Identification of Two Serine Residues Essential for Agonist-Induced 5-HT_{2A} Receptor Desensitization†

John A. Gray,‡ Beth A. Compton-Toth,‡ and Bryan L. Roth*,‡,§,||

Departments of Biochemistry, Psychiatry, and Neurosciences, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106

Received June 19, 2003; Revised Manuscript Received July 15, 2003

ABSTRACT: 5-HT_{2A} serotonin receptors represent the principal molecular targets for LSD-like hallucinogens and atypical antipsychotic drugs. It has been proposed that a dysregulation of 5-HT_{2A} receptor-mediated signaling may contribute to the pathogenesis of schizophrenia and related diseases. A major mechanism for the attenuation of GPCR signaling following agonist activation typically involves the phosphorylation of serine and/or threonine residues by various kinases. Ser/Thr phosphorylation leads to the binding of accessory proteins and the uncoupling of the G proteins, thereby preventing further signaling. The molecular mechanisms by which 5-HT_{2A} receptors are desensitized are unknown, and to date, no residues essential for agonist-mediated desensitization have been identified. Thus, we mutated, individually or in groups, all of the 37 serines and threonines in the cytoplasmic domains of the 5-HT_{2A} receptor and assessed the effects of these mutations on agonist-mediated desensitization. We discovered that mutation of two residues, S421 in the C-terminal tail and S188 in the second intracellular loop, to alanine resulted in a significant block of agonist-induced desensitization. Intriguingly, a single-nucleotide polymorphism, of unreported frequency, at the S421 locus has been reported (S421F); the S421F mutation, like the S421A mutation, significantly attenuated agonist-mediated desensitization. Taken together, these findings indicate that the process of agonist-mediated desensitization of 5-HT_{2A} receptors requires the presence of two nonconserved serine residues located in distinct intracellular loops.

Serotonin$_{2A}$ (5-hydroxytryptamine$_{2A}$, 5-HT_{2A})[1] receptors are important for mediating a large number of physiologic processes in the periphery and in the central nervous system, including platelet aggregation, smooth muscle contraction, and the modulation of mood and perception (*1*). Many drugs of diverse therapeutic classes mediate their actions, at least in part, by interactions with 5-HT_{2A} receptors. Most, but not all, hallucinogens, such as lysergic acid diethylamide and *N*,*N*′-dimethyltryptamine, function as agonists at 5-HT_{2A} receptors (*2*, *3*), while atypical antipsychotic drugs are potent 5-HT_{2A} receptor antagonists (*4*). It is known that acute and chronic exposure to 5-HT_{2A} receptor-active drugs (either agonists or antagonists) causes a decrease in the number and activity of 5-HT_{2A} receptors *in vivo* (*5*−*7*). In culture, studies have demonstrated that short-term agonist exposure results in the desensitization of 5-HT_{2A} receptor-mediated phosphoinositide (PI) hydrolysis in NIH 3T3 cells (*8*), P11 cells (*9*), and HEK-293 cells (*10*).

5-HT_{2A} receptors are characterized by a relatively low affinity (near micromolar) for 5-HT (*11*), suggesting that they may only be activated when 5-HT is released during neuronal exocytosis or during platelet degranulation where the local concentrations of released 5-HT would be high. 5-HT_{2A} receptors are known to desensitize following agonist exposure (*8*−*10*); thus, the stimulatory effect of 5-HT will be brief and not repeated during high-frequency stimulation. These apparent limits on 5-HT_{2A} receptor signaling, the high concentration of 5-HT required for receptor activation and the rapid attenuation of signaling, suggest that prolonged or repeated stimulation of 5-HT_{2A} receptors, possibly due to a failure in the desensitization process, could be pathological.

A general mechanism of G protein-coupled receptor (GPCR) desensitization involves the phosphorylation of intracellular domains of GPCRs by second-messenger kinases (e.g., protein kinase C and protein kinase A) and specific G protein-coupled receptor kinases (GRKs) leading to arrestin binding and G protein uncoupling (*12*, *13*). Arrestins also promote the targeting of desensitized receptors to clathrin-coated pits for their subsequent internalization (*13*). We have recently shown, however, that 5-HT_{2A} receptor internalization, desensitization, and resensitization are all arrestin-independent and unaffected by overexpression of the G protein-coupled receptor kinases GRK2 and GRK5 (*10*, *14*). The basis of this arrestin-independent regulation of 5-HT_{2A} receptors is unknown but is likely due to the apparent lack of agonist-induced 5-HT_{2A} receptor phosphorylation (refs *5*

† This work was supported in part by National Institutes of Health Grants RO1-MH61887 and KO2-MH01366 to B.L.R. J.A.G. was supported in part by National Institutes of Health Medical Scientist Training Program Grant 5T32-GM07250 and Metabolism Training Program Grant 5T32-DK07319.

‡ Department of Biochemistry.

§ Department of Psychiatry.

|| Department of Neurosciences.

[1] Abbreviations: HEK, human embryonic kidney; DMEM, Dulbecco's modified essential medium; 5-HT, 5-hydroxytryptamine (serotonin); PI, phosphoinositide; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; 5-HT_{2A}, 5-hydroxytryptamine$_{2A}$ (serotonin$_{2A}$); GRK, G protein-coupled receptor kinase; ChCl, chelerythrine chloride; GF, GF109203X; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; i1, first intracellular loop; i2, second intracellular loop; i3, third intracellular loop; SNP, single-nucleotide polymorphism.

