0026-895X/95/020200-06$3.00/0

MOLECULAR PHARMACOLOGY, 48:200–205 (1995).

Increased Basal Phosphorylation of the Constitutively Active Serotonin 2C Receptor Accompanies Agonist-Mediated Desensitization

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Received March 1, 1995; Accepted May 3, 1995

SUMMARY

The 5-hydroxytryptamine (5-HT)$_{2C}$ receptor is a G protein-coupled receptor that exhibits constitutive receptor activation, defined as agonist-independent receptor activation of the signal transduction pathway. The present studies were performed to determine whether NIH/3T3 fibroblasts expressing the 5-HT$_{2C}$ receptor exhibited desensitization of agonist-mediated phosphoinositide hydrolysis. Furthermore, 5-HT$_{2C}$ receptor-specific antibodies were used to determine whether the 5-HT$_{2C}$ receptor was phosphorylated in the absence of agonist and whether treatment with an agonist or an inverse agonist altered receptor phosphorylation. Time course studies of basal and serotonin-stimulated phosphoinositide hydrolysis demonstrated that basal values increased in a linear manner, whereas the response to serotonin plateaued within 60 min. In addition, pretreatment with serotonin resulted in a rightward shift of the subsequently determined serotonin dose-response curve. To determine the phosphorylation state of the 5-HT$_{2C}$ receptor, specific antibodies were used to immunoprecipitate the receptor from lysates prepared from ^{32}P-labeled fibroblasts. Phosphorylation of the 5-HT$_{2C}$ receptor was evident under basal conditions, and serotonin treatment increased receptor phosphorylation. The inverse agonist mianserin had no detectable effect on 5-HT$_{2C}$ receptor phosphorylation when added alone but blocked the serotonin-stimulated increase in 5-HT$_{2C}$ receptor phosphorylation. The present study is the first to demonstrate that the 5-HT$_{2C}$ receptor is phosphorylated under basal conditions and phosphorylation is increased by agonist treatment conditions that result in desensitization of receptor signaling. Thus, these studies demonstrate that a cell line exhibiting a high level of constitutive 5-HT$_{2C}$ receptor activity has the ability to undergo agonist-mediated desensitization, consistent with current models of G protein-coupled receptor regulation.

Classical models of G protein-coupled receptors generally require agonist occupancy of the receptor for activation of the signal transduction cascade. Within minutes to hours after agonist treatment, an attenuation of the agonist-stimulated response is seen (1–4). This attenuation has been termed desensitization and is often associated with receptor phosphorylation. Receptor phosphorylation is thought to cause uncoupling of the receptor from the G protein, to result in attenuation of the agonist-stimulated response (1–4).

Recently, several G protein-coupled receptors have been shown to exhibit constitutive activation (5–11). For these receptors, activation of the signal transduction cascade occurs in the absence of agonist. Although desensitization is a well established phenomenon in the field of G protein-coupled receptors, studies that provided the basis for models of desensitization were performed primarily before the description of constitutive receptor activation. Thus, it is not known whether constitutive receptor activation alters the process of agonist-mediated desensitization. Evidence was recently presented suggesting that a constitutively active, mutant β_2-adrenergic receptor is constitutively desensitized in a transfected cell line (12). This mutant β_2-adrenergic receptor (12, 13), as well as a constitutively active, mutant α_2-adrenergic receptor (14), was shown in reconstitution experiments to be phosphorylated by β-adrenergic receptor kinase in an agonist-independent manner. However, it is not known whether constitutively active, wild-type receptors are phosphorylated in an agonist-independent manner in intact cells and, furthermore, whether agonist-mediated desensitization occurs in a system that exhibits constitutive activation of wild-type receptors.

The 5-HT$_{2C}$ receptor has been shown to exhibit constitutive activity, activating the phosphoinositide hydrolysis signaling pathway in the absence of agonist (10). Three classes of receptor ligands for the 5-HT$_{2C}$ receptor are distinguishable in cells expressing constitutively active 5-HT$_{2C}$ receptors, i.e., agonists, which increase constitutive 5-HT$_{2C}$ recep-

This work was supported by National Institutes of Health Grant MH34007 and Training Grant GM07628.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; D-MEM, Dulbecco's modified Eagle medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate.

tor activity, inverse agonists, which decrease constitutive 5-HT$_{2C}$ receptor activity, and neutral antagonists, which have minimal effects on constitutive 5-HT$_{2C}$ receptor activity when added alone but block both agonists and inverse agonists (10, 15). 5-HT$_{2C}$ receptor inverse agonists have higher affinity for the G protein-uncoupled form of the 5-HT$_{2C}$ receptor than the G protein-coupled form, consistent with the interpretation that these ligands stabilize an inactive conformation of the receptor (15).

