–93
2. Barker, E. L., and Blakely, R. D. (1995) in Psychopharmacology: The Fourth Generation of Progress (Bloom, F. E., and Kupfer, D. J., eds) pp. 321–333, Raven Press, New York
3. Ramamoorthy, S. (2002) in Neurotransmitter Transporter: Structure, Function, and Regulation (Reith, M. E. A., ed) pp. 1–23, Humana Press Inc., Totowa, NJ
4. Blakely, R. D., Ramamoorthy, S., Qian, Y., Schroeter, S., and Bradely, C. (1997) Neurotransmitter Transporter: Structure, Function and Regulation (Reith, M. E. A., ed) pp. 29–72, Humana Press Inc., Totowa, NJ
5. Qian, Y., Galli, A., Ramamoorthy, S., Risso, S., DeFelice, L. J., and Blakely, R. D. (1997) J. Neurosci. 17, 45–47
6. Jayanthi, L. D., Ramamoorthy, S., Mahesh, V. B., Leibach, F. H., and Ganapathy, V. (1994) J. Biol. Chem. 269, 14424–14429
7. Ramamoorthy, S., and Blakely, R. D. (1999) Science 285, 763–766
8. Ramamoorthy, S., Giovannetti, E., Qian, Y., and Blakely, R. D. (1998) J. Biol. Chem. 273, 2458–2466
9. Samuvel, D. J., Jayanthi, L. D., Bhat, N. R., and Ramamoorthy, S. (2005) J. Neurosci. 25, 29–41
10. Kilic, F., Murphy, D. L., and Rudnick, G. (2003) Mol. Pharmacol. 64, 440–446
11. Miller, K. J., and Hoffman, B. J. (1994) J. Biol. Chem. 269, 27351–27356
12. Layauf, J., Bondoux, D., Oset-Gasque, M., Emami, S., Mutel, V., Haimart, M., and Gaspach, C. (1994) Am. J. Physiol. 266, 526–536
13. Zhu, C. B., Hewlett, W. A., Feoktistov, I., Biaggioni, I., and Blakely, R. D. (2004) Mol. Pharmacol. 65, 1462–1474
14. Bauman, A. L., Apparsundaram, S., Ramamoorthy, S., Wadzinski, B. E., Vaughan, R. A., and Blakely, R. D. (2000) J. Neurosci. 20, 7571–7578
15. Androulitis-Theotokis, A., and Rudnick, G. (2002) J. Neurosci. 22, 8370–8378
16. Ramamoorthy, S., Bauman, A., Moore, K., Han, H., Yang-Feng, T., Chang, A., Ganapathy, V., and Blakely, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2542–2546
17. Blakely, R., Berson, H., Fremeau, R., Caron, M., Peak, M., Prince, H., and Bradely, C. (1991) Nature 354, 66–70
18. Vaughan, R. A. (2004) J. Pharmacol. Exp. Ther. 310, 1–7
19. Ramamoorthy, S., and Balasubramanian, A. S. (1989) Biochem. J. 258, 777–783
20. Ramamoorthy, S., Melikian, H. E., Qian, Y., and Blakely, R. D. (1998) Methods Enzymol. 296, 347–370
21. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Nature 437, 215–223
22. Beuming, T., Shi, L., Javitch, J. A., and Weinstein, H. (2006) Mol. Pharmacol. 70, 1630–1642
23. Zhu, C. B., Hewlett, W. A., Francis, S. H., Corbin, J. D., and Blakely, R. D. (2004) Eur. J. Pharmacol. 504, 1–6
24. Prasad, H. C., Zach, C.-B., McAuley, I. L., Samuvel, D. J., Ramamoorthy, S., Shelton, R., Hewlett, W. A., Sutcliffe, J. S., and Blakely, R. D. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 11545–11550
25. Zhang, Y. W., and Rudnick, G. (2005) J. Biol. Chem. 280, 30807–30813
26. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–420
27. Drenman, D., and Ryazanov, A. G. (2004) Prog. Biophys. Mol. Biol. 85, 1–32
28. Zhang, Y. W., and Rudnick, G. (2006) J. Biol. Chem. 281, 36213–36220
29. Jayanthi, L. D., Annamalai, B., Samuvel, D. J., Gether, U., and Ramamoorthy, S. (2006) J. Biol. Chem. 281, 23326–23340
30. Ruth, P., Landgraf, W., Keilbach, A., May, B., Egleme, C., and Hofmann, F. (1991) Eur. J. Biochem. 202, 1339–1344
31. Ruth, P. (1999) Pharmacol. Ther. 82, 355–372
32. Okada, M., Kawata, Y., Murakami, T., Wada, K., Nizuno, K., Kondo, T., and Kaneko, S. (1999) Eur. J. Neurosci. 11, 1–9
33. Threlfell, S., Cragg, S. J., Kallo, I., Turi, G. F., Coen, C. W., and Greenfield, S. A. (2004) J. Neurosci. 24, 8704–8710
34. Southam, E., and Garthwaite, J. (1993) Neuropharmacology 32, 1267–1277
35. Boulton, C. L., Irving, A. J., Southam, E., Potier, B., Garthwaite, J., and Collingridge, G. L. (1994) Eur. J. Neurosci. 6, 1528–1535
36. Garthwaite, J., and Boulton, C. L. (1995) Annu. Rev. Physiol. 57, 683–706
37. Simpson, K. L., Waterhouse, B. D., and Lin, R. C. (2003) J. Comp. Neurol. 466, 495–512
38. Fink, K., Schlicker, E., Neise, A., and Goethert, M. (1990) Naunyn-Schmiedebergs Arch. Pharmacol. 342, 513–519
39. Sinner, C., Kaehler, S. T., Philippu, A., and Singewald, N. (2001) Naunyn-Schmiedebergs Arch. Pharmacol. 364, 105–109
40. Kaehler, S. T., Singewald, N., Sinner, C., and Philippu, A. (1999) Brain Res. 835, 346–349
41. Ozaki, N., Goldman, D., Kaye, W. H., Plotnicov, K., Greenberg, B. D., Lappalainen, J., Rudnick, G., and Murphy, D. L. (2003) Mol. Psychiatry 895, 933–936