Deletion of the Serotonin 5-HT$_{2C}$ Receptor PDZ Recognition Motif Prevents Receptor Phosphorylation and Delays Resensitization of Receptor Responses*

Received for publication, February 4, 2000, and in revised form, March 22, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M000922200

Jon R. Backstrom‡, Raymond D. Price, Darcie T. Reasoner, and Elaine Sanders-Bush
From the Department of Pharmacology and the Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6600

Phosphorylation-deficient serotonin 5-HT$_{2C}$ receptors were generated to determine whether phosphorylation promotes desensitization of receptor responses. Phosphorylation of mutant 5-HT$_{2C}$ receptors that lack the carboxyl-terminal PDZ recognition motif (Ser$^{459}$-Ser$^{461}$-Ser$^{463}$-Val-COOH; ΔPDZ) was not detectable based on a band-shift phosphorylation assay and incorporation of $[^{32}P]$. Treatment of cells stably expressing ΔPDZ or wild-type 5-HT$_{2C}$ receptors with serotonin produced identical maximal responses and EC$_{50}$ values for eliciting [H]-inositol phosphate formation. In calcium imaging studies, treatment of cells expressing ΔPDZ or wild-type 5-HT$_{2C}$ receptors with 100 nM serotonin elicited initial maximal responses and decay rates that were indistinguishable. However, a second application of serotonin 2.5 min after washout caused maximal responses that were 5-fold lower with ΔPDZ receptors relative to wild-type 5-HT$_{2C}$ receptors. After 10 min, responses of ΔPDZ receptors recovered to wild-type 5-HT$_{2C}$ receptor levels. Receptors with single mutations at Ser$^{458}$ (S458A) or Ser$^{459}$ (S459A) decreased serotonin-mediated phosphorylation to 50% of wild-type receptor levels. Furthermore, subsequent calcium responses of S459A receptors were diminished relative to S458A and wild-type receptors. These results establish that desensitization occurs in the absence of 5-HT$_{2C}$ receptor phosphorylation and suggest that receptor phosphorylation at Ser$^{459}$ enhances resensitization of 5-HT$_{2C}$ receptor responses.

Serotonin 5-HT$_{2C}$ (formerly 5-HT$_{1C}$) receptors exist as several isoforms throughout the brain, due to RNA editing and alternative splicing, and function to stimulate phospholipase C through activation of the G protein $G_{i/o}$ (1). Whereas RNA editing generates isoforms in the second intracellular loop that modify receptor signaling (2–4), alternative splicing creates truncated nonfunctional receptors (5, 6). In addition, agonists promote phosphorylation of nonedited 5-HT$_{2C}$ receptors (7), raising the possibility that 5-HT$_{2C}$ receptors are also regulated dynamically.

The phosphorylation of G protein-coupled receptors regulates signaling through multiple mechanisms including receptor desensitization, which attenuates second messenger responses. In the case of phospholipase C-linked receptors, phosphorylation has been demonstrated to promote desensitization based on observations that receptors lacking the corresponding phosphorylation sites display sustained phosphoinositide hydrolysis responses relative to wild-type receptors (8–13). Furthermore, a second application of agonist produces amplified phosphoinositide hydrolysis (14) and calcium release (13, 15, 16) by phosphorylation-deficient mutants relative to wild-type receptors, illustrating that mutation of phosphorylation sites involved in desensitization enhances both initial and secondary responses.

Although desensitization of 5-HT$_{2C}$ receptor-mediated responses has been observed in assays that examine phosphoinositide hydrolysis (7, 17), release of intracellular calcium (18, 19), and Ca$^{2+}$-activated currents (20), it is unknown whether phosphorylation of the 5-HT$_{2C}$ receptor is involved in desensitization. To address this issue, we identified a 5-HT$_{2C}$ receptor domain that is required for receptor phosphorylation and performed functional assays with phosphorylation-deficient receptor mutants. First, we determined that phosphorylation of the 5-HT$_{2C}$ receptor requires the carboxyl-terminal PDZ (PSD-95 discs-large ZO-1) recognition motif, a domain present in non-edited and edited isoforms of the 5-HT$_{2C}$ receptor. Next, we found that phosphorylation-deficient receptors display identical initial responses as the wild-type 5-HT$_{2C}$ receptor in phosphoinositide hydrolysis and calcium release assays. However, phosphorylation-deficient receptors exhibit diminished secondary responses and a delayed recovery relative to wild-type 5-HT$_{2C}$ receptors. Cells stably expressing 5-HT$_{2C}$ receptors with a single serine-to-alanine mutation also display decreased receptor phosphorylation and diminished secondary calcium responses. We therefore propose that 5-HT$_{2C}$ receptor phosphorylation promotes resensitization of 5-HT$_{2C}$ receptor-mediated responses.

**Experimental Procedures**

5-HT$_{2C}$ Receptor Antibodies—The production, purification, and characterization of anti-peptide antibodies against rat 5-HT$_{2C}$ receptors have been described (21, 22). Antibodies against a region of the third intracellular loop (referred to below as anti-2C-IC antibodies) were generated against amino acids 270–288 (NH$_{2}$-CKNGGEEENAPNPCPDQK-COOH) of the rat sequence and purified against a shorter peptide (NH$_{2}$-CKNGGEEENAPNPCPDQK-COOH). Antibodies against a region of the carboxyl terminus (referred to below as anti-3C-CT antibodies) were generated against amino acids 419–435 of the rat sequence (NH$_{2}$-RHTNERYKANDPEPGCCOOH, with cysteine added to the carboxyl terminus of peptide) and purified against the same peptide.