The present studies were undertaken to determine whether NIH/3T3 fibroblasts expressing constitutively active 5-HT$_{2C}$ receptors exhibit agonist-mediated desensitization of phosphoinositide hydrolysis. In addition, antibodies specific for the 5-HT$_{2C}$ receptor were used to determine the basal phosphorylation state of the receptor and alterations in the receptor phosphorylation state produced by 5-HT$_{2C}$ receptor agonists and inverse agonists.

Experimental Procedures

Materials. NIH/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). D-MEM, CMRL-1066 medium, Opti-MEM medium, Dulbecco's phosphate-buffered saline, calf serum, penicillin, streptomycin, and ^{14}C-labeled protein molecular weight standards were purchased from Gibco/BRL Life Technologies (Grand Island, NY). Cell culture dishes were purchased from Falcon/Becton Dickinson (Lincoln Park, NJ). Mianserin hydrochloride was purchased from Research Biochemicals (Natick, MA). Alkaline phosphatase-conjugated goat anti-rabbit antibody was purchased from Dako Corp. (Carpinteria, CA). *myo*-[^{3}H]Inositol (20–25 Ci/mmol), ^{35}S-EXPRESS protein labeling mixture (1175 Ci/mmol), [^{32}P]orthophosphoric acid (9000 Ci/mmol), and ENTENSIFY were purchased from New England Nuclear (Boston, MA). Protein A-agarose was purchased from Pierce (Rockford, IL). Gene pulser cuvettes (0.4 cm) and prestained protein molecular weight standards were purchased from Bio-Rad (Melville, NY), and nitrocellulose membranes were from Hoefer Scientific Instruments (San Francisco, CA). Serotonin creatinine sulfate, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylyl phosphate, protein molecular weight standards, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. A stable cell line of NIH/3T3 fibroblasts co-transfected with an expression vector containing the 5-HT$_{2C}$ receptor cDNA (16) and with the pRSV/neo selection vector was used in the present study (10). This cell line, referred to as 3T3/2C, expresses approximately 5 pmol of 5-HT$_{2C}$ receptors/mg of protein (15). Transfected and nontransfected NIH/3T3 fibroblasts were maintained in D-MEM supplemented with 10% calf serum, 5 units/ml penicillin, and 5 μg/ml streptomycin, in a humidified incubator at 37° with 5% CO_2.

Phosphoinositide hydrolysis assay. The accumulation of inositol monophosphate was assayed as described previously (10). Briefly, NIH/3T3 fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (3T3/2C cells) were plated in 11-mm-diameter wells in D-MEM containing 10% calf serum, penicillin, and streptomycin. When confluent, the cells were labeled for 20–24 hr (in the absence of serum) with 1 μCi of *myo*-[^{3}H]inositol/ml of CMRL-1066 medium. Cells were treated with 5-HT$_{2C}$ receptor ligands in the presence of lithium chloride (10 mM) and pargyline (10 μM), and incubations were continued for 10 min, unless otherwise indicated. Reactions were terminated by aspiration of the medium and addition of methanol. [^{3}H]Inositol monophosphate was extracted with chloroform/methanol and isolated by anion exchange chromatography. Radioactivity was quantitated by liquid scintillation counting.

Generation of 5-HT$_{2C}$ receptor-specific antipeptide antibodies. Antibodies were generated in rabbits by using a 19-amino acid peptide from the 5-HT$_{2C}$ receptor, corresponding to amino acid residues 270–288 (17). This peptide is predicted to be in the third intracellular loop of the 5-HT$_{2C}$ receptor, based on hydropathy plots of the deduced amino acid sequence of the cloned 5-HT$_{2C}$ receptor cDNA (16).