10.1021/bi035061z CCC: $25.00
Published on Web 08/21/2003

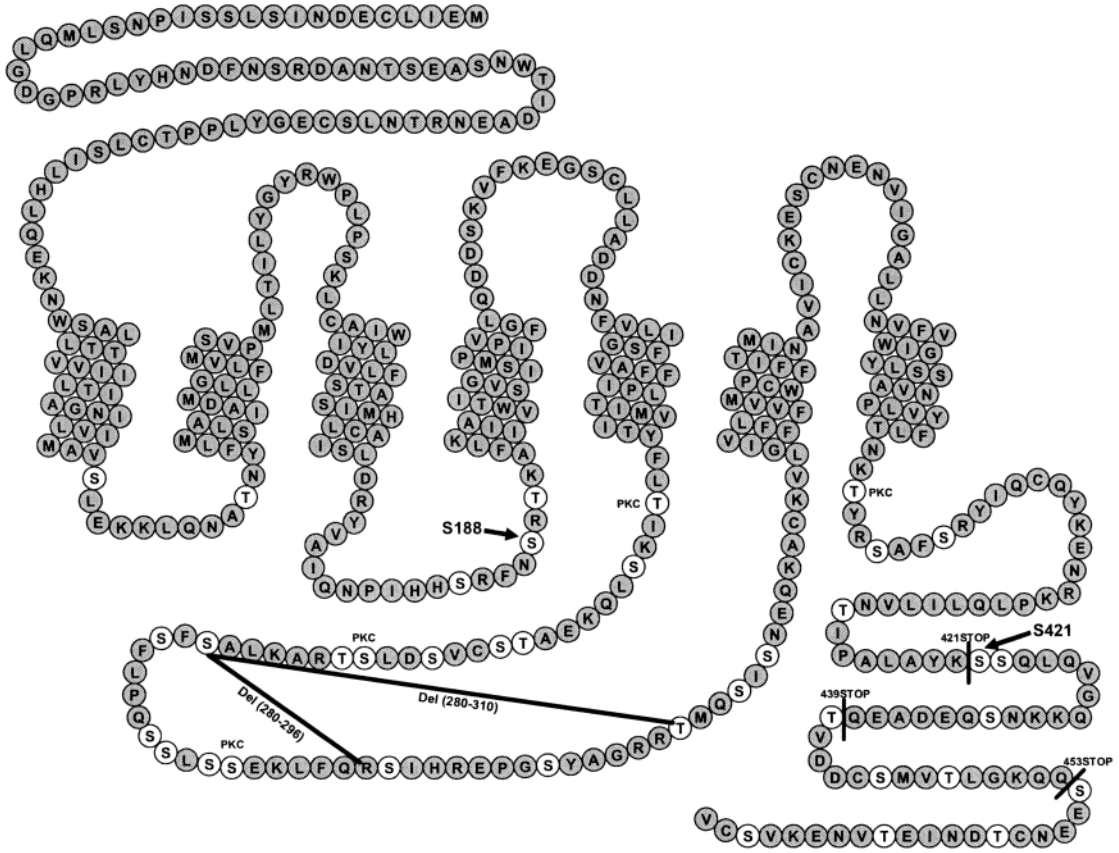

FIGURE 1: Snake diagram of the rat 5-HT_{2A} receptor with labeled mutations. Shown is a diagrammatic representation of the putative transmembrane domains and putative intracellular and extracellular domains of the 5-HT_{2A} receptor. Serine and threonine residues residing in the putative intracellular domains are highlighted. The residues mutated in this study include potential PKC consensus sites T257, S273 (with T274), S291, and T386; S100 and T109 in i1; S184, S188, and T190 in i2; S260A, T266A, T311A, S314/S316, and deletion mutants beginning at S280 and ending at either Q296 or R310 in i3; and S389/S392, T413, and truncation mutants at S421, T439, and S453 in the carboxy-terminal tail. Additional point mutations were introduced at S421, S422, and S432.

and *15* and unpublished observations of B. L. Roth). Though agonist-induced phosphorylation of 5-HT_{2A} receptors has yet to be described, it is likely that phosphorylation plays a role in 5-HT_{2A} receptor desensitization, although phosphorylation may be either constitutive or transient, and the stoichiometry of individual phosphorylation reactions might be negligible since there are 37 Ser and Thr residues in the intracellular domains, many of which could represent potential phosphorylation sites.

To determine a potential role of serine and threonine residues in the agonist-induced desensitization of 5-HT_{2A} receptors, we have systematically mutated, either individually or in groups, all 37 of the serine and threonine residues in the intracellular loops and carboxy-terminal tail of the rat 5-HT_{2A} receptor (Figure 1) and screened them for their effects on agonist-mediated 5-HT_{2A} receptor desensitization in HEK-293 cells. Additionally, we examined the efficacy and potency of 5-HT at each of the mutant receptors compared with wild-type 5-HT_{2A} receptors to screen for any that alter receptor-mediated signaling. As we report here, we found that mutation of only two residues, S188 in the second intracellular (i2) loop and S421 in the carboxy-terminal tail, independently to alanine significantly attenuate agonist-mediated 5-HT_{2A} receptor desensitization.

EXPERIMENTAL PROCEDURES

Materials and Constructs. [^{3}H]Inositol (21.0 Ci/mmol) and [^{3}H]ketanserin (88.0 Ci/mmol) were obtained from PerkinElmer/NEN (Boston, MA). Chelerythrine chloride (ChCl), GF109203X (GF), and phorbol 12,13-dibutyrate (PDBu) were purchased from BioMol Research Laboratories (Plymouth Meeting, PA). An amino-terminal FLAG epitope-tagged rat 5-HT_{2A} receptor was constructed as described previously (*14*). 5-HT_{2A} receptor mutants were made using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Deletion mutants of the third intracellular (i3) loop were made by overlap extension PCR (*16*). All receptor mutants were confirmed by automated fluorescent sequencing of the full-length insert by Cleveland Genomics (Cleveland, OH).

Cell Culture and Transfection. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. HEK-293 cells were transfected in 10 cm dishes at 60−80% confluency with 2 μg of receptor DNA and 4 μg of empty vector (pcDNA3) using Fugene6 (Roche, Indianapolis, IN) exactly as described by the manufacturer.

Assessment of Phosphoinositide (PI) Hydrolysis. Twenty-four hours after transfection, cells were split into poly-L-lysine-coated 24-well plates and grown for an additional 24 h in DMEM supplemented with 5% dialyzed fetal calf serum. Cell cultures were grown at 37 °C in 5% CO_2. Twenty-four hours later, the cells were washed with inositol-free DMEM and incubated for an additional 18 h with inositol-free DMEM containing 1 μCi/mL [^{3}H]inositol and 5% dialyzed

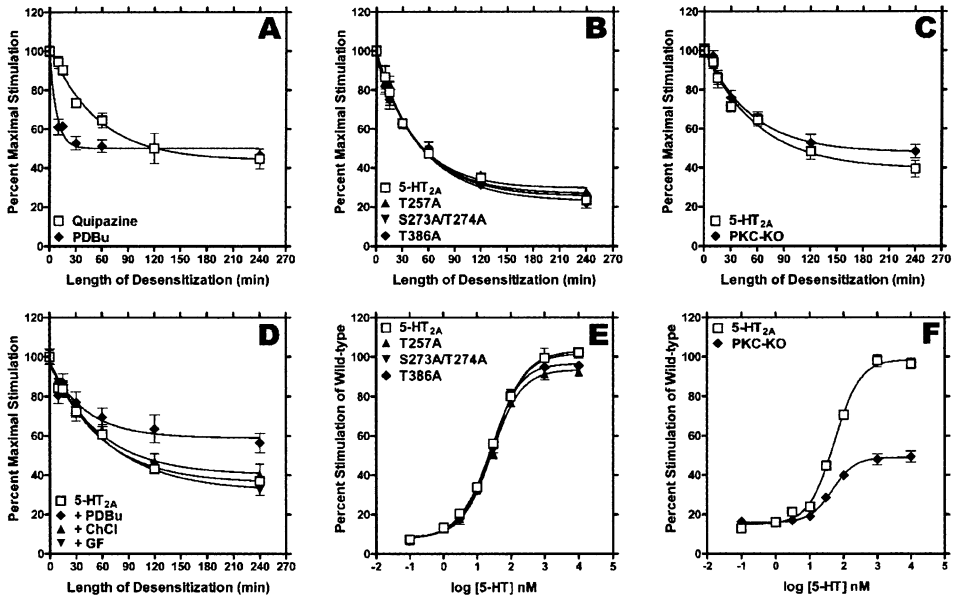

FIGURE 2: Role of PKC in 5-HT$_{2A}$ receptor desensitization in HEK-293 cells. (A) HEK-293 cells transiently transfected with wild-type 5-HT$_{2A}$ receptors were pretreated with either the 5-HT$_{2A}$ receptor agonist quipazine or the phorbol ester PDBu for the indicated times and washed, and 5-HT$_{2A}$ receptor signaling was assessed as described in Experimental Procedures. (B and C) The effect of mutation of consensus PKC sites either individually (B) or together (C) on quipazine-induced 5-HT$_{2A}$ receptor desensitization was assessed in transiently transfected of HEK-293 cells. (D) HEK-293 cells transiently transfected with wild-type 5-HT$_{2A}$ receptors were either pretreated with the phorbol ester PDBu overnight to downregulate PKC or treated for 30 min with pan-specific PKC inhibitors at approximately 10 times their IC$_{50}$ values. Following pretreatments, quipazine-induced 5-HT$_{2A}$ receptor desensitization was assessed as described in Experimental Procedures. (E and F) Dose−response curves for 5-HT at wild-type and mutant 5-HT$_{2A}$ receptors transiently transfected in HEK-293 cells. The data are expressed as a percentage of the maximal response observed with wild-type 5-HT$_{2A}$ receptors. Desensitization data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. All data represent the mean ± standard error of the mean values from at least three independent experiments performed in triplicate.

