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His452Tyr Polymorphism in the Human 5-HT_{2A} Receptor Destabilizes the Signaling Conformation

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ABSTRACT

Naturally occurring variation within the human 5-HT_{2A} receptor results in an amino acid substitution in the carboxyl terminus of the receptor. This single nucleotide polymorphism (SNP), encoding a His452Tyr substitution, occurs at a frequency of 9% in the general population. It is noteworthy that this SNP has been linked to attention deficit hyperactivity disorder and has been associated with schizophrenic patients that do not respond to treatment with clozapine. To evaluate functional consequences of this SNP, agonist-stimulated signaling was investigated in NIH3T3 cells stably expressing either wild-type or 452Tyr variant receptors. The 452Tyr variant of the 5-HT_{2A} receptor had reduced ability to activate phospholipases C and D, suggesting that signaling through both G_q and G_{13} pathways is hindered. This conclusion was supported by assays of G protein coupling, which documented a loss of agonist-induced high affinity binding and a decreased turnover of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate after agonist stimulation. Kinetic analysis of time-course data revealed an altered desensitization phenotype, resulting in a blunted signal downstream of receptor activation. This diminished signaling implies that the His452Tyr variant receptor alters physiological responses, possibly contributing to psychiatric disease.

Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine neurotransmitter that plays a critical role in a wide range of physiological processes, including appetite, mood, aggression, cognition, and motor control (for review, see Hoyer et al., 1994). These physiologic functions are mediated by cell-surface 5-HT receptors, of which there are currently 14 known receptor subtypes. Among these subtypes are the 5-HT_2 family of serotonin receptors, consisting of the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} G protein-coupled receptors. Of particular interest within the central nervous system is the serotonin$_{2A}$ (5-HT_{2A}) receptor, which has been implicated in psychiatric disease (for review, see Roth et al., 1998). The 5-HT_{2A} receptor has been shown to couple to the heterotrimeric G proteins $G_{q/11}$ (Conn and Sanders-Bush, 1986), leading to phospholipase C (PLC) activation, and $G_{12/13}$, which leads to phospholipase D (PLD) activation through Rho stimulation (McGrew et al., 2002).

Single nucleotide polymorphisms (SNPs), substitutions of a novel nucleotide for the wild-type nucleotide within genomic DNA, are the most common type of genetic variation and occur at a frequency of approximately 1% (Cargill et al., 1999). Within the coding region of a gene, SNPs that cause a change in the encoded amino acid (nonsynonymous SNP) may have a deleterious consequence on protein folding, producing an unstable conformation of the protein that is retained within the endoplasmic reticulum (Wenkert et al., 1996). On the other hand, nonsynonymous SNPs may modify the normal functioning of the receptor by several mechanisms, including disruption of receptor-ligand binding, binding of G proteins, and scaffolding/accessory proteins, and/or alteration of post-translational modifications of the receptor.

Genetic abnormalities within the human 5-HT_{2A} receptor may alter serotonin neurotransmission, thereby contributing to mood or anxiety disorders. Herein, we report on a naturally occurring variation within the 5-HT_{2A} receptor, His452Tyr, with a minor allele frequency of 9% (Erdmann et al., 1996). This SNP has been repeatedly associated with schizophrenic patients who do not respond to clozapine treatment (Arranz et al., 1995, 1998a; Masellis et al., 1998), a conclusion that has been confirmed by meta-analysis (Arranz et al., 1998b). In addition, transmission disequilibrium test has revealed a relationship between the 452Tyr SNP and attention deficit hyperactivity disorder (Quist et al., 2000). A decrease in the amplitude of agonist-stimulated intracellular calcium levels has been shown in platelets from persons who

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ABBREVIATIONS: 5-HT, serotonin; PLC, phospholipase C; PLD, phospholipase D; SNP, single nucleotide polymorphism; MDL-100,907, (*R*)-(+)-α(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol; LSD, lysergic acid diethylamide; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; GppNHp, guanosine 5′-[β,γ-imido]triphosphate; HBSS, Hanks' balanced salt solution; PTX, pertussis toxin; PI, phosphoinositide; BSA, bovine serum albumin; LPA, lysophosphatidic acid; ANOVA, analysis of variance.

are heterozygous for this receptor variant (Ozaki et al., 1997). These preliminary investigations were limited by low abundance of the 5-HT_{2A} receptor and multiple, uncontrolled variables associated with using platelets from patients. To more thoroughly investigate the consequence of this polymorphism, a heterologous expression system was used in the current study to examine receptor targeting, ligand binding, signaling, and desensitization. We show that the 452Tyr variant has reduced ability to activate G proteins, resulting in a loss of magnitude in downstream response. In addition, we evaluate the impact of agonist pretreatment on responsiveness of both wild-type and H452Y receptors.

Materials and Methods

Materials. Human 5-HT_{2A} receptor cDNA was a gift from Dr. David Weiner (Acadia Pharmaceuticals, San Diego, CA). MDL-100,907 was a gift from Merrill Dow (Cincinnati, OH); lysergic acid diethylamide (LSD) and 2-bromo-LSD were obtained from the National Institute on Drug Abuse (Bethesda, MD). Methysergide was a gift from Novartis (Basel, Switzerland) and clozapine, from Sandoz Pharma (Basel, Switzerland). [*myo*-^{3}H]Inositol (25 Ci/mmol), [^{3}H]myristic acid (40.2 Ci/mmol), and [^{3}H]ketanserin (88 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{35}S]GTPγS (1009 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents, except as indicated under *Materials and Methods*, were purchased from Sigma Aldrich (St. Louis, MO).

Site-Directed Mutagenesis. Polymerase chain reaction was used to amplify the wild-type pBKS-5-HT_{2A} receptor with recombinant *Pfu* polymerase (Promega, Madison, WI) and synthetic oligonucleotides containing the variant sequence. Mutated receptors were digested with restriction enzymes (New England Biolabs, Beverly, MA) and subcloned into the retroviral vector, pBabe.

Cell Culture. Using the pBABE-puro system (Morgenstern and Land, 1990), human embryonic kidney 293T cells were transfected with three plasmids to generate a replication-deficient retrovirus carrying the RNA encoding the 5-HT_{2A} receptor. In brief, cells were transfected by the calcium phosphate technique and incubated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 35° plus 3% CO_2 for 16 h. Medium containing recombinant RNA virus was removed and filtered, then applied to NIH3T3 fibroblasts, maintained at 37°C plus 5% CO_2, in DMEM supplemented as above. After a 12-h infection, fibroblasts were washed and allowed to recover in normal medium for at least 16 h, at which point cells were placed under selection with 2 μg/ml puromycin, which was gradually decreased to 1 μg/ml for maintenance of stable cell lines.