5-HT$_{2C}$ receptor immunoprecipitation. Fibroblasts were plated on 100-mm plates in D-MEM containing 10% calf serum, penicillin, and streptomycin. When cells were confluent, the medium was changed overnight to D-MEM without serum. Fibroblasts were then incubated for 1 hr at 37° in D-MEM (without methionine and cysteine), followed by radiolabeling with [^{35}S]methionine/cysteine (0.25 mCi/ml) for 4 hr at 37°. Radiolabeled fibroblasts were treated with vehicle or 5-HT$_{2C}$ receptor ligands for 15 min, placed on ice, washed two times with Dulbecco's phosphate-buffered saline, pH 7.5, containing 10 mM sodium fluoride and 10 mM sodium pyrophosphate, and lysed in 50 mM Tris, pH 7.5, containing 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. Lysed cells were scraped into tubes, sonicated, and centrifuged (20,000 × *g* for 30 min). The resultant pellets were solubilized in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris, pH 8, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin) at 4° by passage through a 25-gauge needle and sonication, followed by centrifugation (100,000 × *g* for 1 hr). The resultant supernatants were precleared with 75 μl of Protein A-agarose beads (1:1 in modified RIPA buffer) for 1 hr at 4°. 5-HT$_{2C}$ receptor-specific, affinity-purified antibodies (final concentration, 2–4 μg/ml) were added to the precleared supernatants, and the samples were rotated for 4 hr at 4°. Immune complexes were precipitated by addition of 50 μl of Protein A-agarose beads (1:1 in modified RIPA buffer) and were incubated for 1 hr at 4°, followed by centrifugation (10,000 × *g* for 15 sec). The precipitated beads were washed for 4 × 1 min, 1 × 5 min, and 1 × 1 min with 1 ml of modified RIPA buffer at 4°, followed by elution of precipitated proteins in modified sample buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8, 1% 2-mercaptoethanol, 10 mM sodium fluoride, 10 mM sodium pyrophosphate) at 37° for 45 min. Eluants were subjected to SDS-PAGE, followed by fluorography of the gel using DuPont ENTENSIFY, according to the manufacturer's instructions. Band intensities on the resultant fluorographs were quantitated by volume integration of bands corresponding to the 5-HT$_{2C}$ receptor, using Image 1.49 VDM software (Wayne Rashburn, National Institutes of Health, Bethesda, MD). In immunoprecipitations of fibroblasts transiently transfected with 5-HT$_{2C}$ receptor cDNA, transient transfections of parental NIH/3T3 fibroblasts were performed using electroporation. Briefly, NIH/3T3 fibroblasts (1 × 10^7 cells) with 60 μg of DNA (or vehicle), in 400 μl of Opti-MEM medium, were electroporated using a Bio-Rad GenePulser (960 μF, 270 V). Electroporated cells were resuspended in D-MEM containing 10% calf serum, 5 units/ml penicillin, and 5 μg/ml streptomycin and were plated onto 100-mm plates.

5-HT$_{2C}$ receptor phosphorylation. Fibroblasts were plated on 100-mm plates in D-MEM containing 10% calf serum, penicillin, and streptomycin. When confluent, cells were washed two times with D-MEM (without serum) and then changed overnight to D-MEM without serum. Medium was then changed, twice over a 1-hr period, to serum-free D-MEM without sodium phosphate. Fibroblasts were then labeled with [^{32}P]orthophosphoric acid (1 mCi/ml, in D-MEM without sodium phosphate) for 4 hr, medium was aspirated, and fibroblasts were treated with vehicle or 5-HT$_{2C}$ receptor ligands in serum-free D-MEM for 15 min at 37°. Preparation of lysates and immunoprecipitations were performed as described above. After SDS-PAGE, the gel was dried and exposed to a PhosphorImager cassette. Bands were visualized and then quantitated by volume integration using ImageQuant version 3.3 software (Molecular Dynamics). Dried gels were also exposed to film for autoradiography.

Western blots. CHAPS-soluble proteins were prepared from NIH/3T3 fibroblasts and NIH/3T3 fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (3T3/2C cells) as described previously.[1] Briefly, membrane fractions were prepared in buffer containing 50 mM Tris, pH 7.6, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride,