Generation of Mutant 5-HT$_{2C}$ Receptors—Mutant 5-HT$_{2C}$ receptors were generated as described previously (21). The 5-HT$_{2C}$ receptor was cloned into the expression vector pcDNA3.1 (Invitrogen). The Ser$^{458}$ (S458A) and Ser$^{459}$ (S459A) mutants were generated by site-directed mutagenesis and confirmed by DNA sequencing.
wild-type and mutant 5-HT$_2C$ receptor cDNA in a Bluescript plasmid (Stratagene, La Jolla, PA). Confocal images were captured with a Zeiss LSM410 confocal tors.

The PDZ recognition motif (S$_{458}$) at a dilution of 1:1000 in TBS containing 1% BSA was Cy3-labeled donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) for wild-type and S459A receptors is illustrated in Fig. 1.

FIG. 1. Carboxy-terminal sequences of wild-type and mutant 5-HT$_2C$ receptors. The PDZ recognition motif (SXV) of wild-type and S459A receptors is underlined.

were generated from wild-type rat receptor cDNA (1NI isoform) (2) using recombinant Pu polymerase (Promega, Madison, WI), a forward primer (primer 16.3, 5'-TTGGCATGTGATCTTCGTTTGTCTGTA-3'), and a reverse primer containing an XhoI site. For mutants lacking the PDZ recognition motif (ΔPDZ), polymerase chain reaction was performed with primer 16.3 and the reverse primer 5'TGCCTAGTATTA-AATCCTGCTCGACACCATGTA-3'. For the S458A and S459A mutants, two rounds of polymerase chain reaction were performed with primer 16.3 and overlapping reverse primers. The first round was performed with primer 16.3 and 5'TTACACGGAATCTCTCGTGACACCATAGTA-3' (S458A) or 5'TTACACGGAATCTCTCGTGACACCATGTA-3' (S459A, XbaI) or 5'TTTCTAGATATACACGGCACTAATCTCTGCT-3' (S459A, XbaI). For mutants lacking the carboxyl terminus except for the immunodominant region of the 2C-CT epitope and the conserved cytochrome (Δ375/CT), two rounds of polymerase chain reaction were performed as described in detail elsewhere. Amplified DNA was digested with XhoI and XbaI and ligated into the Stru/IXbaI sites of the wild-type 5-HT$_2C$ receptor cDNA in a Bluescript plasmid (Stratagene, La Jolla, CA). Sequences were confirmed by automated sequencing at the Core Facility of the Center for Molecular Neuroscience at Vanderbilt University. Receptor cDNA was isolated from the Bluescript plasmid with KpnI and XhoI and ligated into the mammalian expression plasmid pCMV2 (a gift of Dr. David Russell). The carboxy-terminal sequences of wild-type, ΔPDZ, Δ375/CT, S458A, and S459A 5-HT$_2C$ receptors are illustrated in Fig. 1.

**Immunocytochemistry—**NIH 3T3 fibroblasts were transiently transfected with wild-type or mutant 5-HT$_2C$ receptor cDNA to determine whether the cellular distribution of mutant receptors differed from that of wild-type 5-HT$_2C$ receptors. Fibroblasts electroporated with 25 μg of receptor cDNA were seeded in eight-well Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) containing Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) with 9% bovine serum (Hyclone Laboratories, Logan, UT), 5 units/ml penicillin, and 5 μg/ml streptomycin. Positive clones were selected with G418 (Geneticin, Life Technologies, Inc.). Stable cell lines were grown in DMEM containing 9% bovine serum, 0.5 mg/ml G418, 5 units/ml penicillin, and 5 μg/ml streptomycin. Positive clones were identified by immunocytochemistry. Mutant clones with receptor levels similar to the wild-type 5-HT$_2C$ receptor cell line were utilized in subsequent functional assays. Mutant ΔPDZ receptors demonstrated identical affinities for the agonists serotonin (5-HT), 2,5-dimethoxy-4-iodoamphetamine (DOI), and lysergic acid diethylamide (LSD) as wild-type 5-HT$_2C$ receptors in competition binding experiments with $[3H]$imidosulergine (data not illustrated).

**Band-shift Phosphorylation Assay—**Cells grown in the presence of serum were washed four times with serum-free DMEM and replaced with 5 ml of DMEM. Tunicamycin (Roche Molecular Biochemicals) was added to a final concentration of 2 μg/ml of medium (7). After 6 h, agonist was added, and incubation was continued for an additional 15 min at 37 °C. Cell supernatants were discarded and replaced with 1 ml of phosphate/EDTA (PE) extraction buffer (50 mM Na$_2$HPO$_4$/Na$_3$PO$_4$, pH 7.2, containing 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 5 mM Na$_3$PO$_4$, and 1 mM Na$_3$VO$_4$) was co-electroporated with 8.6). Cells grown in the presence of 10% SDS-polyacrylamide gels. Proteins were transferred from gels to nitrocellulose membranes Amersham Pharmacia Biotech) in a modified Towbin transfer buffer (25 mM Tris and 192 mM glycine, pH ~8.6). Membranes were probed with anti-2C-IC or anti-2C-CT antibodies, and the immunoreactive bands were detected using alkaline phosphatase-conjugated secondary antibodies (Dako Corp., Carpinteria, CA) as described (21). Band intensities were quantitated on a Macintosh computer using the public domain NIH Image program developed at the United States National Institutes of Health.