Radioligand Binding. Saturation binding was used to determine the receptor density (B_{max}) and the affinity (K_D) of [^{3}H]ketanserin at each receptor. Membranes were prepared by scraping cells off the cell culture dish into binding buffer (50 mM Tris and 10 mM $MgCl_2$, pH 7.4) and briefly homogenizing. The homogenate was centrifuged at 20,000*g* for 20 min, and the pellet was resuspended in binding buffer; protein concentration was determined with Bio-Rad (Hercules, CA) protein assay reagent. Membrane preparations (final concentration of 50 μg/sample) were incubated with a range of [^{3}H]ketanserin concentrations for 30 min at 37°C. Competition binding was carried out on cell membranes prepared as described above and incubated with a final concentration of 1.0 nM [^{3}H]ketanserin and concentrations from 10^{-9} to 10^{-4} M for the competing ligand at 37°C for 30 min. GppNHp was added at a final concentration of 0.1 mM to convert all receptors to the low-affinity state. Nonspecific binding was determined with 0.1 mM methysergide for both saturation and competition binding. Bound radioligand was separated from free by vacuum filtration onto Whatman GF/C glass filters (Brandel, Gaithersburg, MD), which were counted in a liquid scintillation counter. Binding data were analyzed with Prism 3.0 (GraphPad Software, Inc., San Diego, CA) to calculate B_{max} and K_D values from saturation isotherms. For competition binding experiments, IC_{50} values were determined by fitting data to a sigmoidal curve with variable slope using GraphPad Prism; one-site and two-site binding curves were compared using the *F* ratio. IC_{50} values were converted to K_I using the transformation of Cheng and Prusoff (1973).

For whole-cell binding experiments, cells were grown in 12-well plates for 24 h, then serum-starved for 16 h before assay. Cells were washed with binding buffer (50 mM Tris and 10 mM $MgCl_2$), then preincubated in 500 μl of binding buffer plus 50 μl of unlabeled competitor for 10 min at 4°C. After preincubation, 50 μl of [^{3}H]ketanserin (final concentration, 5 nM) was added to each well and incubations were continued at 4°C for 60 min. Nonspecific binding was determined with 10 μM ketanserin. Cells were washed three times with binding buffer and removed from the plates with 1% SDS

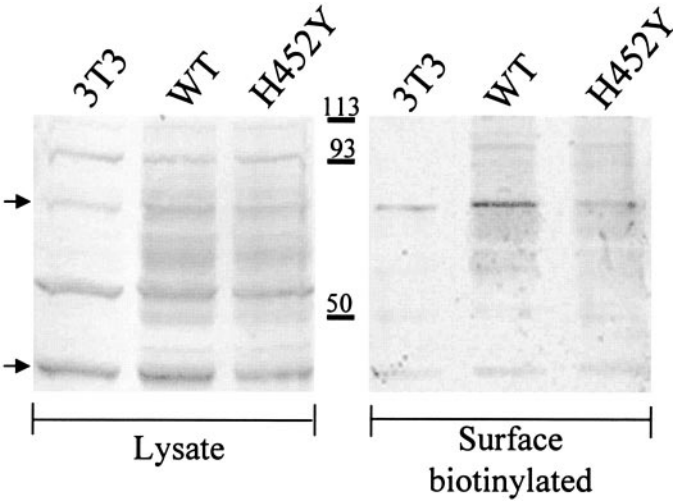

Fig. 1. Cell-surface biotinylation of the wild-type and H452Y variant 5-HT_{2A} receptors expressed in NIH3T3 cells. Membranes were prepared as either surface-biotinylated or total cell lysate from cells expressing wild-type (B_{max}, 7.9 ± 0.9 pmol/mg of protein) or H452Y variant (B_{max}, 6.3 ± 0.3 pmol/mg of protein) receptors. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis. The 5-HT_{2A} receptor migrates as a diffuse band, presumably reflecting multiple glycosylation states (Backstrom and Sanders-Bush, 1997). For each lane, the region between the arrowheads was scanned and quantified by densitometry. Within a treatment condition (lysate or biotinylated), densitometry values for untransfected 3T3 lanes were deemed nonspecific and subtracted from both wild-type and H452Y receptor densitometry values. The ratio of biotinylated cell-surface receptor to total receptor in cell lysate was 0.42 ± 0.04 for wild-type and 0.49 ± 0.09 for H452Y variant receptor. A representative Western blot is shown (n = 4). 3T3, untransfected parental NIH3T3 cells; WT, NIH3T3 cells expressing wild-type 5-HT_{2A} receptor; H452Y, NIH3T3 cells expressing H452Y variant 5-HT_{2A} receptor.

TABLE 1
Affinities of ligands at wildtype and variant receptors

Ligand	K_I	
	Wild-Type	H452Y
	nM	
5-HT	259.2 ± 57.7	256.0 ± 26.2
DOI	14.7 ± 0.8	17.3 ± 2.9
LSD	0.18 ± 0.01	0.18 ± 0.02
Amitriptyline	55.6 ± 9.1	59.4 ± 3.1
Clozapine	11.2 ± 2.4	11.9 ± 1.0
[^{3}H]Ketanserin	1.7 ± 0.2	1.4 ± 0.2
MDL-100,907	0.13 ± 0.02	0.17 ± 0.01
2-Bromo-LSD	1.8 ± 0.3	1.4 ± 0.2

DOI, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane.

in binding buffer. Solubilized cells were counted for radioactivity in a liquid scintillation counter, and data were analyzed with Prism software.

Surface Biotinylation. Cells were washed three times with Hanks' balanced salt solution (HBSS) and labeled with a 1 mg/ml sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Pierce, Rockford, IL) solution at 4°C for 20 min; this incubation was repeated with a fresh biotin solution. The reaction was terminated by aspiration, cells were washed three times with HBSS and scraped into 1% SDS/phosphate-EDTA buffer (0.04 M NaH_2PO_4, 0.01 M Na_2HPO_4, 0.01 M disodium EDTA, and 0.002 M EGTA, pH 7.2) with 0.5 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride. Cells were sonicated, incubated on ice for 30 min, and centrifuged at 16,000*g* for 15 min at 4°C. Parallel samples were processed identically, without biotin labeling, to obtain total cell lysate. Protein concentration was determined by bicinchoninic acid assay (Pierce). Equal amounts of protein were added to 100 μl of immobilized streptavidin (Pierce) and rocked overnight at 4°C. After overnight incubation, streptavidin-biotin complexes were washed three times with 1% SDS/phosphate-EDTA buffer and centrifuged at 400*g* for 2 min. Biotinylated proteins were eluted from the complex by addition of sample buffer containing 50 mM dithiothreitol and incubation at 50°C for 40 min. Samples were resolved on a 10% SDS-polyacrylamide minigel, and proteins were transferred to nitrocellulose (1 A, 1 h). The nitrocellulose membrane was washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and blocked overnight at 4°C in TBST containing 3% BSA. The nitrocellulose membrane was incubated with primary antibody directed against the third intracellular loop of the 5-HT$_{2A}$ receptor (2 mg/ml; Backstrom and Sanders-Bush, 1997) for 1 h, washed three times for 15 min with TBST, and incubated with alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (1:1000; DakoCytomation, Carpinteria, CA) for 1 h. Receptor protein was detected with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphosphate (Pierce) in buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5).