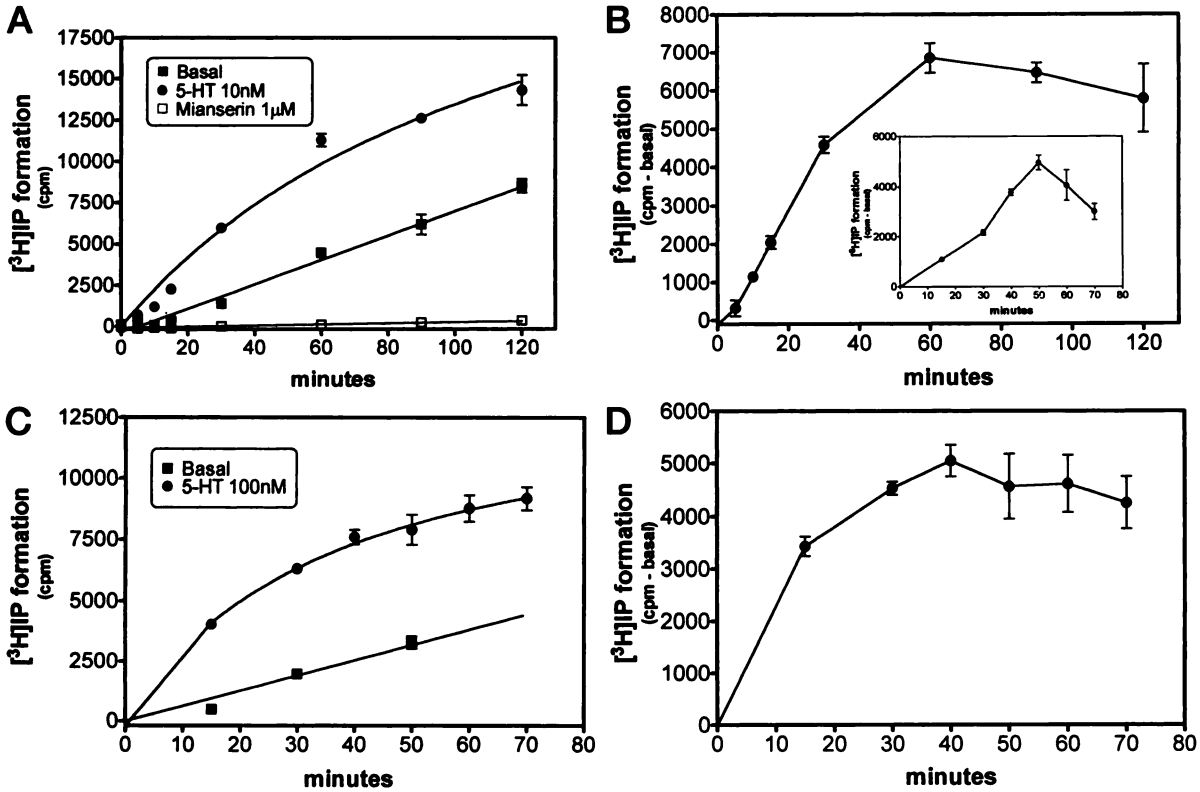

Fig. 1. Time course of basal and serotonin-stimulated phosphoinositide hydrolysis. Phosphoinositide hydrolysis assays were performed with NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (3T3/2C cells), as described in Experimental Procedures. A, *myo*-[^{3}H]Inositol-labeled fibroblasts were incubated with vehicle (■), 10 nM serotonin (●), or 1 μM mianserin (□) for the indicated times. [^{3}H]Inositol monophosphate (*[^{3}H]IP*) formation represents the cpm of [^{3}H]inositol monophosphate generated during the assay. Data are the mean ± standard error of three separate experiments. B, Serotonin (10 nM)-mediated phosphoinositide hydrolysis values, after subtraction of the corresponding basal values, are presented. Data are the mean ± standard error of three separate experiments. *Inset*, values for 1 nM serotonin, after subtraction of the corresponding basal values. Data are the mean ± standard error from a single experiment representative of three separate experiments. C, *myo*-[^{3}H]Inositol-labeled fibroblasts were incubated with vehicle (■) or 100 nM serotonin (●) for the indicated times. [^{3}H]Inositol monophosphate formation represents the cpm of [^{3}H]inositol monophosphate generated during the assay. Data are the mean ± standard error from a single experiment representative of three separate experiments. D, Serotonin (100 nM)-mediated phosphoinositide hydrolysis values, after subtraction of the corresponding basal values, are presented. Data are the mean ± standard error from a single experiment representative of three separate experiments.

1.7 mM diisopropyl fluorophosphate, and 5 μM leupeptin. These membrane fractions were solubilized in CHAPS buffer (50 mM Tris, pH 7.6, 10 mM CHAPS, 0.05 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin) by sonication and then centrifugation (18,000 × *g* for 15 min). The supernatant was mixed with SDS-PAGE sample buffer containing 1% 2-mercaptoethanol, separated by SDS-PAGE (10% polyacrylamide), and electrophoretically transferred to a nitrocellulose membrane in modified Towbin transfer buffer (18) (25 mM Tris, 192 mM glycine, pH ~8.4, 0.05% 2-mercaptoethanol) for 1 hr at 1 A. The membrane was blocked overnight at 4° with blot buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20, 0.05% NaN_3) containing 3% bovine serum albumin. The membrane was then incubated for 1 hr with 5-HT_{2C} receptor-specific, affinity-purified antibodies (5 μg/ml) at room temperature, washed with blot buffer, and incubated for 1 hr with alkaline phosphatase-conjugated goat anti-rabbit antibody diluted 1/1000 in blot buffer containing 1% bovine serum albumin. After washing with blot buffer, the membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 50 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$. Molecular masses of immunoreactive proteins were determined from protein standards (36–205 kDa).

Results

Desensitization of 5-HT_{2C} receptor-mediated phosphoinositide hydrolysis in NIH/3T3 fibroblasts. A time course study of basal and serotonin-stimulated phosphoinositide hydrolysis was performed to determine whether NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (3T3/2C cells) responded to continuous agonist exposure by desensitization of the phosphoinositide hydrolysis response. The phosphoinositide hydrolysis response to 10 nM serotonin (Fig. 1, A and B) plateaued and paralleled basal values within 60 min. A similar time course was found for 1 nM serotonin (Fig. 1B, *inset*), as well as the 5-HT_{2C} receptor agonist *N*,*N*-dimethyltryptamine.[1] Basal phosphoinositide hydrolysis increased in a linear manner for at least 2 hr and was completely blocked by the inverse agonist mianserin (Fig. 1A). The phosphoinositide hydrolysis response to 100 nM serotonin plateaued within 30 min of serotonin treatment (Fig. 1, C and D). In addition, 17-hr pretreatment of fibroblasts with 100 nM serotonin resulted in a significant rightward shift in the subsequent serotonin dose-response curve, with no change in the maximal response (Fig. 2). The shift in the serotonin dose-response curve was independent of changes in total 5-HT_{2C} receptor density, because these fibroblasts do not exhibit receptor down-regulation after treatment with agonist, as determined in studies measuring total

[1] E. Sanders-Bush, unpublished observations.