fetal calf serum. Agonist-mediated desensitization was performed exactly as previously described (*10*). To assess phorbol 12,13-dibutyrate (PDBu)-mediated 5-HT$_{2A}$ receptor desensitization, cells were preincubated for the indicated times in the absence or presence of 1 μM PDBu then washed with inositol-free DMEM. Following all pretreatments, the medium was replaced with 1× Hank's balanced salt solution supplemented with 11 mM glucose and 10 mM LiCl, and the mixture was incubated for 30 min at 37 °C in the presence or absence of 10 μM 5-HT. The reaction was terminated by aspiration and the addition of 1 mL of 10 mM formic acid. After at least 30 min at room temperature, total phosphoinositides were isolated and measured exactly as previously described (*10*).

Data Analysis. PI hydrolysis assays were performed in triplicate and repeated at least three times and were analyzed by nonlinear regression using Prism 3.0 software (GraphPad, San Diego, CA). Statistical significance of the data was determined by a two-tailed paired *t* test, defined as $p < 0.05$, and analyzed with GraphPad Prism.

RESULTS

Role of PKC in 5-HT$_{2A}$ Receptor Desensitization. In initial studies, we characterized the expression levels of FLAG epitope-tagged 5-HT$_{2A}$ receptors transiently transfected into HEK-293 cells. Cotransfection of HEK-293 cells with 2 μg of 5-HT$_{2A}$ receptor plasmid DNA and 4 μg of empty vector consistently yielded 200−400 fmol of 5-HT$_{2A}$ receptor per milligram of total protein (data not shown). Additionally, preincubation of transfected HEK-293 cells with 0.1 μM phenoxybenzamine for 30 min prior to determination of the dose response of 5-HT on PI hydrolysis resulted in a significant reduction in the maximal level of PI hydrolysis without a shift in the dose−response curve (data not shown). Taken together, these results demonstrate that 5-HT$_{2A}$ receptor expression under these conditions in HEK-293 cells does not create a situation of receptor reserve, confirming our prior studies (*17*). The transfection efficiency of HEK-293 cells in these experiments was 55% as assessed by confocal microscopy of HEK-293 cells transfected with pEGFP-N1 (data not shown).

Many prior studies (*18*−*22*) have demonstrated that phorbol ester treatment leads to a reduction in subsequent receptor responses to agonist, implying a role for protein kinase C (PKC) in agonist-mediated desensitization. Thus, in initial experiments, we compared phorbol ester- and agonist-induced 5-HT$_{2A}$ receptor desensitization. As shown in Figure 2A, pretreatment with quipazine, a 5-HT$_{2A}$ receptor agonist, induces a maximum of 65% desensitization with a $t_{1/2}$ for desensitization of 42 min, in agreement with prior studies (*8*, *10*). In comparison, pretreatment with phorbol 12,13-dibutyrate (PDBu), a PKC activator, results in an immediate reduction in the level of 5-HT$_{2A}$ receptor signaling, terminating to a level similar to that for agonist-pretreated cells (Figure 2A). Since the extent of desensitization was similar for the two treatment groups, we further explored the role of PKC in 5-HT$_{2A}$ receptor desensitization. Transfected cells were either treated overnight with phorbol 12,13-dibutryate (PDBu), which downregulates several isoforms of PKC (*8*), or pretreated for 30 min with 5 μM chelerythrine chloride (ChCl) or 0.2 μM GF109203X (GF), each of which is a pan-specific inhibitor of PKC isoforms (IC$_{50}$ values of 0.66 and 0.02 μM, respectively) (*23*, *24*). Following pretreatments, quipazine-induced 5-HT$_{2A}$ receptor

Table 1: Agonist-Induced Desensitization of PI Hydrolysis of Wild-Type and Mutant 5-HT_{2A} Receptors in HEK-293 Cells[a]

	extent of desensitization	$t_{1/2}$ of desensitization
wild type	65.8 ± 3.5	42.0 ± 7.4
S100A	−0.77 ± 3.2	−3.0 ± 5.9
T109A	−2.4 ± 3.8	+2.3 ± 8.1
S184A	+3.3 ± 3.5	+6.7 ± 8.4
S188A/T190A	−49.8 ± 3.4*	+45.3 ± 21.4*
S188A	−36.4 ± 6.3*	+28.0 ± 9.9*
S188D	−33.9 ± 4.1*	+26.5 ± 12.2*
T190A	−3.5 ± 6.4	+13.4 ± 12.9
T257A	−3.4 ± 2.5	−3.3 ± 3.0
S260A	+4.2 ± 4.3	+8.5 ± 8.8
T266A/S270A	+6.8 ± 4.3	−1.5 ± 6.2
S273A/T274A	−1.9 ± 4.1	−0.54 ± 5.3
Del(280−296)	+1.9 ± 2.2	+3.2 ± 3.6
Del(280−310)	+2.9 ± 2.9	−0.57 ± 4.1
T311A	+5.9 ± 4.3	+7.3 ± 8.3
S314A/S316A	−0.75 ± 3.7	+0.04 ± 4.7
T386A	−0.32 ± 3.5	+2.6 ± 4.9
S389A/T392A	+3.8 ± 4.7	−4.9 ± 11.4
T413A	+1.7 ± 3.9	+1.3 ± 10.6
453STOP	+0.79 ± 2.2	+0.62 ± 3.4
439STOP	−2.2 ± 2.6	−4.1 ± 3.5
421STOP	−16.9 ± 3.9*	+53.2 ± 25.2*
S421A/S422A	−16.9 ± 6.8*	+60.6 ± 14.2*
S421A	−13.3 ± 5.1*	+57.9 ± 23.3*
S421F	−18.9 ± 8.6*	+52.6 ± 18.5*
S421D	+1.1 ± 4.0	+9.0 ± 6.0
S422A	−0.76 ± 2.8	+1.2 ± 4.3
S432A	+4.3 ± 3.7	+4.4 ± 5.1
PKC-KO	−7.2 ± 3.9	−5.7 ± 7.6

[a] Because the rate and extent of desensitization vary between experiments, the values for the wild-type receptor represent an average from all experiments while the values for mutant receptors represent the deviation from the wild-type receptor control within an individual experiment.