**Immunoprecipitation of $^{32}$P-Labeled 5-HT$_2C$ Receptors—**Cells stably expressing wild-type or mutant ΔPDZ 5-HT$_2C$ receptors were labeled with $[^{32}]$Porthophosphate as described (7), except cells were incubated with 0.2 μCi of $[^{32}]$P/ml for 2 h. Cells were untreated or treated with 1 μM 5-hydroxytryptamine for 15 min. Membrane fractions of cells were collected and PE extraction buffer, and detergent-soluble proteins were extracted with 0.3 ml of PE extraction buffer containing 150 mM NaCl, 1% Triton X-100, and 0.1% SDS. For immunoprecipitation of receptors, 1 μg of antibody was added to detergent-soluble protein and incubated overnight at 4 °C with rocking. Ten μl of goat anti-rabbit antibody beads (generating by conjugating Fe fragment-specific antibodies (Jackson ImmunoResearch Laboratories, Inc.) to protein G-agarose (Pierce) in 20 ml dimethyl pimelimidate to a final concentration of 4 mg of antibodies/ml of gel) was added, and incubation was continued for 4 h at 4 °C with rocking. The beads were pelleted and washed sequentially with PE extraction buffer containing the following: 0.5% Triton X-100 and 0.5 mM NaCl; 0.25% Triton X-100 and 0.1 mM NaCl; 0.1% Triton X-100; and 0.1% SDS (one wash each). Fifteen μl of PE extraction buffer containing 1% SDS was added to the beads, and the mixture was incubated for 5 min at room temperature. Then, 15 μl of PE extraction

*1 J. R. Backstrom, manuscript in preparation.

*2 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
buffer containing 5% Nonidet P-40 was added to the beads; and after mixing, 90 µl of PE extraction buffer and 2 µl (0.4 units) of recombinant N-glycosidase F (Roche Molecular Biochemicals) were added. After incubation for 2 h at 37°C, 40 µl of 4X sample buffer was added to the suspension, and 70 µl of each fraction was electrophoresed on two 10% gels. One gel was transferred to nitrocellulose, and membranes were probed with anti-2C-IC antibodies; and the other gel was dried and exposed to a PhosphorImager cassette (Molecular Dynamics, Inc.).

**Phosphoinositide Hydrolysis Assay**—Cells stably expressing ΔPDZ (clone ΔPDZ-2) or wild-type 5-HT2C receptors were grown in serum-free medium in the presence of myo-[3H]inositol as described (23). Cells were stimulated with increasing concentrations of serotonin in the presence of 10 mM lithium chloride and 10 μM pargyline for 30 min. [3H]Inositol monophosphates were extracted, isolated by anion-exchange chromatography, and quantitated by liquid scintillation counting. Concentration-response curves and EC50 values were calculated with GraphPad Prism software.

**Calcium Imaging**—Calcium imaging was performed with cells in 35-mm plastic culture dishes. Cells in DMEM containing 9% fetal bovine serum were grown to 50–70% confluence. The medium was replaced with serum-free DMEM and incubated overnight. Cells were loaded with the calcium indicator fura-2/AM (2 µg/ml; Molecular Probes, Inc., Eugene OR) for 1 h at room temperature in HEPES/calcium buffer. Loaded cells were continuously superfused with HEPES/calcium buffer and visualized using a Nikon inverted microscope attached to a Compix calcium imaging system. The imaging system consists of a CCD-72 camera (Dage-MTI, Inc., Michigan City, IN) attached to an IBM compatible computer executing SIMCA C-imaging software (Compix, Inc.). Intracellular calcium was visualized by fluorescence ratio measurements at wavelengths of 340 and 380 nm. The response to the initial challenge with 100 nM serotonin was monitored for 10 min. Cells were then washed with HEPES/calcium buffer for 2.5, 5, or 10 min before a second application of 100 nM serotonin. The half-lives for decay of the initial responses were calculated as the time between maximal and half-maximal responses. The second peak response of a cell was divided by the first peak response to determine the percent initial response for each cell. Responses were obtained from 20–50 cells/experiment. Results from three independent experiments (means ± S.D.) were plotted versus the time interval between agonist challenges.

