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Phosphorylation of $G_{\alpha 11}$ Protein Contributes to Agonist-Induced Desensitization of 5-HT_{2A} Receptor Signaling

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ABSTRACT

Agonist treatment causes desensitization of many G protein-coupled receptor systems. Recent advances have delineated changes in receptors in the desensitization response; however, the role of G proteins remains unclear. We investigated the role of phosphorylation of $G\alpha_{q/11}$ proteins in agonist-induced desensitization of serotonin 2A (5-HT_{2A}) receptors. In an embryonic rat cortical cell line (A1A1v), 24-h treatment with 100 nM (−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), a 5-$HT_{2A/2C}$ receptor agonist, decreased DOI-stimulated inositol phosphate accumulation and increased the phosphorylation of $G\alpha_{q/11}$ proteins, as demonstrated by immunoprecipitation of $G\alpha_{q/11}$ and both incorporation of ^{32}P phosphate and labeling with a S/T/Y phosphorylation-dependent antibody. Treatment with DOI for 30 min induced desensitization but did not increase phosphorylation of $G\alpha_{q/11}$ proteins, suggesting that different mechanisms are involved in desensitization after short- and long-term treatments. Mutation of S154A in a protein kinase C (PKC) and calcium/calmodulin dependent kinase (CaMK) consensus site in $G\alpha_{11}$ significantly reduced DOI-stimulated phosphorylation of $G\alpha_{11}$ and DOI-induced desensitization of 5-HT_{2A} receptor signaling. Inhibition of PKC and CaMK attenuated phosphorylation of $G\alpha_{q/11}$ proteins and DOI-induced desensitization of 5-HT_{2A} receptors. Expression of $G\alpha_{11}$ S154D, a phosphorylation mimic, reduced DOI-stimulated inositol phosphate accumulation. DOI treatment for 24 h also produced heterologous desensitization, as indicated by decreased bradykinin-stimulated inositol phosphate accumulation. These data suggest that phosphorylation of $G\alpha_{11}$ protein by PKC and CaMK contributes to agonist-induced homologous desensitization of 5-HT_{2A} receptor signaling as well as heterologous desensitization. The phosphorylation of $G\alpha$ protein represents a novel mechanism involved in regulation of receptor signaling and agonist-induced desensitization of G protein-coupled receptors.

Alterations in serotonin 2A (5-HT_{2A}) receptor signaling have been implicated in the etiology of a number of psychiatric disorders, such as schizophrenia, depression, and obsessive-compulsive disorder (Roth, 1994; Baxter et al., 1995; Naughton et al., 2000). Several drugs currently used to treat psychiatric disorders target 5-HT_{2A} receptors. However, regulation of 5-HT_{2A} receptor signaling, specifically desensitization, is currently not well understood.

Desensitization can occur as a result of receptor uncoupling from G proteins, internalization (sequestration of the receptor away from the cell surface), or down-regulation (reduced ligand-bound receptor); each has been reported for 5-HT_{2A} receptors. Internalization of 5-HT_{2A} receptors was induced by agonist stimulation in vivo and in cell culture models, although the mechanisms involved in internalization of 5-HT_{2A} receptors are cell type-specific (Grotewiel and Sanders-Bush, 1994; Gray et al., 2001; Hanley and Hensler, 2002). Blockade of receptor internalization prevented agonist-induced desensitization of endogenous 5-HT_{2A} receptors in C6 glioma cells but not in human embryonic kidney 293 cells transfected with 5-HT_{2A} receptors. Receptor binding and autoradiographic studies have shown a 40% average decrease in agonist and antagonist binding in several brain regions, including cortical areas after sustained agonist treatment (Buckholtz et al., 1988; McKenna et al., 1989; Smith et al., 1999; Anji et al., 2000; Valdez et al., 2002). McKenna et al. (1989) found a greater reduction in the B_{max} of the high affinity (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl-labeled receptors than the reduction in

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ABBREVIATIONS: 5-HT, serotonin (5-hydroxytryptamine); DOI, (−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; PLC, phospholipase C; PKC, protein kinase C; CaMK, Ca^{2+}-calmodulin dependent kinase; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; KN-93, 2-(*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl))amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; TBS, Tris-buffered saline; IOD, integrated optical density; PI, phosphoinositol; IP, inositol phosphate; ANOVA, analysis of variance; MDL 100,907, (±)-α -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol; ANOVA, analysis of variance.