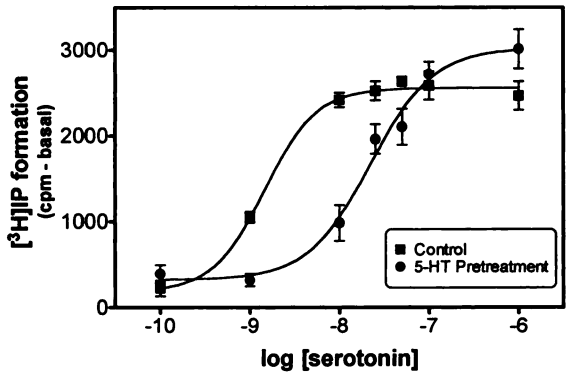

Fig. 2. Dose-dependent effects of serotonin on phosphoinositide hydrolysis. Phosphoinositide hydrolysis assays were performed with NIH/3T3 fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (3T3/2C cells), as described in Experimental Procedures. *myo*-[^{3}H]Inositol-labeled fibroblasts were incubated with vehicle (■) or 100 nM serotonin (●) overnight. Fibroblasts were then washed six times with 750 μl of D-MEM (without serum), for 3 min/wash, followed by incubation with increasing concentrations of serotonin for 10 min. [^{3}H]Inositol monophosphate (*[^{3}H]IP*) formation represents the cpm of [^{3}H]inositol monophosphate generated during the assay. Data are the mean ± standard error from a single experiment representative of three separate experiments. EC$_{50}$ values were determined from the curves generated using the equation for a sigmoidal curve (GraphPad PRISM; Intuitive Software for Science). EC$_{50}$ values (mean ± standard error) were as follows: control, 5.5 ± 4 nM; pretreated, 23 ± 1 nM. The difference in EC$_{50}$ values was significant (two-tailed p value = 0.021), as determined by unpaired Student's t test (GraphPad INSTAT2). The E_{max} value (mean ± standard error, three experiments) for cells pretreated with serotonin was 97 ± 15% of control.

5-HT$_{2C}$ receptor density by radioligand binding.[2] Although the plateau in serotonin-mediated phosphoinositide hydrolysis and the rightward shift in the serotonin dose-response curve could be due to desensitization of other components in the phosphoinositide hydrolysis signaling pathway, these results were consistent with classical characteristics of agonist-mediated receptor desensitization.

Immunoprecipitation of 5-HT$_{2C}$ receptors. Affinity-purified antibodies from rabbits immunized with the 5-HT$_{2C}$ receptor peptide conjugate[1] were used in Western blots to evaluate antibody specificity. The antibodies labeled bands specific for fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (3T3/2C cells) that were not present in nontransfected NIH/3T3 fibroblasts (Fig. 3). The immunoreactive proteins contain *N*-linked sugars, which account for the broad banding pattern.[1]

Immunoprecipitations using these affinity-purified antibodies with lysates prepared from [^{35}S]methionine/cysteine-labeled NIH/3T3 fibroblasts transiently transfected with 5-HT$_{2C}$ receptor cDNA yielded a banding pattern similar to that in the Western blots, with no bands present in lysates prepared from parental fibroblasts (Fig. 4). Immunoprecipitations from the stable cell line expressing the 5-HT$_{2C}$ receptor (3T3/2C cells) yielded similar results (see Fig. 6A). Thus, the affinity-purified antibodies immunoprecipitated the 5-HT$_{2C}$ receptor.

5-HT$_{2C}$ receptor phosphorylation. To determine the effects of 5-HT$_{2C}$ receptor ligands on the phosphorylation state of the 5-HT$_{2C}$ receptor, immunoprecipitations were performed with lysates prepared from ^{32}P-labeled NIH/3T3 fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (3T3/2C cells), after treatment with vehicle, agonist, or inverse agonist. The 5-HT$_{2C}$ receptor exhibited basal phosphorylation that was increased by the agonist serotonin but was unaffected by the inverse agonist mianserin (when added alone) (Fig. 5, A and B). The serotonin-mediated increase in

[2] E. Barker and E. Sanders-Bush, unpublished observations.