**RESULTS**

**Characterization of 5-HT2C Receptor Phosphorylation**—Fibroblasts stably expressing the wild-type 5-HT2C receptor were grown in serum-free medium containing tunicamycin to generate newly synthesized, unglycosylated 5-HT2C receptors. Cells were either untreated or treated with serotonin or with serotonin in the presence of the antagonist mianserin. Membrane proteins were solubilized with CHAPS detergent and electrophoresed on SDS-polyacrylamide gels. Immunoblots were probed with 5-HT2C receptor antibodies against a region of the third intracellular loop (anti-2C-IC) or carboxyl terminus (anti-2C-CT). Untreated cells contained an immunoreactive protein with a mass of 40 kDa (Fig. 2A, first lane). Treatment of cells with serotonin caused formation of an additional 41-kDa immunoreactive protein (Fig. 2A, second lane). The serotonin-mediated shift in mass from 40 to 41 kDa was blocked by preincubation with the antagonist mianserin (Fig. 2A, third lane). The amount of 41-kDa protein was dependent on serotonin concentration (Fig. 2B) and was maximal after treatment with 100 nM serotonin. Experimental controls were performed on glycosylated receptors that were treated with N-glycosidase F to remove N-linked sugars before electrophoresis. Although this approach also demonstrated an agonist-mediated increase in receptor mass, treatment of cells with tunicamycin produced better resolution between the 40- and 41-kDa bands. Next, we did treatment of cell extracts with N-glycosidase F. The possibility that the 41-kDa immunoreactive protein was a phosphorylated form of 40-kDa receptors was examined. Alkaline phosphatase or buffer was added to CHAPS-soluble extracts prepared from cells pretreated with serotonin (Fig. 2C). Increasing concentrations of alkaline phosphatase progressively decreased the mass of the 41-kDa protein to 40 kDa. Additional experiments were performed to determine the time course of serotonin-mediated receptor phosphorylation and dephosphorylation. To examine phosphorylation, cells were treated with serotonin for increasing times before extracting CHAPS-soluble protein. Formation of the phosphorylated 41-kDa protein was complete by 10 min of agonist treatment, the earliest time point examined (Fig. 3A, second lane). Treatment of cells with 100 nM 5-hydroxytryptamine (5-HT) for 15 min caused the appearance of an additional 41-kDa protein (second lane). Preincubation of cells with the antagonist mianserin (1 μM) prevented formation of the 41-kDa protein (third lane). B, shown is the concentration dependence of serotonin to cause formation of the 41-kDa protein. Data represent the means ± S.D. from three independent experiments, where responses to 1 μM serotonin were assigned a value of 100%. C, CHAPS-soluble extracts were incubated with the indicated amounts of alkaline phosphatase. Increasing concentrations of alkaline phosphatase progressively decreased the mass of the 41-kDa protein to 40 kDa. Data presented in A and C are representative of five and two independent experiments, respectively.

**Immunocytochemistry of Mutant 5-HT2C Receptors**—To determine which region of the 5-HT2C receptor was phosphorylated, tryptic digests from intact cells were evaluated in the band-shift phosphorylation assay using anti-peptide antibodies against the third intracellular loop (anti-2C-IC) and the carboxyl terminus (anti-2C-CT). The results were consistent with phosphorylation within the carboxyl terminus (data not shown). Thus, two mutant 5-HT2C receptor constructs were created that lack either two (ΔPDZ) or all (Δ375/CT) potential phosphorylation sites in the carboxyl terminus (Fig. 1). The
Δ375/CT receptor was created by deletion of all 86 amino acids and replacement with a region of the 2C-CT epitope and the conserved cysteine 13 residues from the seventh transmembrane domain. To determine whether the cellular distribution of truncation mutants differs from that of wild-type 5-HT₂C receptors, fibroblasts were transiently transfected with receptor cDNA, and immunoreactive receptors were detected with anti-2C-IC antibodies. Receptors that lack the terminal PDZ recognition motif (Ser⁴⁵⁸-Ser-Val-COOH; ΔPDZ) were distributed throughout transiently transfected cells (Fig. 4B) in a pattern similar to that of wild-type receptors (Fig. 4A). In contrast, receptors that lack 72 residues of the 86-amino acid tail (Δ375/CT) were localized primarily in punctate structures surrounding the nucleus (Fig. 4C). Identical results were observed when receptors were labeled with anti-2C-CT antibodies (data not shown). Since the intracellular distribution of Δ375/CT receptors would make it difficult to interpret results from phosphorylation assays, only ΔPDZ receptor cell lines were evaluated further.

Phosphorylation Assays—Fibroblasts stably expressing ΔPDZ or wild-type 5-HT₂C receptors were untreated or treated with 1 μM serotonin and examined in two phosphorylation assays. Phosphorylation was not detected in the band-shift assay using two independent ΔPDZ clones (ΔPDZ-2 and ΔPDZ-19) (Fig. 5A, lanes 1 and 2), whereas wild-type receptors were phosphorylated as determined by the increase in mass to 41 kDa (lane 3). To confirm the lack of phosphorylation of the ΔPDZ receptor, ³²P incorporation studies were performed with glycosylated receptors in the absence of tunicamycin. Cells labeled with ³²P were treated with 1 μM serotonin or vehicle (water), and receptors were immunoprecipitated with anti-2C-IC antibodies. The immunoprecipitates were treated with N-glycosidase F, electrophoresed, and either exposed to a PhosphorImager cassette to determine incorporation of ³²P (Fig. 5B) or blotted onto nitrocellulose and probed with anti-2C-IC antibodies to determine the total amount of receptors (data not shown). Consistent with the results from the band-shift assay, incorporation of ³²P was not detected in ΔPDZ receptors from either cell line (Fig. 5B, lanes 1 and 2), whereas the wild-type receptor was phosphorylated (lane 3).