B_{max} of ketanserin-labeled 5-HT_{2A} receptors after long-term agonist treatment. The greater decrease in the DOI-labeled receptors would reflect a reduction in G proteins coupling to 5-HT_{2A} receptors.

Our recent in vivo data are consistent with a reduction in G protein coupling. We previously found a decrease in serotonin (5-HT)-stimulated phospholipase C (PLC) activity in the frontal cortex of rats after 4 to 7 days of DOI treatment (Damjanoska et al., 2004). However, sustained DOI treatment had no effect on guanosine 5′-*O*-(3-thio)triphosphate-stimulated PLC activity, suggesting that an alteration at the receptor or an alteration in receptor-G protein interaction mediates the desensitization of 5-HT_{2A} receptors. In addition, this desensitization cannot fully be explained by an alteration in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins (Damjanoska et al., 2004).

Previous studies suggested that second messenger-dependent kinases, such as protein kinase C (PKC) and Ca^{2+}-calmodulin dependent kinase (CaMK) are important in desensitization of 5-HT_{2A} receptors in cell culture models. Short-term studies demonstrated that PKC is important in 5-HT_{2A} receptor desensitization in Chinese hamster ovary cell lines that stably express human 5-HT_{2A} receptors (Berg et al., 2001) and HEK293 cells (Bhattacharyya et al., 2002). PKC inhibitors and CaMK inhibitors prevent agonist-induced 5-HT_{2A} receptor system desensitization in some (Berg et al., 2001) but not all cultured cells (Hanley and Hensler, 2002). Although kinases can mediate desensitization of 5-HT_{2A} receptor signaling, the target for phosphorylation is not known. Among the many potential targets, phosphorylation of 5-HT_{2A} receptors and $G\alpha_{q/11}$ proteins could lead to desensitization. Mutation of two serine residues to alanine residues on 5-HT_{2A} receptors attenuated agonist-induced desensitization of 5-HT_{2A} receptors; however, mutation of serine and threonine residues in PKC consensus sites had no effect on agonist-induced desensitization (Gray et al., 2003). Furthermore, in a variant of Chinese hamster lung fibroblasts, 5-HT_{2A} receptors are not phosphorylated after PKC-mediated 5-HT_{2A} receptor desensitization (Vouret-Craviari et al., 1995), suggesting that PKC-catalyzed phosphorylation of another protein such as $G\alpha_q$ or $G\alpha_{11}$ proteins could be involved. In addition, CaMK has been shown to be involved in agonist-induced desensitization of 5-HT_{2A} receptor signaling (Gray et al., 2001), although it is also not clear which protein or proteins this kinase phosphorylates to mediate the desensitization of 5-HT_{2A} receptor signaling. To summarize these previous studies, PKC and CaMK are necessary for desensitization of 5-HT_{2A} receptor signaling, but it is not clear which proteins are phosphorylated by these kinases to mediate the desensitization response. $G\alpha_{q/11}$ proteins could be the necessary substrate for desensitization, because each contains several consensus sites for phosphorylation by either CaMK or PKC subtypes (α, β, γ, μ, ζ).

Few studies have investigated alterations in the signaling pathway downstream of 5-HT_{2A} receptors that may contribute to receptor desensitization. In the present study, we found that sustained treatment with DOI caused an increase in phosphorylation of $G\alpha_{q/11}$ proteins and desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. Mutation of serine 154 to alanine in the $G\alpha_{11}$ subunit, a PKC and CaMK site, significantly attenuated DOI-induced desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. These data suggest that phosphorylation of $G\alpha_{11}$ proteins contributes to sustained agonist-induced desensitization of 5-HT_{2A} receptor signaling.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), Lipofectamine Plus, and [^{32}P]phosphate were supplied by Invitrogen (Carlsbad, CA). DOI and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). KN-93 was supplied by Tocris Cookson Inc. (Ellisville, MO). [*myo*-^{3}H]Inositol was supplied by PerkinElmer Life and Analytical Sciences (Boston, MA). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). All other reagents were of the highest grade available.