Table 2: Relative Efficacies and Potencies of 5-HT at Wild-Type and Mutant 5-HT_{2A} Receptors for Stimulating PI Hydrolysis in HEK-293 Cells

	relative efficacy	EC_{50}[a] (nM)	log EC_{50} (nM)
wild type	1.000 ± 0.000	51.8	1.714 ± 0.073
S100A	1.027 ± 0.024	52.7	1.722 ± 0.055
T109A	1.058 ± 0.039	3.9*	0.593 ± 0.087*
S184A	1.025 ± 0.016	27.6*	1.440 ± 0.036*
S188A/T190A	0.605 ± 0.013*	135.6*	2.132 ± 0.050*
S188A	0.631 ± 0.016*	99.7*	1.999 ± 0.057*
S188D	0.527 ± 0.034*	285.8*	2.456 ± 0.019*
T190A	1.045 ± 0.028	52.2	1.718 ± 0.069
T257A	1.006 ± 0.018	58.6	1.768 ± 0.059
S260A	1.047 ± 0.025	49.8	1.697 ± 0.062
T266A/S270A	0.994 ± 0.033	106.9*	2.029 ± 0.081*
S273A/T274A	1.016 ± 0.015	54.9	1.740 ± 0.040
Del(280−296)	1.034 ± 0.014	47.0	1.672 ± 0.034
Del(280−310)	0.807 ± 0.026*	41.0	1.612 ± 0.088
T311A	1.010 ± 0.021	41.8	1.621 ± 0.050
S314A/S316A	1.024 ± 0.017	44.4	1.647 ± 0.050
T386A	0.999 ± 0.016	57.0	1.756 ± 0.046
S389A/T392A	1.002 ± 0.027	57.9	1.763 ± 0.058
T413A	0.980 ± 0.016	47.8	1.679 ± 0.035
453STOP	0.998 ± 0.019	53.3	1.727 ± 0.046
439STOP	1.016 ± 0.014	56.4	1.751 ± 0.030
421STOP	0.846 ± 0.012*	52.7	1.722 ± 0.035
S421A/S422A	1.001 ± 0.015	61.1	1.786 ± 0.030
S421A	0.994 ± 0.028	47.7	1.678 ± 0.025
S421F	1.007 ± 0.022	52.0	1.716 ± 0.029
S421D	1.001 ± 0.024	53.4	1.727 ± 0.032
S422A	1.034 ± 0.027	48.5	1.686 ± 0.034
S432A	0.996 ± 0.011	54.9	1.739 ± 0.025
PKC-KO	0.490 ± 0.015*	46.1	1.663 ± 0.082

[a] EC_{50} is the concentration of 5-HT that produces a half-maximal response.

desensitization was assessed as described in Experimental Procedures. As shown in Figure 2D, neither ChCl nor GF had any effect on 5-HT_{2A} receptor desensitization, while overnight treatment with PDBu reduced the extent of desensitization at 2−4 h, but had no effect between 10 min and 1 h. These results are consistent with our previous work (*8*) demonstrating that downregulating various PKC isoforms by overnight treatment with phorbol esters had no effect on rapid (10 min to 2 h) or delayed (>6 h) phases of agonist-induced desensitization, whereas the intermediate phase (2−4 h) was attenuated by downregulating PKC.

For quite some time, PKC has been implicated in 5-HT_{2A} receptor desensitization in cell culture (*5*), though it appears that the role of PKC is complex and may or may not involve direct 5-HT_{2A} receptor phosphorylation. Thus, to investigate the possible role of PKC-induced phosphorylation, we mutated each of the potential PKC phosphorylation sites in the intracellular loops and C-terminal tail of the 5-HT_{2A} receptor (Figure 1) and assessed the effects of the mutations on 5-HT_{2A} receptor desensitization. Three potential PKC phosphorylation sites, based on the consensus motif [ST]x-[RK] (where x is any amino acid), are found in the third intracellular (i3) loop (T257, S273, and S291), and one is found in the C-terminal tail (T386). T257, S273, and T386 were each mutated to alanine, while S291 was contained in a deletion mutant described below. The S273A/T274A double mutant, rather than single mutants, was constructed to facilitate analysis. As shown in Figure 2B and Table 1, mutation of the various consensus PKC phosphorylation sites had no effect on 5-HT_{2A} receptor desensitization. Additionally, these mutations had no effect on the potency or efficacy of 5-HT (Figure 2E and Table 2). As multiple PKC sites may be necessary to affect 5-HT_{2A} receptor desensitization, we constructed a receptor mutant with all of the PKC consensus sites (T257, S273, S291, and T386) mutated to alanine (PKC-KO). As shown in Figure 2C and Table 1, the PKC-KO mutant was desensitized in a manner similar to that of the wild-type receptor, with a decrease in agonist efficacy (E_{max}) and no change in agonist potency (EC_{50}) (Figure 2F and Table 2).

We also examined the effect these PKC consensus site mutants had on the PDBu-induced attenuation of 5-HT_{2A} receptor desensitization. As shown in Figure 3, these mutants were affected by PDBu treatment to the same extent as wild-type 5-HT_{2A} receptors. We also mutated S188 and T190 in i2 to alanine as these residues are flanked by positively charged residues and thus could conceivably constitute a noncanonical PKC phosphorylation motif. However, as shown in Figure 3B, the S188A/T190A mutation had no effect on the extent of PDBu-induced attenuation of 5-HT_{2A} receptor signaling. Taken together, these results suggest that 5-HT_{2A} receptor desensitization in HEK-293 cells is independent of the direct action of the phorbol ester-sensitive PKC isoforms on the 5-HT_{2A} receptor.

Role of Serine and Threonine Residues in i1 and i2 in Agonist-Mediated 5-HT_{2A} Receptor Desensitization. To examine the role of other serine and threonine residues in the first (i1) and second (i2) intracellular loops of the 5-HT_{2A} receptor, we individually mutated each of these residues to alanine and screened the mutants for effects on agonist-