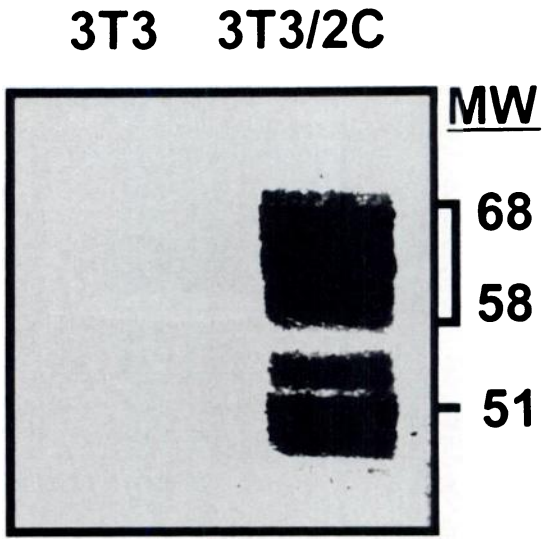

Fig. 3. Specific immunodetection of 5-HT$_{2C}$ receptors by affinity-purified antibodies. CHAPS-soluble membrane proteins prepared from NIH/3T3 fibroblasts (*3T3*) and NIH/3T3 fibroblasts stably transfected with 5-HT$_{2C}$ receptor cDNA (*3T3/2C*) were subjected to Western blot assays as described in Experimental Procedures. Molecular weights (*MW*) (× 10^{-3}) are indicated. The presented blot is representative of three separate experiments.

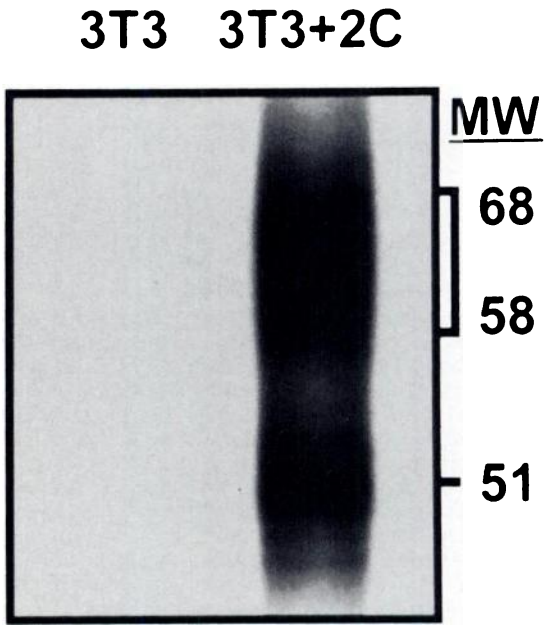

Fig. 4. Immunoprecipitation of 5-HT$_{2C}$ receptors by affinity-purified antibodies. NIH/3T3 fibroblasts were electroporated in the presence of vehicle or 5-HT$_{2C}$ receptor cDNA as described in Experimental Procedures. Twenty-four hours after electroporation, immunoprecipitations were performed with solubilized cell lysates from [^{35}S]methionine/cysteine-labeled fibroblasts, as described in Experimental Procedures. *3T3*, NIH/3T3 fibroblasts electroporated in the presence of vehicle; *3T3+2C*, NIH/3T3 fibroblasts transiently transfected with 5-HT$_{2C}$ receptor cDNA. Molecular weights (*MW*) (× 10^{-3}) are indicated and were determined using ^{14}C-labeled protein molecular weight standards. The presented fluorograph is representative of two separate experiments.