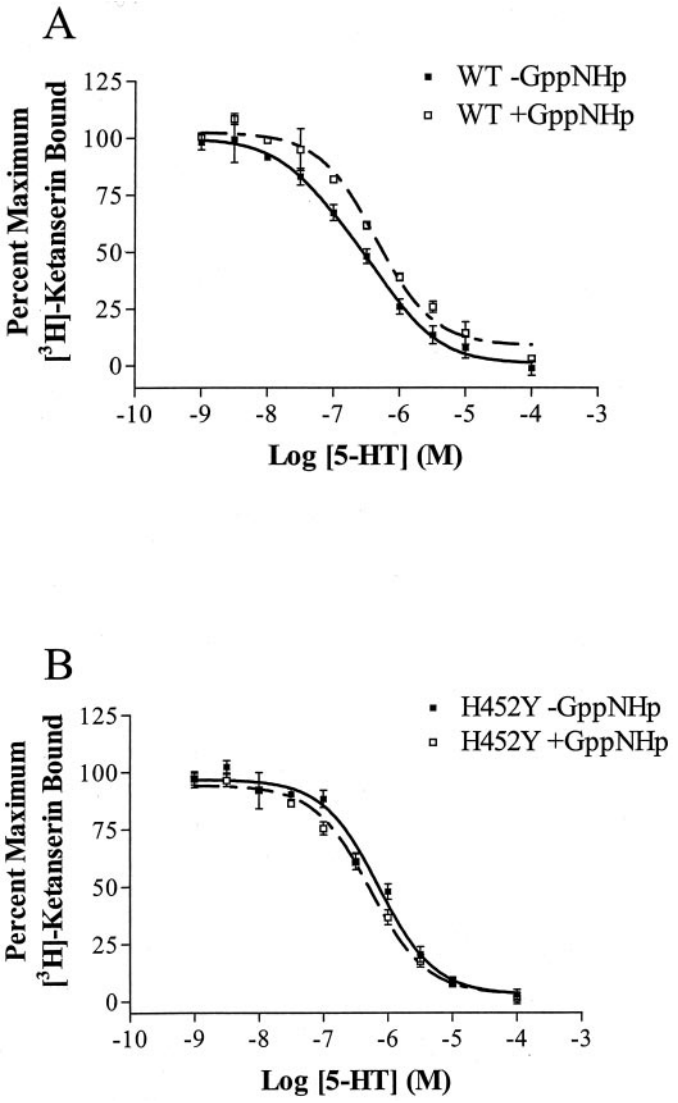

Fig. 2. Competition binding with 5-HT in the presence of GppNHp. [^{3}H]Ketanserin binding was determined in membrane preparations from wild-type and H452Y receptor-expressing cell lines. The ability of increasing concentrations of 5-HT, from 10^{-9} to 10^{-4} M, to compete for [^{3}H]ketanserin (1 nM) is plotted. A single experiment, representative of four, is shown for each receptor. A, the 5-HT competition curve was best fit by a two-site model; 29.7 ± 2.4% of the receptor population exists in the high affinity state (K_I = 11.9 ± 3.7 nM). Coincubation with the nonhydrolyzable GTP-analog GppNHp (0.1 mM) shifts the 5-HT competition curve to a single, low-affinity state. Maximum bound cpm values were 1567 ± 185 for wild-type receptor. B, the H452Y variant receptor was best fit by a one-site model, indicating only one affinity state. Addition of GppNHp does not change the characteristics of the curve. Maximum bound cpm values were 1883 ± 216 for H452Y variant receptor.

[^{35}S]GTPγS Assay. Cells were shifted to serum-free medium for 16 to 20 h before assay; for experiments involving pertussis toxin (PTX), 125 ng/ml of PTX was added at this time. The protocol for assessment of [^{35}S]GTPγS turnover in membranes was adapted from Cussac et al. (2002) and Adlersberg et al. (2000). After three washes with HBSS, cells were homogenized in [^{35}S]GTPγS assay buffer (20 mM HEPES, 100 mM NaCl, and 3 mM $MgCl_2$, pH 7.4), and centrifuged at 20,000*g* for 20 min. The prepared membranes were preincubated in assay buffer supplemented with 0.5 mM dithiothreitol and 50 μM GDP for 15 min at room temperature to allow GDP loading of the G proteins. For experiments involving antagonists, ligands were added for an additional 10 min. Membrane preparations (final concentration, 75–100 μg/sample) were incubated with 10^{-5} M 5-HT and 0.2 nM [^{35}S]GTPγS for 60 min at RT with shaking. Nonspecific binding was determined in the presence of 0.1 mM unlabeled GTPγS. Reactions were stopped by the addition of 500 μl of ice-cold assay buffer and were centrifuged at 16,000*g* for 15 min at 4°C. Membrane pellets were resuspended in 250 μl of assay buffer and counted for radioactivity in a Tri-Carb 2900TR liquid scintillation counter. Data were analyzed with Prism ver. 3.0.

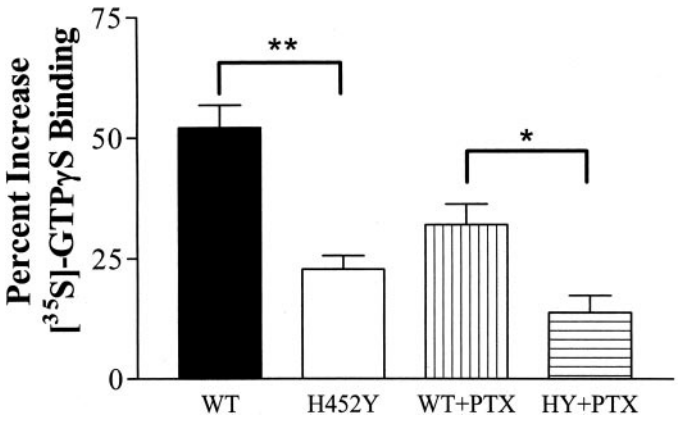

Fig. 3. 5-HT-stimulated G protein turnover measured by [^{35}S]-GTPγS turnover. Membranes prepared from cells expressing wild-type (B_{max}, 7.9 ± 0.9 pmol/mg of protein) or H452Y variant (B_{max}, 6.3 ± 0.3 pmol/mg of protein) 5-HT$_{2A}$ receptor were treated with 10^{-5} M 5-HT in the presence of the nonhydrolyzable GTP analog [^{35}S]GTPγS. Cells were pretreated with PTX (16 h, 125 ng/ml) to evaluate the $G_{i/o}$ component of both wild-type and H452Y receptors. Basal cpm values were 2820 ± 358 for wild-type receptor and 2375 ± 555 for H452Y variant receptor. Data represent the mean ± S.E. of five experiments. **, $P < 0.01$; *, $P < 0.05$ determined by one-way ANOVA.