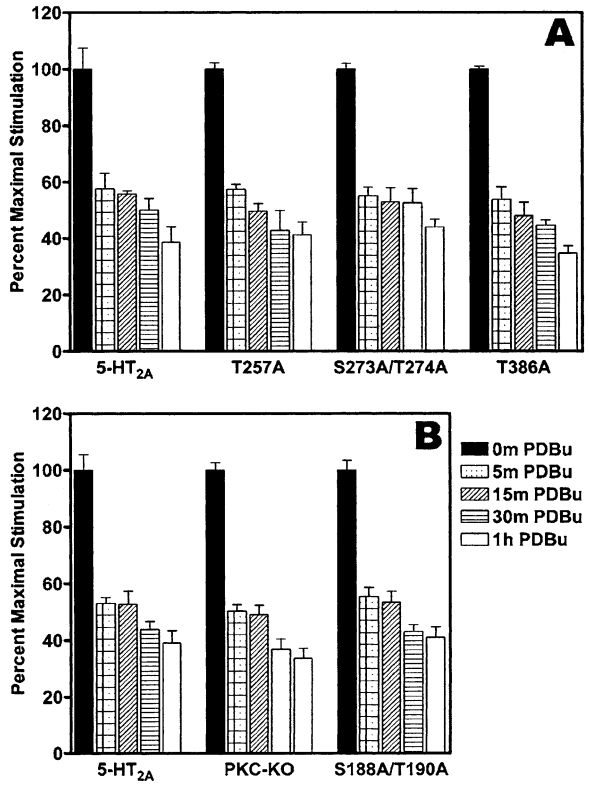

FIGURE 3: Effect of PKC consensus site mutants on PDBu-induced 5-HT$_{2A}$ receptor desensitization. (A) HEK-293 cells transiently transfected with wild-type or PKC consensus site mutant 5-HT$_{2A}$ receptors were pretreated with the phorbol ester PDBu for the indicated times and washed, and 5-HT$_{2A}$ receptor signaling was assessed as described in Experimental Procedures. (B) PDBu-induced attenuation of a complete PKC consensus site mutant (T257A, S273A, S291A, and T386A) and an S188A/T190A mutant that may constitute a noncanonical PKC phosphorylation site. Data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. All data represent the mean ± standard error of the mean values from at least three independent experiments performed in triplicate.

induced 5-HT$_{2A}$ receptor desensitization. In i1, S100 and T109 were individually mutated to alanine, and as shown in Figure 4A and Table 1, neither of these mutants affected the rate or extent of agonist-induced 5-HT$_{2A}$ receptor desensitization. Additionally, neither the S100A nor the T109A mutant affected the efficacy of 5-HT-stimulated PI hydrolysis, and the S100A mutant had no effect on potency (Figure 4E and Table 2). Interestingly, however, the T109A mutant resulted in a greater than 10-fold increase in the potency of 5-HT in stimulating PI hydrolysis (Figure 4E and Table 2).

In i2, we made S184A and S188A/T190A mutants, and as shown in Figure 4B and Table 1, S184A had no effect on the rate or extent of 5-HT$_{2A}$ receptor desensitization while the S188A/T190A double mutant resulted in a significant inhibition of 5-HT$_{2A}$ receptor desensitization. We next examined the effects of these mutations on signaling. As shown in Figure 4F and Table 2, S184A resulted in a small but significant decrease in EC_{50} with no effect on efficacy while S188A/T190A caused an almost 3-fold increase in EC_{50} and a 39.5% decrease in the maximal response. We next made the single S188A and T190A mutants and, as shown in Figure 4C and Table 1, discovered that the S188A mutant greatly attenuated 5-HT$_{2A}$ receptor desensitization while the T190A mutation had no effect. Additionally, the S188A mutant, but not the T190A mutant, had the same effects on agonist potency and efficacy as the S188A/T190A double mutants (Figure 4G and Table 2). These results demonstrate that S188 is essential for 5-HT$_{2A}$ receptor desensitization in HEK-293 cells. As mutation of a serine to aspartic acid is a commonly used approach for mimicking a phosphorylated residue, we constructed the S188D mutant and assessed the effects of this mutation on signaling and desensitization. As shown in Figure 4D and Table 1, the S188D mutant inhibited 5-HT$_{2A}$ receptor desensitization to an extent similar to that of the S188A mutants. Additionally, the S188D mutant resulted in an even greater increase in EC_{50} and a decrease in efficacy compared to those of the S188A mutant (Figure 4H and Table 2).

Role of Additional Serine and Threonine Residues in i3 in 5-HT$_{2A}$ Receptor Desensitization. In addition to the potential PKC phosphorylation sites discussed above (T257A and S273A/T274A), we mutated the remaining serine and threonine residues in the third intracellular loop (i3) of the 5-HT$_{2A}$ receptor and assessed the effects of these mutations on agonist-induced desensitization. Gq-coupled receptors are characterized by relatively long i3s which are important for G protein coupling (*25*, *26*) and have been suggested to play an important role in 5-HT$_{2A}$ receptor desensitization (*27*). Thus, to examine the possible involvement of other serine and threonine residues in 5-HT$_{2A}$ receptor desensitization, we deleted residues 280−296 and 280−310 in i3 of the receptor (Figure 1). We chose residues 280−296 due to a cluster of six serine residues and the larger deletion to capture an additional three serine and threonine residues. As shown in Figure 5A and Table 1, deletion of residues 280−296 or 280−310 had no effect on the time course or extent of 5-HT$_{2A}$ receptor desensitization in HEK-293 cells. Additionally, these deletions did not affect the potency of 5-HT (Figure 5D and Table 2), although the deletion of residues 280−310 had a small effect on agonist efficacy. In addition to these residues, we mutated each of the remaining serine and threonine residues in i3 and screened them for effects on agonist-induced 5-HT$_{2A}$ receptor desensitization. As shown in panels A and B of Figure 5 and Table 1, the S260A, T266A/S270A, T311A, and S314A/S316A mutants had no effect on the rate or extent of agonist-induced 5-HT$_{2A}$ receptor desensitization in HEK-293 cells. These mutants had no effect on the efficacy of 5-HT-stimulated PI hydrolysis (panels D and E of Figure 5 and Table 2). Additionally, S260A, T311A, and S314A/S316A had no effect on 5-HT potency, while the T266A/S270A mutant caused a modest (2-fold) increase in EC_{50} (panels D and E of Figure 5 and Table 2). Taken together, these results suggest that serines and threonines in i3 are not important for 5-HT$_{2A}$ receptor desensitization.

Role of the 5-HT$_{2A}$ Receptor Carboxyl-Terminal Tail in Receptor Desensitization. The C-terminal tail of GPCRs represents a common site for GRK-mediated phosphorylation, and accordingly, we initially constructed a series of C-terminal truncation mutants by inserting stop codons at residues 421, 439, and 453 (Figure 1). These sites were chosen because of relatively equal intervals and the location of the serines and threonines at those positions. Truncations were not made further into the tail as we predicted that they would likely interfere with receptor function. Thus, the other