Phosphoinositide Hydrolysis Assays—To test the hypothesis that phosphorylation of the 5-HT₂C receptor promotes desensitization, a phosphoinositide hydrolysis assay was performed with cell lines expressing either ΔPDZ receptors (ΔPDZ-2) or wild-type 5-HT₂C receptors (Fig. 6). Both receptors demonstrated an ~4-fold increase in basal inositol monophosphate accumulation with identical EC₅₀ values (ΔPDZ, 4.7 ± 1.1 nM; and wild-type, 4.7 ± 1.2 nM; n = six experiments). Thus, deletion of the PDZ recognition motif did not alter the EC₅₀ or maximal response during a single 30-min application of serotonin relative to the wild-type 5-HT₂C receptor. To determine
whether treatment of cells with serotonin promotes desensitization of a subsequent response, we utilized a protocol that was previously demonstrated to promote desensitization of wild-type 5-HT$_{2C}$ receptor responses (7). Cells were either untreated or treated with 100 nM serotonin, a concentration that causes maximal receptor phosphorylation (Fig. 2), for 16 h. After washing four times with serum-free DMEM, phosphoinositide hydrolysis was determined with increasing concentrations of serotonin (10$^{-10}$ to 10$^{-5}$ M) to generate concentration-response curves. Table I illustrates that pretreatment of cells with serotonin caused a 3-fold increase in the EC$_{50}$ of wild-type receptors and an 8-fold increase in the EC$_{50}$ of PDZ receptors. In these experiments, cells were treated with serotonin for 16 h; however, maximal 5-HT$_{2C}$ receptor phosphorylation occurs within 10 min of serotonin treatment (Fig. 3). Therefore, calcium imaging was utilized to examine dynamic 5-HT$_{2C}$ receptor responses in a more relevant time frame.

**Calcium Imaging**—Calcium imaging was performed with cells expressing ΔPDZ (ΔPDZ-2) or wild-type 5-HT$_{2C}$ receptors to examine the dynamics of receptor desensitization. Cells were treated with 100 nM serotonin for 10 min, washed for 2.5 min, and restimulated with 100 nM serotonin. Wild-type 5-HT$_{2C}$ receptors (Fig. 7A) and ΔPDZ receptors (Fig. 7B) demonstrated a robust, transient release of intracellular calcium. Furthermore, wild-type and ΔPDZ receptors displayed similar maximal responses (Fig. 7) and indistinguishable half-lives for decay of the initial responses (wild-type, 62.4 ± 7.9 s; and ΔPDZ, 75.2 ± 9.5 s; n = 10; p = 0.31 by unpaired Student’s t test). After washing for 2.5 min, a second application of serotonin to cells expressing wild-type receptors promoted calcium release at 37 ± 9% of the initial response (Figs. 7A and 8). In contrast, a second application of serotonin to cells expressing ΔPDZ receptors produced responses that were only 13 ± 9% of the initial signal (Figs. 7B and 8). To further investigate this observation, longer washout periods were examined. Fig. 8 illustrates that after washing for 5 min, secondary responses of wild-type and ΔPDZ receptors were 50 ± 21 and 7 ± 10% of the initial response, respectively. After washing for 10 min, secondary responses of wild-type and ΔPDZ receptors were indistinguishable.

**Analysis of S458A and S459A Receptors**—Since ΔPDZ receptors lack two potential phosphorylation sites (Ser$^{458}$-Ser-Val-COOH), serine-to-alanine point mutations were created, and cell lines expressing S458A or S459A receptors were examined in phosphorylation and calcium release assays. For the phosphorylation assay, cells were treated with 1 μM serotonin, and the levels of phosphorylated receptors were determined in the band-shift assay (Fig. 9A). Both S458A and S459A receptors decreased serotonin-mediated phosphorylation to 50% of wild-type receptor levels (Fig. 9B, white bars). Identical results were obtained with 100 nM serotonin (data not shown). For the calcium release assay, cells were treated with 100 nM serotonin for 10 min, washed for 5 min, and restimulated with 100 nM serotonin. Whereas the peak secondary responses of S458A receptors were similar to those of wild-type receptors, those of S459A receptors were diminished (Fig. 9B, gray bars). Thus, mutation of Ser$^{459}$ decreased receptor phosphorylation and diminished subsequent calcium responses relative to wild-type 5-HT$_{2C}$ receptors.

**DISCUSSION**

Treatment of cells expressing serotonin 5-HT$_{2C}$ receptors with serotonin promotes phosphoinositide hydrolysis (24), release of intracellular calcium (18, 19, 25), and receptor phos-

---

**FIG. 6. Phosphoinositide hydrolysis assays of ΔPDZ and wild-type 5-HT$_{2C}$ receptors.** Cells stably expressing ΔPDZ (ΔPDZ-2) or wild-type 5-HT$_{2C}$ receptors were incubated in serum- and inositol-free medium containing myo-[3H]inositol for 16 h. Cells were treated with 10$^{-10}$ to 10$^{-5}$ M serotonin in triplicate wells for 30 min. Radiolabeled inositol monophosphates were isolated by anion-exchange chromatography and subjected to scintillation counting. The data (mean ± S.D.) from four independent experiments were statistically analyzed by two-way analysis of variance, followed by unpaired two-tailed Student’s t test.

**FIG. 7. Calcium imaging of ΔPDZ and wild-type 5-HT$_{2C}$ receptors.** Cells stably expressing either wild-type or ΔPDZ 5-HT$_{2C}$ receptors were incubated in serum-free medium overnight and then loaded with fura-2 for 1 h. Cells were challenged by a 10-min application of 100 nM serotonin (as illustrated by the bars), washed for 2.5 min, and challenged with serotonin again (arrows). A, calcium responses of cells expressing wild-type receptors showed a robust signal in response to a second serotonin challenge. B, ΔPDZ receptors did not resensitize to wild-type receptor levels in response to a second challenge with serotonin. Traces are representative of at least three independent experiments.