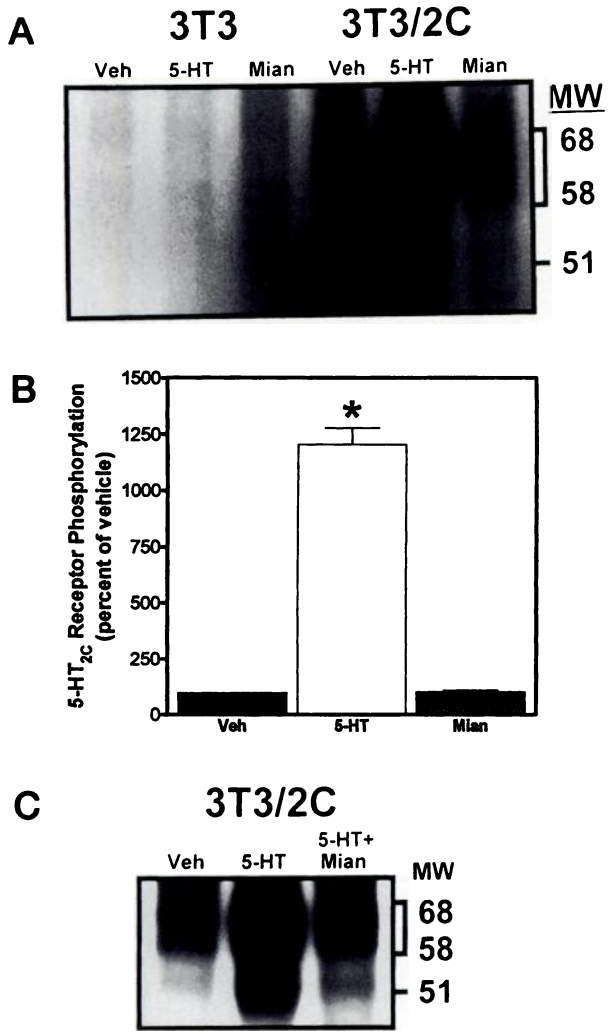

Fig. 5. Serotonin-induced increases in 5-HT_{2C} receptor phosphorylation. A, [^{32}P]Orthophosphoric acid-labeled nontransfected NIH/3T3 fibroblasts (*3T3*) and NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (*3T3/2C*) were treated with vehicle (*Veh*), 100 nM serotonin (*5-HT*), or 100 nM mianserin (*Mian*) for 15 min and subjected to immunoprecipitation as described in Experimental Procedures. A representative autoradiograph (of three experiments) is shown. B, Band intensities from PhosphorImager cassette imaging of A were quantitated by volume integration (ImageQuant version 3.3; Molecular Dynamics) and normalized to the response to vehicle. *Bars*, mean ± standard error of three experiments. Data were analyzed using one-way analysis of variance, followed by Dunnett's *post hoc* test comparing each treatment with vehicle. *, Significant difference, $p < 0.01$. C, [^{32}P]Orthophosphoric acid-labeled NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (3T3/2C cells) were treated with vehicle (*Veh*), 100 nM serotonin (*5-HT*), or 100 nM serotonin in the presence of 1 μM mianserin (*5-HT+Mian*) for 15 min and subjected to immunoprecipitation as described in Experimental Procedures. A representative autoradiograph (of two experiments) is shown. Molecular weights (*MW*) (× 10^{-3}) are indicated and were determined as in Fig. 4.

5-HT_{2C} receptor phosphorylation was blocked by mianserin (Fig. 5C), consistent with the interpretation that phosphorylation was mediated through 5-HT_{2C} receptor activation.

To determine whether serotonin or mianserin treatment altered the ability of the affinity-purified antibodies to immunoprecipitate the 5-HT_{2C} receptor, [^{35}S]methionine/cysteine-labeled fibroblasts were treated with vehicle, serotonin, or mianserin and then subjected to immunoprecipitation (Fig. 6A). Treatment with serotonin or with mianserin had no

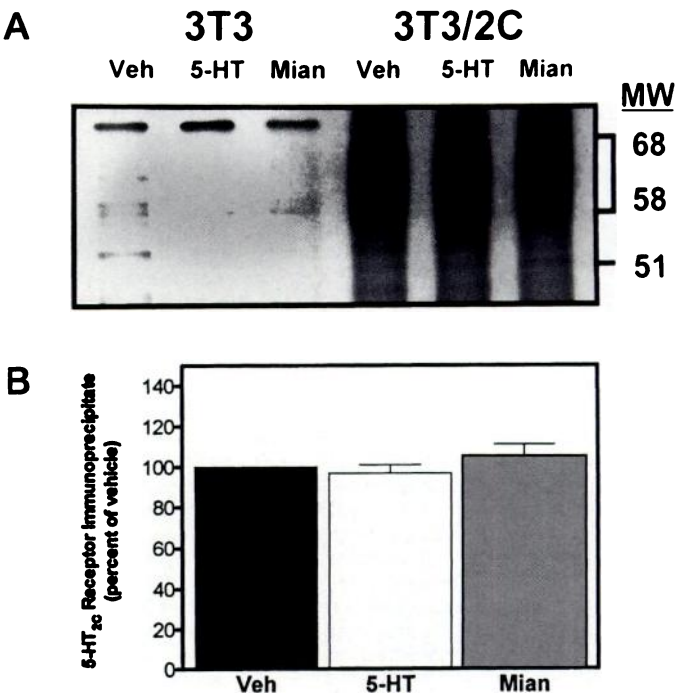

Fig. 6. Lack of effect of serotonin or mianserin on 5-HT_{2C} receptor immunoprecipitation. [^{35}S]Methionine/cysteine-labeled nontransfected NIH/3T3 fibroblasts (*3T3*) and NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (*3T3/2C*) were treated with vehicle (*Veh*), 100 nM serotonin (*5-HT*), or 100 nM mianserin (*Mian*) for 15 min and subjected to immunoprecipitation as described in Experimental Procedures. A, A representative fluorograph (of three experiments) is shown. Molecular weights (*MW*) (× 10^{-3}) are indicated and were determined as in Fig. 4. B, Band intensities from fluorographs were quantitated by volume integration (Image 1.49 VDM; Wayne Rashburn, National Institutes of Health) and normalized to the response to vehicle. *Bars*, mean ± standard error of three experiments. Data were analyzed using one-way analysis of variance, followed by Dunnett's *post hoc* test comparing each treatment with vehicle. The differences were not statistically significant ($p > 0.05$).

effect on the amount of receptor immunoprecipitated, compared with vehicle treatment (Fig. 6B). Therefore, we conclude that the serotonin-mediated increase in ^{32}P labeling of the 5-HT_{2C} receptor reflected enhanced phosphorylation of the receptor, rather than an increase in the amount of immunoprecipitated receptor protein.