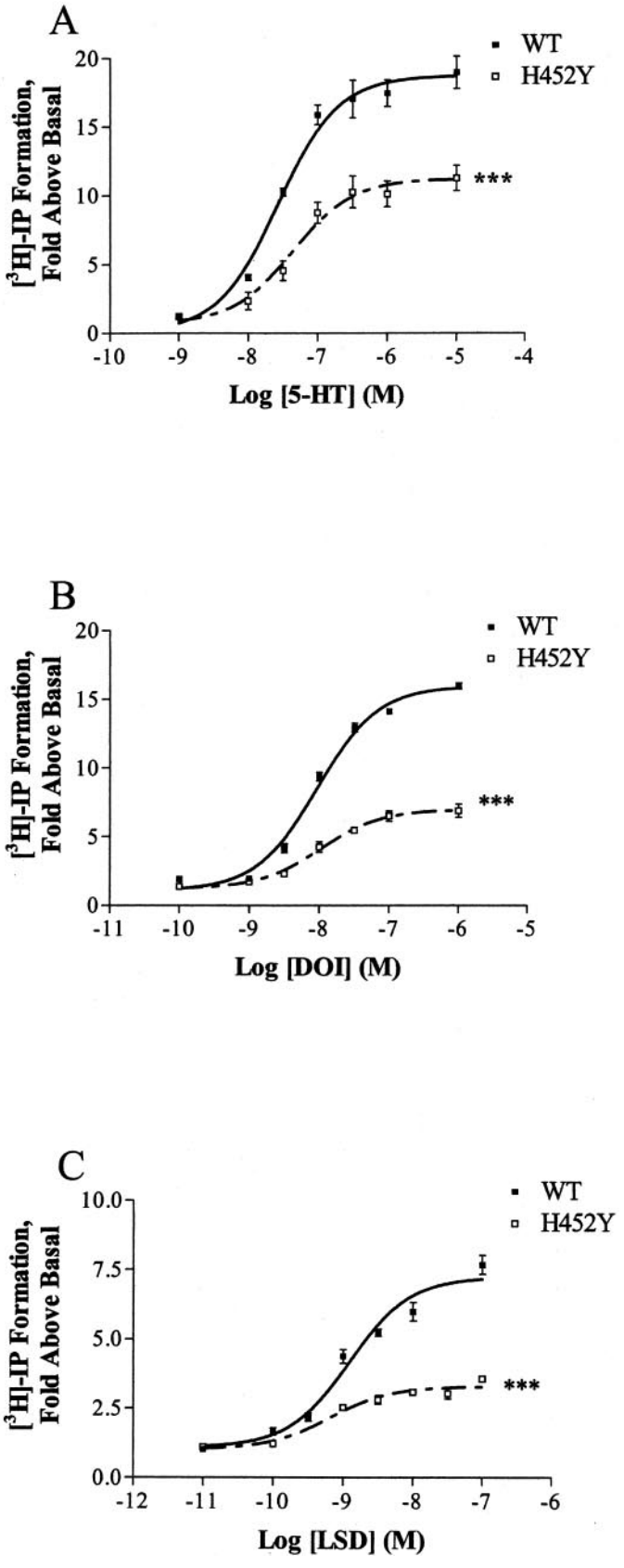

Fig. 4. Agonist-induced activation of PLC at wild-type and H452Y 5-HT_{2A} receptors. Cells were incubated in inositol-free DMEM with 1 μCi/ml [^{3}H]inositol for 16 h before stimulation with a range of agonist concentrations. [^{3}H]Inositol monophosphates were isolated by anion exchange chromatography. Basal cpm values were 1083 ± 82 for the wild-type receptor and 1317 ± 167 for the H452Y variant receptor. A, the maximum response after 5-HT stimulation is diminished from 18 ± 0.4-fold above basal for the wild-type receptor to 11.2 ± 0.4-fold above basal for the H452Y variant. ***, $P < 0.001$ determined by t test. The EC_{50} values are unchanged at approximately 40 nM. B, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI) response is also reduced from a 15.7 ± 0.01-fold increase for the wild-type receptor to a 7.0 ± 1.0-fold increase for the H452Y variant; ***, $P < 0.001$ determined by t test, with EC_{50} values of approximately 10 nM. C, LSD-induced response is reduced in the H452Y variant to a maximum response of 3.5 ± 0.4-fold compared with the wild-type response of 8.1 ± 0.4-fold above basal, ***, $P < 0.001$ determined by t test. EC_{50} values are unchanged at approximately 1.0 nM. Each data panel depicts one experiment, representative of three to five trials.

Phosphoinositide Hydrolysis Assay. Stably transfected cells were plated in 24-well plates and grown for 24 h; cells were washed with inositol-free DMEM and then incubated in serum-free, inositol-free DMEM containing 1 μCi of [*myo*-^{3}H]inositol/ml (20–25 Ci/mmol) for 16 h before assay. For experiments involving PTX treatment, 125 ng/ml PTX was also added 16 h before assay. Lithium chloride (10 mM) and pargyline (10 μM) were added to the cells and incubated at 37°C for 15 min before addition of agonist for 30 min at 37°C. For desensitization experiments, cells in 24-well plates were preincubated in inositol-free DMEM and 1 μCi of [*myo*-^{3}H]inositol/ml for 16 h. Then, 10^{-5} M 5-HT was added to each well for 10 min, 30 min, 1, 2, 4, 12, or 24 h at 37°C. After this preincubation, cells were washed three times with inositol-free DMEM, and 0.5 ml of fresh inositol-free DMEM was added back to each well. Lithium chloride and pargyline were added for 15 min, followed by 5-HT (concentration range of 10^{-9} to 10^{-4} M) for a 30-min incubation. All reactions were stopped by removal of media and addition of 50 μl of methanol per well. [^{3}H] Inositol monophosphates were isolated as described previously (Barker et al., 1994). Data were analyzed with GraphPad Prism software to generate dose response curves and calculate maximum and EC_{50} values.