**TABLE I**

Agonist pretreatment increases the EC$_{50}$ of serotonin for ΔPDZ and wild-type 5-HT$_{2C}$ receptors

| 5-HT$_{2C}$ receptor | EC$_{50}$<sub>nm</sub> | p value<sup>a</sup> | p value<sup>b</sup> |
|----------------------|-----------------|-----------------|-----------------|
| Wild-type            | 4.9 ± 1.4       | 14 ± 4.6        | 0.0091          |
| ΔPDZ                 | 4.8 ± 1.5       | 41 ± 13         | 0.0015          |
| Wild-type            | 4.9 ± 1.4       | 14 ± 4.6        | 0.0091          |
| ΔPDZ                 | 4.8 ± 1.5       | 41 ± 13         | 0.0015          |

<sup>a</sup> Comparison between values obtained without and with serotonin.

<sup>b</sup> Comparison between wild-type and ΔPDZ, values obtained with serotonin.
Phosphorylation of the 5-HT$_{2C}$ Receptor

Phosphorylation of mutant receptors expressed as percent of wild-type S459A 5-HT$_{2C}$ receptors.

A

Responses relative to wild-type 5-HT$_{2C}$ receptors.

(peak 2) by the first response (peak 1). Cells expressing 2.5 and 5 min. **, representative of at least three independent experiments. *

Cells were challenged twice with 100 nM serotonin (see Fig. 7) at the interval indicated on the x axis. A ratio (shown on the y axis) was generated by dividing the peak height of the second calcium response (peak 2) by the first response (peak 1). Cells expressing ΔPDZ receptors demonstrated slower functional desensitization of calcium responses at 2.5 and 5 min. **, p < 0.005; *, p < 0.05 (unpaired two-tailed Student’s t test). Error bars represent S.D. Data for each time point are representative of at least three independent experiments.

B

Cell lines expressing identical densities of S458A or S459A receptors as the wild-type 5-HT$_{2C}$ receptor line were stably expressing either wild-type or ΔPDZ 5-HT$_{2C}$ receptors were incubated in serum-free medium overnight and then loaded with fura-2 for 1 h. Cells were challenged twice with 100 nM serotonin (see Fig. 7) at the interval indicated on the x axis. A ratio (shown on the y axis) was generated by dividing the peak height of the second calcium response (peak 2) by the first response (peak 1). Cells expressing ΔPDZ receptors demonstrated slower functional desensitization of calcium responses at 2.5 and 5 min. **, p < 0.005; *, p < 0.05 (unpaired two-tailed Student’s t test). Error bars represent S.D. Data for each cell line are representative of at least three independent experiments.

FIG. 8. ΔPDZ receptors display delayed recovery of calcium responses relative to wild-type 5-HT$_{2C}$ receptors. Cells stably expressing either wild-type or ΔPDZ 5-HT$_{2C}$ receptors were incubated in serum-free medium overnight and then loaded with fura-2 for 1 h. Cells were challenged twice with 100 nM serotonin (see Fig. 7) at the interval indicated on the x axis. A ratio (shown on the y axis) was generated by dividing the peak height of the second calcium response (peak 2) by the first response (peak 1). Cells expressing ΔPDZ receptors demonstrated slower functional desensitization of calcium responses at 2.5 and 5 min. **, p < 0.005; *, p < 0.05 (unpaired two-tailed Student’s t test). Error bars represent S.D. Data for each time point are representative of at least three independent experiments.

FIG. 9. Phosphorylation and calcium responses of S458A and S459A 5-HT$_{2C}$ receptors. Cell lines expressing identical densities of S458A or S459A receptors as the wild-type 5-HT$_{2C}$ receptor line were selected for these studies. A, band-shift phosphorylation assay of mutant and wild-type (WT) 5-HT$_{2C}$ receptors. Cells in serum-free medium were untreated (−) or treated (+) with 1 μM serotonin for 15 min. B, phosphorylation of mutant receptors expressed as percent of wild-type 5-HT$_{2C}$ receptor levels (left axis, white bars) and calcium responses of each cell line expressed as percent of initial response (right axis, gray bars). For the calcium release assay, cells were treated with 100 nM serotonin for 10 min, washed for 5 min, and restimulated with 100 nM serotonin. Error bars represent S.D. Data for each cell line are representative of at least three independent experiments. **, p < 0.05.

Phosphorylation of the 5-HT$_{2C}$ Receptor

Although subsequent responses to agonist application are attenuated (desensitized) relative to the first response (7, 17–19), it is not known whether phosphorylation of the 5-HT$_{2C}$ receptor plays a role in desensitization. Therefore, we created 5-HT$_{2C}$ receptor phosphorylation-deficient mutants and examined serotonin responses in phosphoinositide hydrolysis and calcium release experiments. Here, we demonstrate that deletion of the terminal three amino acids, which include a PDZ recognition motif (Ser$_{458}$-Ser-Val-COOH; ΔPDZ), abrogates serotonin-mediated phosphorylation and, unexpectedly, delays the recovery (resensitization) of desensitized 5-HT$_{2C}$ receptor responses.