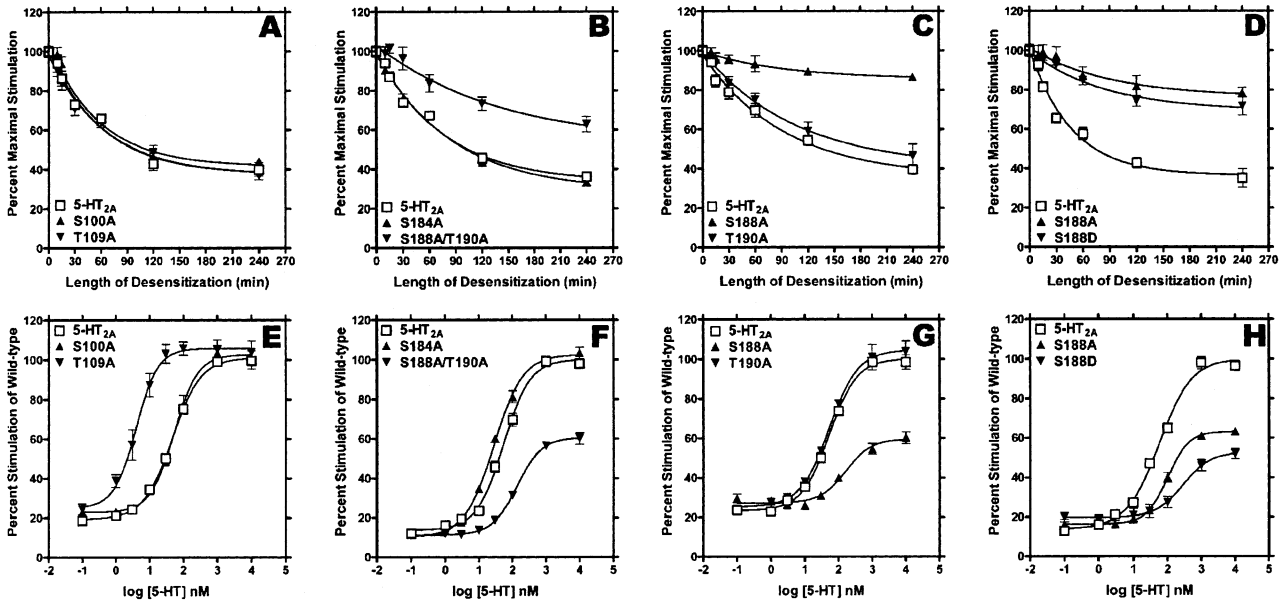

FIGURE 4: Effect of i1 and i2 mutants on agonist-induced 5-HT_{2A} receptor desensitization. To examine the role of serines and threonines in i1 and i2 in 5-HT_{2A} receptor desensitization, S100 and T109 in i1 and S184 and S188/T190 in i2 were mutated to alanine. (A) HEK-293 cells were transiently transfected with wild-type 5-HT_{2A} receptors or S100A or T109A mutants, and agonist-induced receptor desensitization was assessed as described in Experimental Procedures. (B) Agonist-induced desensitization of S184A and S188A/T190A. All points for the S188A/T190A double mutant, except at 10 min, are statistically different from those of the wild-type receptor ($p < 0.05$). (C) Since the S188A/T190A mutant inhibited desensitization, the single alanine mutants S188A and T190A were made and agonist-induced receptor desensitization was assessed. All points for the S188A mutant, except at 10 min, are statistically different from those of the wild-type receptor ($p < 0.05$). (D) Agonist-induced desensitization of an aspartic acid mutant of S188 was compared with that of S188A. All points for both the S188A and S188D mutants, except at 10 min, are statistically different from those of the wild-type receptor ($p < 0.05$). (E−H) Dose−response curves for 5-HT at wild-type and mutant 5-HT_{2A} receptors transiently transfected in HEK-293 cells. The data are expressed as a percentage of the maximal response observed with wild-type 5-HT_{2A} receptors. Desensitization data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. All data represent the mean ± standard error of the mean values from at least three independent experiments performed in triplicate.

serine and threonine residues in the receptor tail were mutated individually or in pairs. Threonine at position 386 was a potential PKC phosphorylation site and has been described above. As shown in Figure 5C and Table 1, S389A/S392A and T413A had no effect on the rate or extent of agonist-induced 5-HT_{2A} receptor desensitization in HEK-293 cells. Additionally, the S389A/S392A and T413A mutants had no effect on the efficacy or potency of 5-HT (Figure 5F and Table 2).

When the carboxy-terminal truncation mutants were examined, neither the 453STOP mutant nor the 439STOP mutant had any effect on agonist-induced 5-HT_{2A} receptor desensitization while the 421STOP mutant significantly reduced both the rate and extent of desensitization (Figure 6A and Table 1). Additionally, none of these truncation mutants altered the efficacy or potency of 5-HT (Figure 6E and Table 2). These results suggested that residues between positions 421 and 439 are important for agonist-induced 5-HT_{2A} receptor desensitization. To identify the residue(s) that is important for the reduced level of desensitization with the 421STOP mutant, we made a S421A/S422A double mutant and a S432A mutant. As shown in Figure 6B and Table 1, the S421A/S422A double mutant completely accounted for the reduced level of desensitization seen with the 421STOP mutant while the S432A mutant, which comprises both a consensus casein kinase II and PKA phosphorylation site, had no effect on 5-HT_{2A} receptor desensitization. Since the double mutant reduced the level of desensitization, we made the corresponding single mutations (S421A and S422A) to determine if one residue or both were important for the inhibition of 5-HT_{2A} receptor desensitization. As shown in Figure 6C and Table 1, S421A, but not S422A, accounted for all of the reduced level of desensitization seen with the double mutant. Again, none of these mutations significantly affected the potency of 5-HT (panels F and G of Figure 6 and Table 2). Thus, it is clear that the mutation of S421 to alanine accounts for all of the reduced level of 5-HT_{2A} receptor desensitization seen with the 421STOP mutant.

As mentioned above, mutation of a serine to aspartic acid is a commonly used approach for approximating a phosphorylated residue. Thus, we made the S421D mutant and assessed its effects on signaling and desensitization in HEK-293 cells. As shown in Figure 6D and Table 1, the S421D mutation displayed wild-type agonist-induced desensitization patterns in HEK-293 cells. Interestingly, the S421D mutant behaved in a manner identical to that of wild-type receptors in response to 5-HT (Figure 6H and Table 2). Finally, a nonsynonymous single-nucleotide polymorphism (SNP) of unknown frequency at position 421 has recently been reported in the SNP database (rs1058576 at contig position 15983618) wherein S421 is mutated to phenylalanine (S421F). We constructed the S421F mutant and found that the S421F mutant behaved in a manner identical to that of the S421A mutant (Tables 1 and 2). These results indicate that the S421F SNP is functionally important.

DISCUSSION

The major finding of this study is that two nonconserved serine residues, one in the C-terminal tail (S421) and the