PLD Assay. Cells were plated in 100-mm plates; 2 days after plating, the medium was replaced with DMEM supplemented with 0.5% fatty acid-free bovine serum albumin (BSA) and 1 μCi/ml [^{3}H]myristic acid. After 16 h, cells were washed three times with HBSS and medium was replaced with DMEM supplemented with 0.25% fatty acid-free BSA and incubated at 37°C for 50 min. Cells were then treated with 0.3% butanol for 10 min before stimulation with 10^{-5} M 5-HT for 15 min. The formation of [^{3}H]phosphatidylbutanol was used as an index of PLD activity as described previously (McGrew et al., 2002). Data were analyzed with GraphPad Prism software.

Results

Wild-Type and H452Y Variant Receptors Are Expressed at Similar Levels. Saturation binding experiments were performed to assess receptor densities of stably transfected NIH3T3 cell lines. Multiple cell lines were used for experimentation; unless otherwise indicated, wild-type 5-HT_{2A} receptor expression was 1.5 ± 0.02 pmol/mg and the H452Y variant expression was 1.3 ± 0.09 pmol/mg. Cell-surface biotinylation experiments were performed to investigate potential alterations in protein targeting (Fig. 1). Because our antibody against the human 5-HT_{2A} receptor has low affinity, cells expressing a higher density were used for these experiments. The ratio of biotinylated cell-surface receptor to total receptor in cell lysate was not altered, suggesting that targeting to the plasma membrane is comparable for the two proteins. As an alternate method for assessing cell-surface receptor expression, 5-HT (a hydrophilic ligand) was used to compete for binding of the hydrophobic ligand [^{3}H]ketanserin in intact cells. Serotonin (10 μM) displaced

21.8 ± 1.4% and 24.0 ± 2.8% of the [^{3}H]ketanserin binding in wild-type and 452Tyr variant cells, respectively. This prominent intracellular localization agrees with previous studies of the rat receptor (Cornea-Hebert et al., 1999).

H452Y Variant Receptors Do Not Efficiently Activate G Proteins. Competition binding was employed to determine the affinity of various ligands for the wild-type and H452Y receptors. All ligands tested had equal affinity for wild-type and H452Y variant receptors (Table 1). The 5-HT competition curve of wild-type receptor was shallow and best-fit by a two-site model, suggesting multiple affinity states of the receptor (Fig. 2A). Upon addition of the nonhydrolyzable GTP analog GppNHp, the competition curve was shifted to the right, indicative of elimination of the receptor high affinity state. When the same experiments were performed with membranes prepared from cells expressing the H452Y receptor, the 5-HT competition curve had normal steepness, and no difference was observed with addition of GppNHp (Fig. 2B). These results suggest that the H452Y receptor is less able to activate G proteins. To further test this hypothesis, the turnover of GTP was investigated using the radiolabeled, nonhydrolyzable GTP analog [^{35}S]GTPγS. A 5-HT-stimulated increase in [^{35}S]GTPγS was observed for both wild-type and H452Y receptors; however, the response in cells expressing the H452Y receptor was greatly diminished (Fig. 3). Pertussis toxin treatment reduced the response of both wild-type and H452Y receptors by approximately 40% (Fig. 3), indicating that this measure of signaling reflects coupling of the receptor to multiple G proteins. It is noteworthy that the PTX-insensitive component, presumably G_q and $G_{12/13}$, was significantly reduced in H45ZY cells (Fig. 3). 5-HT–stimulated [^{35}S]GTPγS was blocked by the highly selective 5-HT$_{2A}$ antagonist MDL-100,907 (data not shown), confirming that this response reflects 5-HT$_{2A}$ receptor activation.

H452Y Variant Receptors Fail to Generate a Maximal Response after Agonist Stimulation. Because it is possible to overcome diminished G protein activity through downstream signal amplification, it was necessary to test endpoints of the 5-HT$_{2A}$ receptor signaling cascade. The 5-HT$_{2A}$ receptor has been reported to couple to both $G_{q/11}$ and $G_{12/13}$ (McGrew et al., 2002). To examine signaling downstream of G_q, agonist-stimulated PI hydrolysis was evaluated. The H452Y variant had a significantly diminished response after 5-HT-stimulation compared with the wild-type 5-HT$_{2A}$ receptor (Fig. 4A). The PI responses of both wild-type and variant receptors were blocked by the 5-HT$_{2A}$ receptor antagonists ketanserin and MDL-100,907, and neither response was sensitive to PTX treatment (data not shown). Experiments conducted with the partial agonists (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (Fig. 4B) and LSD (Fig. 4C) yielded similar results; the H452Y variant receptor exhibited a 45% decrease in maximum response compared with wild-type receptor for each of the agonists tested. To ensure that the blunted signal was not caused by a generalized alteration in cell signaling or some other nonspecific cellular difference between stable cell lines, lysophosphatidic acid (LPA) was used to activate the endogenous LPA receptor. The PI hydrolysis response to LPA was similar between the two cell lines; wild-type activation was 1.61 ± 0.04-fold above basal and 452Tyr response was 1.69 ± 0.08-fold above basal. The reduction in maximum PI hydrolysis response for the H452Y receptor was reproduced in multiple cell lines, including the high density cell lines used for the cell-surface biotinylation experiments (data not shown).

PLD activation was used to evaluate signaling through the G protein $G_{12/13}$. Treatment of wild-type cells with a maximum concentration of 5-HT elicited a 10-fold increase in PLD activity. As with the PLC signal, the H452Y variant exhibited significant decrease in the maximum PLD response compared with wild-type receptor (Fig. 5).

Desensitization of the H452Y Variant Receptor Is Altered. To determine whether desensitization was the underlying cause of the H452Y variant phenotype, both wild-type and H452Y cell lines were pretreated with 5-HT for varying periods of time, and the subsequent PI hydrolysis response to 5-HT was measured. As illustrated in Fig. 6, A and B, the rate of desensitization at early times seems to differ, and this was confirmed by kinetic analyses (Fig. 6C). The decay of wild-type receptor response was best fit to a biphasic model, whereas the H452Y variant exhibited only a single phase of decay (Fig. 6C). The rate constants defining desensitization were different between the two receptor types; the wild-type receptor had an early, rapid phase of desensitization with a rate constant of 110 h^{-1} and a slower phase of desensitization with a rate constant of 0.2 h^{-1}. The desensitization rate of the H452Y variant receptor was defined by a single rate constant of 0.6 h^{-1}. In addition, pretreatment of the wild-type receptor with 5-HT for 10 or 30 min yielded a dose-response curve that was identical to the dose-response curve of the vehicle-treated H452Y receptor (Fig. 7A), further supporting the hypothesis that the mutant receptor exists in a partially desensitized state. Finally, the ultimate consequence of desensitization was to depress the response of both the wild-type and H452Y receptors to approximately 2.5-fold above basal response (Fig. 7B) indicating that structural hindrance of G protein coupling does not explain the 452Tyr phenotype. This desensitized response was receptor-specific and could be blocked by the antagonist MDL-100907 (Fig. 7B).