Immunoreactive 5-HT$_{2C}$ receptors have masses of 51–68 kDa; and after treatment of cells with tunicamycin to prevent N-linked glycosylation, receptors are detected with masses of 40 and 41 kDa (21). Since two bands are detected from cells maintained in medium containing serum (21), we examined the possibility that serotonin, present in serum, alters the migration of receptors. Here, we demonstrate that the 41-kDa protein is a phosphorylated form of 40-kDa 5-HT$_{2C}$ receptors. Cells in serum-free medium contain 5-HT$_{2C}$ receptors with a mass of 40 kDa, whereas treatment of cells with the agonist serotonin causes the appearance of the 41-kDa protein. This agonist effect is blocked by co-incubation with mianserin, a 5-HT$_{2A}$/2C receptor antagonist, and reversed by either agonist washout or treatment of cell extract with alkaline phosphatase. These results establish that the 41-kDa protein reflects phosphorylated 5-HT$_{2C}$ receptors. We exploited the band-shift phosphorylation assay to identify a domain of 5-HT$_{2C}$ receptor that is required for receptor phosphorylation.

Two truncated 5-HT$_{2C}$ receptors were generated that lack either all potential phosphorylation sites in the carboxyl terminus (Δ375/CT) or the terminal two serine residues (ΔPDZ). The mutant Δ375/CT receptor was created by truncation after the seventh transmembrane domain to remove all 86 amino acids of the carboxyl terminus and replacement with a region of the 2C-CT epitope that lacks serine, threonine, and tyrosine residues. The cysteine located 13 residues from the predicted membrane domain was conserved in this construct because this residue has been shown to be important for the function of 5-HT$_{2A}$ receptors (26). The mutant ΔPDZ receptor lacks only three amino acids (Ser$_{458}$-Ser-Val-COOH). Mutant 5-HT$_{2C}$ receptor cDNA was transiently expressed in fibroblasts, and the distribution of immunoreactive receptors was compared with that of wild-type 5-HT$_{2C}$ receptors. Two different phenotypes were observed: Δ375/CT receptors were predominantly intracellular, whereas ΔPDZ and wild-type 5-HT$_{2C}$ receptors were distributed in a similar pattern throughout cells. Cell lines expressing ΔPDZ receptors were therefore a valid system for evaluating the contribution of the PDZ recognition motif in phosphorylation assays.

The band-shift phosphorylation assay and incorporation of $^{32}$P were used to examine phosphorylation of two independent cell lines expressing ΔPDZ 5-HT$_{2C}$ receptors. In the absence of serotonin, wild-type and ΔPDZ receptors had an apparent mass of 40 kDa. Treatment of cells with serotonin caused the appearance of the phosphorylated 41-kDa form in wild-type receptors, whereas no increase in mass was detected in ΔPDZ receptors. In support of the results obtained with the band-shift assay, serotonin-mediated incorporation of $^{32}$P was not detected in ΔPDZ receptors, whereas wild-type receptors demonstrated robust phosphorylation. These results further confirm that the band-shift phosphorylation assay reflects 5-HT$_{2C}$ receptor phosphorylation and establish that the PDZ recognition motif is required for receptor phosphorylation.

Previously, we demonstrated that overnight pretreatment of cells stably expressing wild-type 5-HT$_{2C}$ receptors with serotonin increases the EC$_{50}$ value for stimulating phosphoinositide hydrolysis without changing the maximal response to serotonin (7). To determine whether receptor phosphorylation is involved in the observed desensitization, cells stably expressing ΔPDZ or wild-type 5-HT$_{2C}$ receptors were either untreated or treated with 100 nM serotonin overnight, washed, and then stimulated with increasing concentrations of serotonin. Interestingly, pretreatment with serotonin promoted a significantly greater increase in the EC$_{50}$ value of serotonin for ΔPDZ receptors (8-fold) than for wild-type 5-HT$_{2C}$ receptors (3-fold). This result is not consistent with the hypothesis that 5-HT$_{2C}$ receptor phosphorylation promotes desensitization and raises the intriguing possibility that phosphorylation may actually attenuate desensitization or promote resensitization of 5-HT$_{2C}$
responses. In contrast, the PDZ recognition motif of wild-type 5-HT2C receptor phosphorylation, significant differences were observed. After washing cells with agonist-free medium, dramatically different recovery responses were observed. After 5 min, wild-type 5-HT2C receptor responses recovered to 50% of the initial response, whereas ΔPDZ receptor responses recovered to only 7% of the initial response. After 10 min, which corresponds to the half-life for reversal of receptor responses, which is the time of pretreatment with serotonin, responses of wild-type and ΔPDZ receptors were not observed. Therefore, calcium imaging was used to examine the dynamics of 5-HT2C receptor desensitization and resensitization within a time frame that corresponds to calcium responses. Both ΔPDZ and wild-type 5-HT2C receptors displayed robust, transient increases in intracellular calcium with similar maximal responses. In the continued presence of serotonin, responses of wild-type and ΔPDZ receptors decayed with indistinguishable half-lives of ∼1 min. These results lend additional support, within a relevant time frame, that desensitization of 5-HT2C receptor occurs in the absence of receptor phosphorylation. After washing cells with agonist-free medium, dramatically different recovery responses were observed. After 5 min, wild-type 5-HT2C receptor responses recovered to 50% of the initial response, whereas ΔPDZ receptor responses recovered to only 7% of the initial response. After 10 min, which corresponds to the half-life for reversal of wild-type 5-HT2C receptor phosphorylation, significant differences between ΔPDZ and wild-type 5-HT2C receptors were not observed. Thus, the results from phosphoinositide hydrolysis and calcium release experiments suggest that phosphorylation of the 5-HT2C receptor enhances resensitization of 5-HT2C receptor responses rather than altering desensitization kinetics.