Site-Directed Mutagenesis and Plasmid Construction. The mammalian expression vector pcDNA 3.1(+) containing a cytomegalovirus promoter was purchased from Invitrogen. The pcDNA3.1(+) clones for human wild-type $G\alpha_{11}$, and $G\alpha_q$ were obtained from the UMR cDNA Resource Center (http://www.cdna.org). The sequence similarity between rat $G\alpha_{11}$ and human $G\alpha_{11}$ is 96.1%. The sequence similarity between rat $G\alpha_q$ and human $G\alpha_q$ is 99.4%. $G\alpha_{11}$ contains five consensus PKC and/or CaMK phosphorylation sites (Ser154, Ser156, Ser268, Thr54, and Thr76). $G\alpha_q$ contains four consensus PKC and/or CaMK phosphorylation sites (Ser154, Ser268, Thr54, and Thr76). These consensus sites are identical in rat and human proteins. The codons in $G\alpha_q$ and $G\alpha_{11}$ genes that encode these serine or threonine residues were mutated to codons that encode alanine residues using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutation of a serine or threonine to alanine is an approach commonly used to examine the role of phosphorylated residues. Codon 154 in $G\alpha_{11}$ was mutated to aspartic acid to mimic serine phosphorylation. DNA constructs used for transfection were purified from TOP10 *Escherichia coli* (Invitrogen) using Bio-Rad Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing. Sequencing is performed on an ABI Prism 3100 4-capillary automated genetic analyzer (Agencourt Bioscience Corporation, Beverly, MA) by Loyola University Medical Center Core Facility.

Cell Culture and Transfections. A1A1v neuronal cells endogenously express the 5-HT_{2A} receptor signaling system and were kindly provided by Dr. William Clarke and Kelly Berg (University of Texas Health Science Center, San Antonio, TX). The A1A1v cells were grown in poly-L-ornithine-coated plates in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. Serum was heat-inactivated and charcoal-treated to remove monoamines (Berg et al., 1994; Scalzitti et al., 1998). Cells were plated onto 24-well plates at a density of 2×10^4 cells/well. Cells were transiently transfected with one of the following cDNAs: either pcDNA3.1(+) empty vector, wild-type $G\alpha_q$, $G\alpha_{11}$, mutant $G\alpha_q$, or mutant $G\alpha_{11}$ using Lipofectamine Plus (Invitrogen) according to the manufacturer's recommendations. Total DNA of 4 μg/dish or 0.15 μg/well was used in each transfection. Overexpression of proteins was verified 48 h after transfection by Western blots. Samples containing 10 μg of protein were separated by SDS-polyacrylamide gel electrophoresis, and immunodetection was performed with either anti-$G\alpha_{11}$ (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-$G\alpha_q$ (1:500; Santa Cruz Biotechnology).

To determine the percentage of cells transiently transfected, cells were transiently transfected with either $G\alpha_{11}$-EE (Glu-Glu)-tagged (UMR, Rolla, MO) or pcDNA 3.1-EGFP (gift from Dr. Rory A. Fisher, University of Iowa, Iowa City, IA). Cells were transfected with $G\alpha_{11}$-EE tagged and 48 h later used for immunocytochemistry to determine transfection efficiency. Expression of $G\alpha_{11}$-EE was detected by fluorescein isothiocyanate-labeled Glu-Glu monoclonal antibody (1:500; Covance Research Products, Princeton, NJ). Vecta-

shield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA) was used to label cell nuclei, and the transfection ratio was determined by comparing fluorescein isothiocyanate- or green fluorescent protein-labeled cells with the total number of 4,6-diamidino-2-phenylindole-labeled cells.