To determine whether the hydroxyl group on the 452Tyr residue was the site of a novel phosphorylation event, the

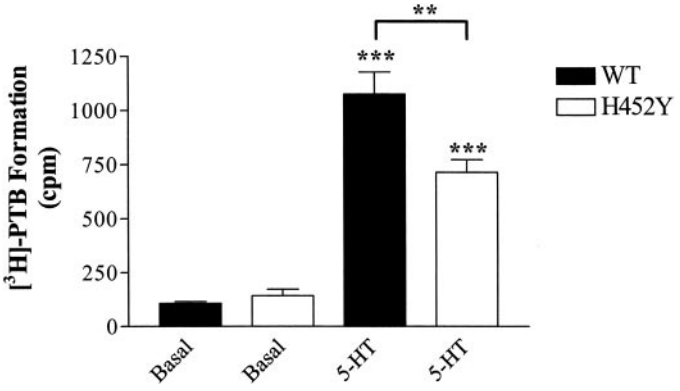

Fig. 5. Serotonin-induced activation of PLD at wild-type and H452Y variant receptors. Cells were incubated in DMEM supplemented with 0.5% fatty acid-free BSA and 1 μCi/ml [^{3}H]myristic acid for 16 h, washed three times, and incubated in DMEM with 0.25% fatty acid-free BSA for 50 min. Cells were then treated with 0.3% 1-butanol for 10 min before 15-min treatment with 10^{-5} M 5-HT. [^{3}H]Phosphatidylbutanol (PTB) was isolated using thin-layer chromatography and subjected to scintillation counting. Data represent the mean ± S.E. of five experiments; **, $P < 0.01$ (5-HT treatment of wild-type versus H452Y); ***, $P < 0.001$ (5-HT treatment compared with basal) determined by one-way ANOVA.

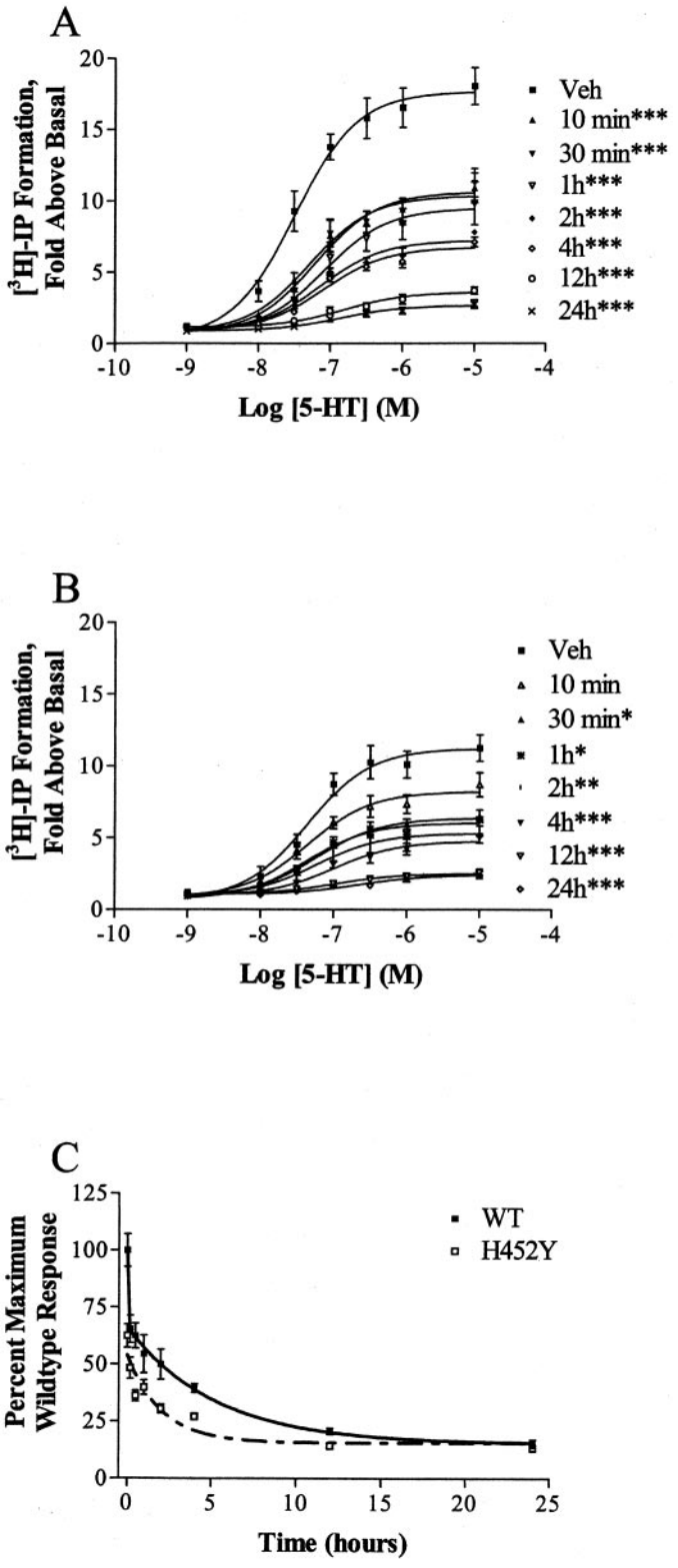

Fig. 6. Agonist-induced desensitization of wild-type and H452Y variant receptors. Cells were incubated in DMEM with 1 μCi/ml [^{3}H]inositol for 16 h before pretreatment with 10^{-5} M 5-HT for 10 or 30 min, or 1, 2, 4, 12, or 24 h. Cells were then extensively washed and treated with LiCl/pargyline for 15 min, followed by a 30-min exposure to a range of 5-HT concentrations. A, the maximum wild-type response initially drops off very rapidly after brief agonist exposure. Longer pretreatment times slow the desensitization. Data represent the mean ± S.E. of three to five experiments; ***, $P < 0.001$ compared with WT-Veh maximum response (determined by one-way ANOVA). B, the maximum H452Y response diminishes slowly with agonist pretreatment, and there is no early, rapid loss of response as observed with wild-type receptor. Data represent the mean ± S.E. of three to five experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with HY-Veh response (determined by one-way ANOVA). C, the desensitization of the wild-type response has an early, rapid phase and a late, slower phase best fit by biphasic exponential decay with rate constants of 110 h^{-1} and 0.2 h^{-1}. The desensitization of the 452Tyr variant is monophasic with a rate constant of 0.6 h^{-1}. Desensitization is expressed as a percentage of the maximal response observed in the wild-type, vehicle-treated cells. Data represent the mean ± S.E. of at least three experiments.