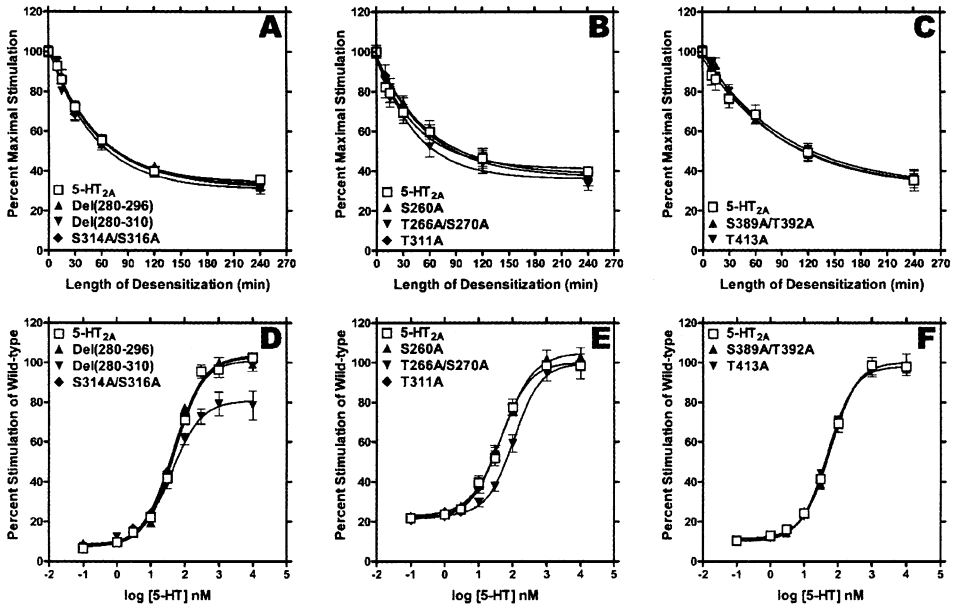

FIGURE 5: Effect of i3 and proximal carboxy-terminal tail mutants on 5-HT$_{2A}$ receptor desensitization. (A) HEK-293 cells were transiently transfected with wild-type 5-HT$_{2A}$ receptors or an S260A, T266A/S270A, or T311A mutant, and agonist-induced receptor desensitization was assessed as described in Experimental Procedures. (B) Deletion mutants of i3 of the 5-HT$_{2A}$ receptor were constructed in a region of high serine and threonine content in the center of i3. Additionally, an S314A/S316A mutant was analyzed. HEK-293 cells were transiently transfected with the wild-type receptor, the Del(280−296) and Del(280−310) deletion mutants, or S314A/S316A, and agonist-induced receptor desensitization was assessed as described in Experimental Procedures. (C) Agonist-induced desensitization of S389A/T392A and T413A mutants near the proximal end of the carboxy-terminal tail of the 5-HT$_{2A}$ receptor. Desensitization data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. (D−F) Dose−response curves for 5-HT at wild-type and mutant 5-HT$_{2A}$ receptors transiently transfected in HEK-293 cells. The data are expressed as a percentage of the maximal response observed with wild-type 5-HT$_{2A}$ receptors. All data represent the mean ± standard error of the mean values from at least three independent experiments performed in triplicate.

other in the putative i2 (S188), are essential for agonist-mediated desensitization of the 5-HT$_{2A}$ receptors. Additionally, we report that a newly discovered SNP of unknown frequency (S421F) greatly attenuates agonist-mediated desensitization; these results indicate that the S421F SNP has profound functional consequences on 5-HT$_{2A}$ receptor regulation. We also found, via alanine and deletion mutagenesis, that all of the other serine or threonine residues predicted to reside in the intracellular loop regions of the 5-HT$_{2A}$ receptor are not required for agonist-mediated desensitization. Since many of these residues are located in consensus phosphorylation sites for well-known kinases (e.g., PKC isoforms, PKA, and CKII to name a few), our findings imply that if the 5-HT$_{2A}$ receptor is a substrate for phosphorylation it must (1) be phosphorylated by a novel kinase or (2) be phosphorylated by a kinase which utilizes a novel site (e.g., S421 or S188) or that (3) phosphorylation by conventional kinases has no effect on agonist-mediated desensitization. To begin to address these possibilities, we have scanned the 5-HT$_{2A}$ sequence for potential phosphorylation sites using multiple public domain kinase site prediction algorithms and have not found any that predict that S421 is likely to be phosphorylated. One public domain algorithm (http://scansite.mit.edu/) **did** predict that S188 had a low probability of being a substrate for phosphorylation by CLK-2.

For many years, it has been clear that 5-HT$_{2A}$ receptors are desensitized following PKC activation (*18*−*22*), though these effects are cell type-specific. 5-HT$_{2A}$ receptor desensitization by PKC is often assessed following direct activation of PKC by pretreatment of cells with phorbol esters (*18*, *21*, *22*). Thus, prior studies assessed the heterologous desensitization of the 5-HT$_{2A}$ receptor by PKC activation and not the homologous desensitization of the receptor following agonist pretreatment. Interestingly, however, it has been shown that 5-HT$_{2A}$ receptors desensitized by PKC activation are **not** phosphorylated (*15*), suggesting that PKC acts downstream of the 5-HT$_{2A}$ receptor. While past studies have directly activated PKC, we examined the role of PKC in the homologous desensitization of 5-HT$_{2A}$ receptors in HEK-293 cells, by treating the cells with either pan-selective PKC inhibitors or by downregulating several PKC isoforms by overnight treatment with the phorbol ester PDBu. We found that the PKC inhibitors had no effect on homologous desensitization while overnight PDBu treatment significantly reduced the level of desensitization during the "intermediate phase" of desensitization (e.g., 2−4 h after agonist exposure). These results were similar to those from our previous study where we found that downregulating various PKC isoforms by overnight treatment with phorbol esters had no effect on rapid (10 min to 2 h) or delayed (>6 h) phases of agonist-induced desensitization, but that the intermediate phase (2−4 h) was attenuated by downregulating PKC (*8*). It is likely that the attenuation seen during this intermediate phase is the result of either nonspecific effects of overnight phorbol ester treatment or nonreceptor proximal effects of downregulating PKC isoforms. Additionally, we show that mutation of each of the putative consensus PKC phosphorylation sites in the intracellular domains of the 5-HT$_{2A}$ receptor had no effect on agonist-mediated desensitization in HEK-293 cells. Taken together, our results imply that PKC does not play a role in homologous 5-HT$_{2A}$ receptor desensitization in HEK-293 cells. Additionally, we show that mutation of all of the PKC consensus sites in the intracellular loops of the 5-HT$_{2A}$ receptor did not alter the ability of 5-HT2A