Immunoprecipitation of G$\alpha_{q/11}$ Proteins. After treatment, cells grown in 100-mm dishes were washed twice in cold Tris-buffered saline, pH 7.4, and lysed in Tris assay buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X100, 1 mM dithiothreitol, 50 mM NaF, 2 mM activated sodium orthovanadate, and protease inhibitor cocktail (Sigma) containing 104 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.08 μM aprotinin, 2 μM leupeptin, 4 μM bestatin, 1.5 μM pepstatin A, and 1.4 μM E-64, pH 7.4. After sonication and 30-min rotation at 4°C, the homogenate was centrifuged for 15 min at 20,000*g*. The supernatant was saved from each sample and stored at −80°C before use in the immunoprecipitation assay. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

For immunoprecipitation of G$\alpha_{q/11}$ proteins, 1000 to 1500 μg of protein from each sample was brought up to a total 800-μl volume with a Tris assay buffer. Within an assay, the same amount of protein was used for all samples. The samples were then precleared using 25 μl of recombinant protein G (rProtein G) agarose (Invitrogen, Carlsbad, CA) for 1 h. The samples were centrifuged for 10 min at 10,600*g*, and the supernatant was incubated overnight at 4°C with either 2 μg of G$\alpha_{q/11}$ antibody (Santa Cruz Biotechnology) or 2 μg of normal rabbit IgG (Santa Cruz Biotechnology) as a control for nonspecific binding. The immuno-complexes were precipitated using 30 μl of the rProtein G agarose at 4°C for 1 h. The agarose-immuno complexes were washed three times using the Tris assay buffer and centrifuged after each wash at 1100*g* for 3 min. After the last wash, the agarose-immuno complex was resuspended in 2× electrophoresis sample buffer containing bromphenol blue and heated for 10 min at 100°C. The samples were centrifuged at 15,300*g* for 5 min, and the supernatant containing the G$\alpha_{q/11}$ proteins was removed.

The immunoprecipitated G$\alpha_{q/11}$ proteins were resolved by loading 15 μl of supernatant from each sample onto an SDS-polyacrylamide gel containing 0.1% SDS, 10% acrylamide/bisacrylamide (30:0.2), 4.6 M urea, and 375 mM Tris, pH 8.7. The proteins were electrophoretically transferred from the gels onto nitrocellulose membranes. The membranes were incubated at room temperature in blocking buffer (TBS solution containing 5% bovine serum albumin and 0.1% Tween 20) for 1 h and then incubated overnight at 4°C with phospho-Ser/Thr/Tyr antibody (1:200; Spring Bioscience, Fremont, CA) diluted in a TBS solution containing 5% bovine serum albumin and 0.1% Tween 20. The membranes were washed after the overnight incubation, followed by 1-h incubation at room temperature with a horseradish peroxidase-labeled anti-mouse antibody (1:50,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in the same TBS/bovine serum albumin solution as the primary antibody. Levels of G$\alpha_{q/11}$ proteins were examined to verify equal loading of protein in each lane [using G$\alpha_{q/11}$ antibody (1:500) and a horseradish peroxidase-labeled anti-rabbit antibody (1:100,000); Santa Cruz Biotechnology].

Films were analyzed densitometrically using the Scion Image program (Scion Corp., Frederick, MD). For normalization, the integrated optical density (IOD) of phosphorylated G$\alpha_{q/11}$ protein bands on each film was divided by the mean IOD of saline-treated animals and by the IOD of the respective G$\alpha_{q/11}$ protein bands (independent of their phosphorylation state). The G$\alpha_{q/11}$ protein usually resolved as a doublet (i.e., two bands very close together); however, on some films, a single larger band was evident. Both bands or the single larger band was included in the measurement of the G$\alpha_{q/11}$ protein bands.

In Vivo Labeling with [^{32}P]phosphate. For in vivo phosphorylation experiments, A1A1v cells were cultured and transfected as described above. Twenty-four hours after transfection, cells were labeled with [^{32}P]phosphate (1 mCi/100-mm dish) for 24 h, treated with the indicated amount of DOI during [^{32}P]phosphate labeling, and subjected to immunoprecipitation, gel electrophoresis, and analyses with autoradiography. Cells from two 100-mm dishes were combined for each sample. Levels of G$\alpha_{q/11}$ proteins were examined to verify the loading of protein in each lane. For normalization, the IOD of phosphorylated G$\alpha_{q/11}$ protein bands was divided by the IOD of the respective G$\alpha_{q/11}$ protein bands.