His452 residue was mutated to phenylalanine to mimic the structure of tyrosine without the possibility of phosphorylation. The 452Phe mutation caused diminished G protein coupling, as measured by competition binding in the presence or absence of GppNHp (Fig. 8A), and a decrease in the maximum response to 5-HT (Fig. 8B) as measured by PI hydrolysis.

Discussion

The human 5-HT_{2A} receptor has been shown to contain multiple SNPs within the coding region of the gene (Warren et al., 1993; Arranz et al., 1995; Erdmann et al., 1996; Ozaki et al., 1996; Spurlock et al., 1998; Cargill et al., 1999). The present study focused on the most common nonsynonymous polymorphism, H452Y, which occurs at a frequency of 9% in the general population (Erdmann et al., 1996; Ozaki et al., 1997; Minov et al., 2001). This SNP lies within the cytoplasmic tail of the receptor, a region that has been implicated in binding of accessory/scaffolding proteins and GPCR desensitization and internalization (for review, see Freedman and Lefkowitz, 1996; Ferguson, 2001).

The current studies demonstrate that the 452Tyr variant of the 5-HT_{2A} receptor has reduced ability to activate PLC and PLD, suggesting that signaling through both $G_{q/11}$ and $G_{12/13}$ pathways is hindered. This conclusion was supported by assays of G protein coupling, which document loss of agonist-induced high affinity binding and a decreased turnover of [^{35}S]GTPγS after agonist stimulation. To ensure that there was no general signaling deficiency with the H452Y cell line, the phosphoinositide hydrolysis response of the endogenous LPA receptor was measured and found to be unaltered. Furthermore, multiple pairs of wild-type and H452Y variant cell lines, expressing a variety of receptor densities, were generated, and the percentage decrease in maximum agonist-induced response was reproduced, regardless of receptor density.

The decreased maximum responsiveness of the 452Tyr variant could be caused by defective targeting of the variant receptor, resulting in a loss of receptor at the cell surface. To address this possibility, Western analysis of the 5-HT_{2A} receptor was performed using biotinylation as a marker of cell-surface protein. Precise quantification was difficult because of the low affinity of the human 5-HT_{2A} receptor antibody, but the relative cell-surface expression levels of wild-type versus H452Y variant receptor were measurable and found to be unchanged. To lend further support to the data obtained by Western analysis, whole-cell binding experiments were performed. These results indicated that there was no change in cell-surface expression between wild-type and H452Y variant receptor as evidenced by equal displacement of hydrophobic [^{3}H]ketanserin by hydrophilic 5-HT.

Based on these data, we conclude that protein processing and membrane targeting is not disrupted by the presence of the SNP.

To investigate a possible alteration in the desensitization kinetics of the H452Y receptor, a time course of desensitization was generated. Both 10- and 30-min agonist pretreatment of the wild-type receptor caused a 40% reduction in maximum [^{3}H]IP formation, in agreement with earlier studies (Berg et al., 2001) showing that the human 5-HT$_{2A}$ receptor rapidly desensitizes in response to agonist treatment. However, the H452Y variant receptor did not display an early, rapid phase of desensitization; the desensitization time course of the 452Tyr variant was monophasic and resembled the slower, late phase of wild-type receptor desensitization. In addition, 10- and 30-min pretreatment of the wild-type receptor with 5-HT yielded a desensitized response that mimicked the 452Tyr variant vehicle-treated response. These data imply differential desensitization of the 452Tyr variant receptor. If receptor-G protein coupling were structurally hindered, the differential H452Y signal would be retained regardless of the extent of desensitization. However the phosphoinositide hydrolysis responses of the wild-type and 452Tyr variant receptors were not significantly different after long-term desensitization treatments, which is not con-

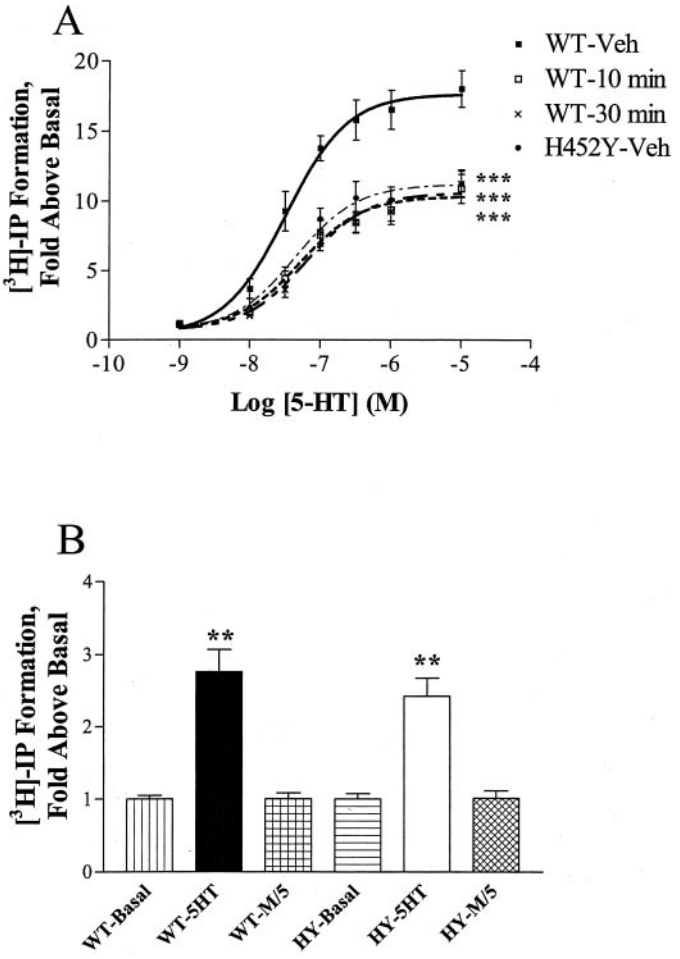

Fig. 7. Receptor response after short- or long-term desensitization. Cells were incubated in DMEM with 1 μCi/ml [^{3}H]inositol for 16 h before pretreatment with 10^{-5} M 5-HT for 10 or 30 min (A) or 24 h (B). Cells were then extensively washed and treated with LiCl/pargyline for 15 min, followed by a 30-min exposure to a range of 5-HT concentrations. Data represent the mean ± S.E. of three experiments. A, pretreatment of wild-type receptors with 10^{-5} M 5-HT for 10 or 30 min (WT-10 and WT-30) generated a 40% decrease in signal compared with vehicle-treated wild-type receptor (WT-Veh). This desensitized response was equivalent to the vehicle-treated H452Y response (H452Y-Veh). ***, $P <$ 0.001 compared with WT-Veh maximum response (determined by one-way ANOVA). B, 24-h 5-HT pretreatment reduces the response of both wild-type and 452Tyr variants to similar levels. Desensitized wild-type response is 2.7 ± 0.3-fold above basal; desensitized 452Tyr response is 2.5 ± 0.3-fold above basal. This desensitized response is blocked by coincubation of MDL-100907 with 5-HT during the final 30 min. M/5: coincubation with MDL-100907 and 5-HT; **, $P < 0.01$ compared with WT-Veh, WT-M/5, H452Y-Veh or H452Y-M/5 (determined by one-way ANOVA).