Since ΔPDZ 5-HT2C receptors lack two potential phosphorylation sites, single point mutants were created at Ser458, the PDZ recognition motif serine, or Ser459 to determine if mutation of either residue alters receptor phosphorylation and/or calcium responses. Cell lines expressing identical densities of S458A or S459A 5-HT2C receptors were first examined in the brain-shift phosphorylation assay. Both S458A and S459A receptors decreased serotonin-mediated phosphorylation to 50% of wild-type 5-HT2C receptor levels, suggesting that both Ser458 and Ser459 are phosphorylated. Next, calcium release responses were examined. Interestingly, responses of S459A receptors to a second application of serotonin were diminished relative to wild-type 5-HT2C receptors, whereas responses of S458A receptors reproduced the wild-type phenotype. These results are consistent with a major role of Ser459 rather than the PDZ recognition motif per se in resensitization of 5-HT2C receptor responses. In contrast, the PDZ recognition motif of β2-adrenergic receptors (Ser-Leu-Leu-COOH) is involved in efficient recycling of internalized receptors to the cell surface, and mutations within this motif enhance agonist-mediated receptor degradation (27). Our observations are consistent with a role of Ser459 in resensitization of 5-HT2C receptor responses and suggest that phosphorylation at this site regulates resensitization.

Acknowledgments—We thank Ann Westphal and Antoinette Poinexter for expert technical assistance and Dr. Colleen Niswender for providing technical guidance and wisdom.

REFERENCES
1. Chang, M., Zhang, L., Tam, J. P., and Sanders-Bush, E. (2000) J. Biol. Chem. 275, 7021–7029
2. Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., and Emeson, R. B. (1997) Nature 387, 303–308
3. Herrick-Davis, K., Grinde, E., and Niswender, C. M. (1999) J. Neurochem. 73, 1711–1717
4. Niswender, C. M., Copeland, S. C., Herrick-Davis, K., Emeson, R. B., and Sanders-Bush, E. (1999) J. Biol. Chem. 274, 9472–9478
5. Canton, H., Emeson, R. B., Barker, E. L., Backstrom, J. R., Lu, J. T., Chang, M. S., and Sanders-Bush, E. (1996) Mol. Pharmacol. 50, 799–807
6. Xie, K., Zhu, L., Zhao, L., and Chang, L. S. (1998) Genomics 35, 551–561
7. Westphal, R. S., Backstrom, J. R., and Sanders-Bush, E. (1995) Mol. Pharmacol. 48, 200–205
8. Alblas, J., van Ettten, I., Khaman, A., and Moolenaar, W. H. (1995) J. Biol. Chem. 270, 8944–8951
9. Diviani, D., Lattion, A. L., and Coteecchia, S. (1997) J. Biol. Chem. 272, 28712–28719
10. Lattion, A. L., Diviani, D., and Coteecchia, S. (1994) J. Biol. Chem. 269, 22887–22893
11. Rao, R. V., Roettger, B. F., Hadac, E. M., and Miller, L. J. (1997) J. Biol. Chem. 272, 7021–7029
12. Smith, R. D., Hunyady, L., Olivares-Reyes, J. A., Mihalik, B., Jayadev, S., and Catt, K. J. (1998) Mol. Pharmacol. 54, 935–941
13. Takeo, T., Honda, Z., Sakanaoka, C., Isumi, T., Kameyama, K., Haga, K., Haga, T., Kurokawa, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 22453–22458
14. Spurney, R. F. (1998) J. Biol. Chem. 273, 28496–28503
15. Mueller, S. G., White, J. R., Schraw, W. P., Lam, V., and Richmond, A. (1997) J. Biol. Chem. 272, 9207–9214
16. Prossnitz, E. R. (1997) J. Biol. Chem. 272, 15213–15219
17. Bridden, S. J., Leslie, R. A., and Elliott, J. M. (1998) Br. J. Pharmacol. 123, 727–734
18. Akiyoshi, J., Nishizono, A., Yamada, K., Nagayama, H., Mifune, K., and Fuji, T. (1995) J. Neurochem. 64, 2473–2479
19. Watson, J. A., Elliott, A. C., and Brown, P. D. (1995) Cell Calcium 17, 120–128
20. Boddeke, H. W., Hoffman, B. J., Palacios, J. M., and Hoyer, D. (1995) Neunyn-Schmiedebergs Arch. Pharmacol. 348, 221–224
21. Backstrom, J. R., Westphal, R. S., Canton, H., and Sanders-Bush, E. (1995) Mol. Brain Res. 33, 311–318
22. Backstrom, J. R., and Sanders-Bush, E. (1997) J. Neurosci. Methods 77, 109–117
23. Barker, E. L., Westphal, R. S., Schmidt, D., and Sanders-Bush, E. (1994) J. Biol. Chem. 269, 11687–11690
24. Conn, P. J., Sanders-Bush, E., Hoffman, B. J., and Hartig, P. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4086–4088
25. Porter, H. P., Benwell, K. R., Lamb, H., Malcolm, C. S., Allen, N. H., Revel, D. F., Adams, D. R., and Sheardawn, M. J. (1999) Br. J. Pharmacol. 128, 13–20
26. Buck, F., Meyerhof, W., Werr, H., and Richter, D. (1991) Biochem. Biophys. Res. Commun. 178, 1421–1428
27. Cao, T. T., Deacon, H. W., Reeczek, D., Bretherer, A., and von Zastrow, M. (1999) Nature 401, 286–290