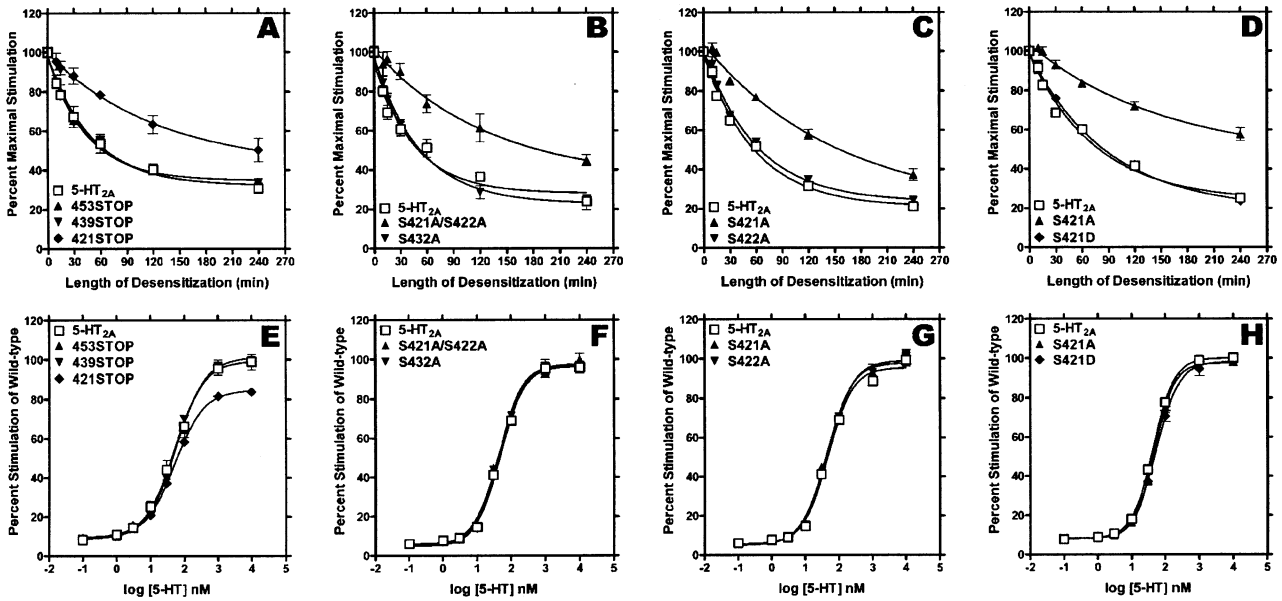

FIGURE 6: Effect of distal carboxy-terminal tail mutants on 5-HT_{2A} receptor desensitization. To examine the role of serines and threonines in the C-terminal tail of the 5-HT_{2A} receptor in receptor desensitization, truncation mutants were made by introducing STOP codons at amino acid positions 421, 439, and 453 by site-directed mutagenesis. (A) HEK-293 cells were transiently transfected with wild-type 5-HT_{2A} receptors or the 421STOP, 439STOP, or 453STOP mutant, and agonist-induced receptor desensitization was assessed as described in Experimental Procedures. All points for the 421STOP mutant, except at 10 min, are statistically different from those of the wild-type receptor ($p < 0.05$). (B) Since the 421STOP mutant attenuated 5-HT_{2A} receptor desensitization while the 439STOP mutation had no effect, we made a double S421A/S422A mutant and an S432A mutant, transiently transfected them into HEK-293 cells, and assessed agonist-induced receptor desensitization. All points for the S421A/S422A mutant are statistically different from those of the wild-type receptor ($p < 0.05$). (C) Since the S421A/S422A mutant accounted for the attenuated 5-HT_{2A} receptor desensitization seen with the 421STOP mutants while the S432A mutant had no effect, S421A and S422A single mutants were made and transiently transfected into HEK-293 cells, and agonist-induced receptor desensitization was assessed. All points for the S421A mutant are statistically different from those of the wild-type receptor ($p < 0.05$). (D) Since the S421A mutant inhibited 5-HT_{2A} receptor desensitization, we mutated this residue to aspartic acid which is commonly used to approximate a phosphorylated amino acid. HEK-293 cells were transiently transfected with wild-type 5-HT_{2A} receptors or the S421A or S421D mutant, and agonist-induced receptor desensitization was assessed. All points for the S421A mutant, except at 10 min, are statistically different from those of the wild-type receptor ($p < 0.05$). (E−H) Dose−response curves for 5-HT at wild-type and mutant 5-HT_{2A} receptors transiently transfected in HEK-293 cells. The data are expressed as a percentage of the maximal response observed with wild-type 5-HT_{2A} receptors. Desensitization data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. All data represent the mean ± standard error of the mean values from at least three independent experiments performed in triplicate.

receptors to be desensitized by PKC activation. These studies, however, do not rule out possible contributions of atypical PKC isoforms or phosphorylation at noncanonical PKC phosphorylation sites.

We also found that a single residue (S421) in the carboxy-terminal tail of the 5-HT_{2A} receptor is important for mediating homologous desensitization in HEK-293 cells. We currently have no direct evidence of phosphorylation of S421, and indeed, no evidence of any agonist-induced phosphorylation of 5-HT_{2A} receptors has ever been reported (*5*, *15*). Indeed, one could argue that our results are consistent with notions that S421 contributes to 5-HT_{2A} receptor desensitization by either phosphorylation-dependent or phosphoarylation-independent mechanisms. Thus, for instance, the S421A mutation attenuated agonist-mediated desensitization while the S421D mutation, which approximates a phosphoserine, restored the ability of the receptor to be desensitized by agonist pretreatment. On the other hand, the S421D mutant had no effect on receptor signaling. Our *a priori* prediction was that **if** the S421D mutant mimics a phosphorylated serine then the S421D mutant receptor may be "pre-desensitized" and show a diminished level of agonist-mediated signaling. Since the S421D mutant does not affect receptor signaling or the rate or extent of desensitization, our results are also consistent with the hypothesis that receptor activation stimulates accessory protein binding and not phosphorylation. For many GPCRs, phosphorylation occurs by a specific GRK which then facilitates arrestin binding (*13*). We have previously shown, however, that 5-HT_{2A} receptor internalization, desensitization, and resensitization are all arrestin-independent and are unaffected by overexpression of the G protein-coupled receptor kinases GRK2 and GRK5 in HEK-293 cells (*10*, *14*). Thus, if S421 is a kinase substrate, the kinase important for a putative phosphorylation event and the potential accessory protein involved in 5-HT_{2A} receptor desensitization in HEK-293 cells both remain unknown. Indeed, S421 does not represent any known phosphorylation consensus motifs, though many serine/threonine kinases do not have consensus phosphorylation sequences. Alternatively, it is possible that S421 is subject to some other type of post-translational modification since it is well-known that serines may be post-translationally modified in a number of ways, including acetylation, amidation, palmitoylation, and glycosylation. Further studies will be necessary to distinguish between these alternatives.

We also demonstrated that a nonsynonymous SNP of unknown frequency (S421F) has profound functional consequences in that the S421F mutation attenuates agonist-mediated desensitization. The functional consequences of such a mutation in humans are unknown. One could speculate

that since the 5-HT_{2A} receptor is essential for a number of physiological processes a deficit in desensitization is likely to have functional consequences.

In addition to the effect of S421 on 5-HT_{2A} receptor desensitization, we also demonstrated a role for i2 in the homologous desensitization of the 5-HT_{2A} receptor in HEK-293 cells. The S188A mutant at the distal end of i2 resulted in a significant attenuation of agonist-induced 5-HT_{2A} receptor desensitization. Unlike the S421 mutants though, the S188A mutant caused a 3-fold increase in EC_{50} and a 40% reduction in 5-HT efficacy. These findings imply that S188 may be important for G protein coupling, and thus, post-translational modification of this residue might directly disrupt G protein coupling efficiency, leading to desensitization **independent** of accessory protein binding. Indeed, conserved residues in this region of i2 have been implicated in determining the G_α subunit specificities of GPCRs (*28*), and homologous residues have been suggested to directly contact G_α subunits (*28*). In this regard, μ-opioid receptors have recently been shown to desensitize less rapidly than δ-opioid receptors because of the less efficient activation of arrestins, because their primary phosphorylation site is on a threonine residue in i2 rather than in the carboxy-terminal tails as in δ-opioid receptors (*29*, *30*). While arrestins are not involved in 5-HT_{2A} receptor desensitization in HEK-293 cells (*10*), these studies provide precedence for the notion that selected i2 residues might be involved in GPCR desensitization. In this regard, it is important to note that 5-HT_{2A} receptors desensitize at a slow rate compared with many GPCRs (e.g., β2-adrenergic receptors and others), similar to the μ-opioid receptors (*10*, *29*).

In conclusion, we report the discovery of two nonconserved serine residues in the intracellular domains of the 5-HT_{2A} receptor critical for agonist-mediated receptor desensitization. We also report that a newly discovered SNP (S421F) has functional consequences on agonist-mediated desensitization. Studies in progress are aimed at determining if S188 and S421 are subject to post-translational modifications (e.g., phosphorylation or acetylation) and whether they serve as docking sites for accessory proteins involved in GPCR desensitization.

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BI035061Z