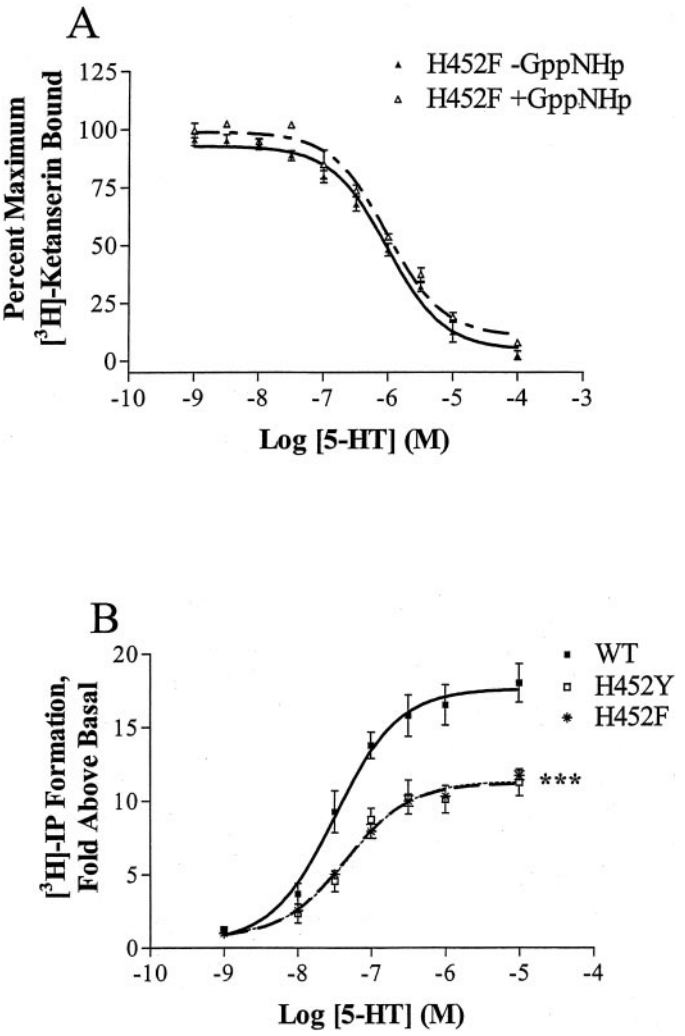

Fig. 8. Competition binding and PI hydrolysis with H452F mutant receptor. The H452F receptor was stably expressed in NIH3T3 cells with a B_{max} of 1.3 ± 0.08 pmol/mg of protein. A, [^{3}H]ketanserin binding was determined in membrane preparations from wild-type and H452F receptor-expressing cell lines. The ability of increasing concentrations of 5-HT, from 10^{-9} to 10^{-4} M, to compete for [^{3}H]ketanserin (1 nM) is plotted. The competition curve is of normal steepness and the curve is unchanged by 0.1 mM GppNHp (K_I of 315 ± 29 nM), indicating that the receptor exists in a single, low-affinity state. Maximum bound cpm values were 2648 ± 405 for the H452F receptor. B, cells expressing wild-type, H452Y, or H452F receptor were incubated in inositol-free DMEM with 1 μCi/ml [^{3}H]inositol for 16 h before stimulation with a range of agonist concentrations. [^{3}H]Inositol monophosphates were isolated by anion exchange chromatography. The H452F receptor reproduces the H452Y phenotype with a maximum response of only 11.4 ± 1.1 fold above basal and an EC$_{50}$ value of 38.6 ± 8.6 nM. Basal cpm values were 1163 ± 78 for the H452F receptor. For each panel, data are from one experiment and represent three trials; ***, $P < 0.001$ determined by t test.

sistent with a structural alteration as the sole mechanism for the diminished downstream signal. We conclude, therefore, that altered desensitization provides a molecular explanation of the 452Tyr receptor phenotype.

In classic models of GPCR regulation, agonist-induced desensitization is accompanied by phosphorylation and internalization of the receptor. However, phosphorylation of the 5-HT_{2A} receptor has not been reported; this is probably a result of the unavailability of antibodies. Our attempts to develop a human 5-HT_{2A} receptor antibody for immunoprecipitation, which is necessary for investigations of phosphorylation state, have been unsuccessful. The tyrosine at residue 452 may be the site of a novel phosphorylation event that attenuates receptor signaling. To indirectly investigate this, the His452 residue was mutated to phenylalanine. Phenylalanine is structurally similar to tyrosine, but lacks the hydroxyl group necessary for phosphorylation of the amino acid. The His452Phe mutant exhibited a response identical to the 452Tyr receptor, indicating that the phenotype of the variant receptor was not related to phosphorylation of residue 452. Down-regulation of the receptor cannot be assessed as a measure of differential desensitization because 5-HT_{2A} receptors expressed in NIH3T3 cells have been shown to undergo desensitization without down-regulation or a loss of cell-surface expression (Grotewiel and Sanders-Bush, 1994; Roth et al., 1995).

We conclude that the H452Y SNP in the human 5-HT_{2A} receptor has markedly reduced intracellular signaling capacity. This SNP occurs at an appreciable frequency in the general population (Erdmann et al., 1996; Ozaki et al., 1996; Minov et al., 2001) and is the subject of intensive efforts for association analyses. The 452Tyr allele has been linked to attention deficit hyperactivity disorder (Quist et al., 2000) and has repeatedly been demonstrated to predict drug responsiveness of schizophrenic patients (Arranz et al., 1995, 1998b; Masellis et al., 1998). Our demonstration of functional alterations downstream of this variant receptor enhance the significance of existing association data and move us closer to establishing a relationship between the biological effects of genetic alterations and the biological basis of brain disease.

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