Discussion

The 5-HT_{2C} receptor is a member of the G protein-coupled receptor superfamily (16) that is coupled to activation of the phosphoinositide hydrolysis signaling pathway (19). Expression of the cloned receptor in NIH/3T3 fibroblasts results in agonist-independent (constitutive) receptor activation that is increased by agonists, such as serotonin, and decreased by antagonists, such as mianserin, that have negative intrinsic activity and are referred to as inverse agonists (10, 15). In addition, 5-HT_{2C} receptor inverse agonists bind with higher affinity to the G protein-uncoupled form of the receptor than to the G protein-coupled form (15), consistent with the interpretation that inverse agonists stabilize an inactive conformation of the receptor. Constitutive receptor activation is a property of several other members of the G protein-coupled receptor superfamily, including the α_2-adrenergic (7), β_2-adrenergic (6), δ-opioid (5, 8), B_2 bradykinin (11), and D_{1B}

dopamine (9) receptors. In addition, antagonists with negative intrinsic activity have been shown to decrease constitutive activity of these receptors (5–9, 11).

The present studies demonstrated that the 5-HT$_{2C}$ receptor was phosphorylated under basal conditions and, furthermore, phosphorylation of the receptor was increased by the agonist serotonin but was unaffected by the inverse agonist mianserin. Mianserin may alter phosphorylation of a specific residue that was undetectable under the present assay conditions. However, the results are consistent with the interpretation that the inverse agonist-mediated decrease in phosphoinositide hydrolysis is independent of marked changes in overall receptor phosphorylation. In contrast, in a reconstituted system, agonist-independent β-adrenergic receptor kinase-mediated phosphorylation of a constitutively active, mutant β_2-adrenergic receptor is inhibited by an antagonist with negative intrinsic activity (13). This discrepancy in antagonist effects on receptor phosphorylation could be due to differences in assay conditions or in the mechanisms of constitutive receptor activation.

Consistent with classical models of G protein-coupled receptor desensitization, treatment of the 5-HT$_{2C}$ receptor with an agonist caused a decrease in the response to subsequent agonist stimulation. This was evident by the plateau in the time course of serotonin-stimulated phosphoinositide hydrolysis and a rightward shift in the serotonin dose-response curve after pretreatment with serotonin. Therefore, we conclude that NIH/3T3 fibroblasts expressing constitutively active 5-HT$_{2C}$ receptors exhibited agonist-mediated desensitization of phosphoinositide hydrolysis. Serotonin also increased phosphorylation of the 5-HT$_{2C}$ receptor before, or coincident with, desensitization of serotonin-mediated phosphoinositide hydrolysis. Although the present studies have not ruled out alterations in phosphoinositide hydrolysis signaling components as contributing factors in the observed agonist-mediated desensitization, our findings are consistent with models of G protein-coupled receptor desensitization in which receptor phosphorylation plays a role. In addition, desensitization of 5-HT$_{2C}$ receptor signaling has been shown to be inhibited by the protein serine/threonine phosphatase calcineurin (20), also suggesting that phosphorylation plays a role in the mechanism of desensitization. The 5-HT$_{1A}$ receptor (21) has been shown to be phosphorylated under conditions of desensitization, suggesting that phosphorylation plays a role in 5-HT$_{1A}$ receptor desensitization as well.

Recently, a constitutively active, mutant β_2-adrenergic receptor was shown to be phosphorylated *in vitro* by β-adrenergic receptor kinase and also to be constitutively desensitized in a transfected cell line (12). Those results demonstrate that a constitutively active receptor is subject to modification by the cellular machinery involved in regulating receptor desensitization. Although it is likely that the stoichiometric ratio between receptor and G protein plays an important role in the level of constitutive receptor activity, it is also conceivable that the level of constitutive activity may be regulated by the cell. For example, the cell may regulate the level of constitutive activity by manipulating the basal levels of receptor phosphorylation and desensitization. Determination of the sites within the 5-HT$_{2C}$ receptor that are phosphorylated under basal conditions, as well as after agonist treatment, will allow the construction of mutant 5-HT$_{2C}$ receptors to begin to address the role of receptor phosphorylation in the mechanism of constitutive 5-HT$_{2C}$ receptor activation.

Acknowledgments

The authors wish to thank Ann Westphal for expert technical assistance, Dr. David Julius (University of California, San Francisco) for providing the 5-HT$_{2C}$ receptor cDNA clone, and Drs. Matt Kennedy and Jeff Keefer for informative discussions.

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