Phosphoinositol Hydrolysis. Cells were plated into 24-well plates with DMEM and 10% dialyzed fetal bovine serum. Approximately 18 to 24 h before the assay, cells were labeled with 1 μCi/ml [*myo*-^{3}H]inositol in serum-free and inositol-free medium. PI hydrolysis assays were performed as described by Berg et al. (1994). In brief, cells were washed with Hanks' balanced salt solution containing 20 mM $LiCl_2$ and 20 mM HEPES, pH 7.4. After a 15-min preincubation in Hanks' balanced salt solution, the stimulation of PI hydrolysis was initiated by addition of DOI or bradykinin at 37°C. Reaction was stopped after 30 min by the addition of ice-cold 10 mM formic acid. The accumulation of total ^{3}H-labeled inositol phosphates (IP) (inositol monophosphate, inositol bisphosphate, and inositol triphosphate) was determined by ion exchange chromatography.

Statistical Analyses. All data are presented as group mean ± S.E.M. The data were analyzed using a one- or two-way ANOVA followed by a Newman-Keul's post hoc analysis. GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used for all statistical analyses. The data for the [^{32}P]phosphate labeling resulted in unequal variances between groups, so the Wilcoxon Mann-Whitney *U* test was used as a nonparametric test in place of a Student's *t* test. Inhibition data were analyzed using Prism software (GraphPad Software, Inc. San Diego, CA). A probability level of $p < 0.05$ was considered to be statistically significant for all statistical tests.

Results

Identification of Possible Phosphorylation Sites in Gα_q and Gα_{11} Proteins. Possible phosphorylation sites on Gα_q and Gα_{11} proteins were searched for based on consensus sequences for various protein kinases and the amino acid sequences of Gα_q and Gα_{11} proteins in the Swiss-Pro database using programs available on http://scansite.mit.edu. We found that Gα_q and Gα_{11} proteins contain several consensus sites for phosphorylation by CaMK and for a number of PKC subtypes (α, β, γ, μ, ζ) (Fig. 1).

Effect of Sustained DOI Treatment on DOI-Stimulated IP Accumulation and Phosphorylated Levels of G$\alpha_{q/11}$ Proteins in A1A1v Cells. The A1A1v cell line was derived from a rat cortical culture and expresses the 5-HT$_{2A}$ receptor coupled to the stimulation of PI hydrolysis (Berg et al., 1994). [^{3}H]Ketanserin binding assay revealed that the total density of 5-HT$_{2A}$ receptor sites in A1A1v cells is 159.53 ± 7.39 fmol/mg of protein (data not shown). Treatment of A1A1v cells with increasing concentrations of DOI (10 nM to 100 μM) produced a concentration-dependent increase in IP accumulation with an EC_{50} of approximately 1 μM and E_{max} at 100 μM DOI (Fig. 2A). DOI is an agonist that has comparable affinity for 5-HT$_{2A}$ receptors and 5-HT$_{2C}$ receptors. To determine whether DOI-induced IP accumulation in A1A1v cells was mediated by 5-HT$_{2A}$ receptors, we used the 5-HT$_{2A}$ selective antagonist MDL 100,907 (pK_i = 9.07), which has a much lower affinity for 5-HT$_{2C}$ receptors (pK_i = 7.06) (Kehne et al., 1996). As shown in Fig. 2B, MDL 100,907 caused a concentration-dependent (0.5–100 nM) inhibition of DOI-induced IP accumulation. This inhibition data fit a one-site binding model as determined using Graph-

Pad Prism 4. The concentration required for 50% inhibition (IC_{50}) was 5.35 ± 0.86 nM.

Treatment of A1A1v cells with 100 nM DOI for 24 h resulted in a significant attenuation in 5-HT_{2A} receptor signaling. The E_{max} values of IP accumulation stimulated by agonist (100 μM DOI) was decreased by 51.1 ± 10.1% compared with the vehicle-treated group ($p < 0.01$). There was no change in the concentration of DOI eliciting an EC_{50} response after treatment with DOI for 24 h (Fig. 2A). Using immunoprecipitation and Western blotting, we found that 24 h of treatment with 100 nM DOI increased the levels of phosphorylated $G\alpha_{q/11}$ proteins in the A1A1v cell line probed by phospho-Ser/Thr/Tyr antibody (Fig. 2C). The levels of phosphorylation of $G\alpha_{q/11}$ proteins after 24 h of sustained DOI treatment were significantly ($p < 0.01$) increased 65% above control levels. In vivo labeling of cells with [^{32}P]phosphate showed the same pattern of results (Fig. 2D). Treatment with DOI for 24 h increased levels of phosphorylation of $G\alpha_{q/11}$ proteins to 278% of control levels as measured by [^{32}P] phosphate incorporation. Furthermore, 24 h of sustained DOI treatment did not alter the total levels of $G\alpha_{q/11}$ proteins (data not shown).

We also checked the effect of short exposures of DOI on 5-HT_{2A} receptor-mediated IP accumulation and levels of phosphorylation of $G\alpha_{q/11}$ proteins. As shown in Fig. 2E, treatment of A1A1v cells with 100 nM DOI for 15 or 30 min resulted in a significant attenuation in the E_{max} values of IP accumulation stimulated by agonist (100 μM DOI) by 39.98 and 36.06% compared with the vehicle-only group ($p < 0.01$), respectively. In vivo labeling of cells with [^{32}P]phosphate showed that there is no change of the levels of phosphorylation of $G\alpha_{q/11}$ proteins after 30-min DOI treatment compared with vehicle-treated cells ($p > 0.05$) (Fig. 2F).

Role of Serine and Threonine Residues of $G\alpha_{11}$ on DOI-Induced 5-HT_{2A} Receptor Desensitization. $G\alpha_{11}$ contains five consensus PKC and/or CaMK phosphorylation sites: Ser154, Ser156, Ser268, Thr54, and Thr76 (Fig. 1). To explore the functional consequence of phosphorylation at these residues on DOI-induced desensitization of 5-HT_{2A} receptors in A1A1v cells, we individually mutated each of these serine and threonine residues in the $G\alpha_{11}$ subunit to alanine. Expression vectors encoding full-length wild-type or mutant $G\alpha_{11}$ were constructed. The overexpression of wild-type or mutated $G\alpha_{11}$ was verified in cell homogenates 48 h after transfection by Western blot analysis (Fig. 3A). Western blot analysis revealed an increase in the level of $G\alpha_{11}$ subunit in wild-type or mutant $G\alpha_{11}$ of approximately 10- to 20-fold compared with control vector group.

To investigate the effects of the PKC and CaMK consensus site mutations in $G\alpha_{11}$ on DOI-mediated desensitization of 5-HT_{2A} receptors, A1A1v cells were first transfected with either vector, wild-type $G\alpha_{11}$, or mutated $G\alpha_{11}$. After 24 h, transfected cells were treated with either vehicle or 100 nM DOI for another 24 h. Finally, the DOI-stimulated IP accumulation was used to estimate the effect of mutation and DOI treatment. The percentage of transiently transfected cells was approximately 50%. The two-way ANOVA for mutation and DOI treatment indicated significant main effects for the mutations ($F_{6,30} = 5.54$, $p < 0.01$) and for DOI treatment ($F_{1,30} = 106.19$, $p < 0.01$). The interaction effect between mutation and DOI treatment was not statistically significant. IP accumulation after agonist stimulation was not significantly different between cells transfected with wild-type $G\alpha_{11}$ or vector. Furthermore, IP accumulation after agonist stimulation was not significantly different in cells overexpressing wild-type $G\alpha_{11}$ compared with cells overexpressing each of the mutant $G\alpha_{11}$ (Fig. 3B). However, some of the mutations in $G\alpha_{11}$ protein had an effect on agonist-stimulated IP accumulation compared with the vector-transfected group. Overexpression of $G\alpha_{11}$ T54A, T76A, and S154A resulted in a significant ($p < 0.05$) increase in the maximum IP accumulation compared with the vector-transfected group.

We also examined the effect of these consensus site mutants on the DOI-induced desensitization of 5-HT_{2A} receptor signaling. As shown in Fig. 3B, 24 h of treatment with 100

$G_{\alpha 11}$ - amino acid sequence

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1   mtlesmiacc lsdevkeskr inaeiekqlr rdkrdarrel kllllgtges gksTfikqmr
61  iihgagysee dkrgfTklvy qniftamqav vramdtlkir ykyeqnkana llirevdvek
121 vttfehqyvn aiktlwsdpg vqecydrrre fqlSdSakyy ltdvdriatv gylptqqdvl
181 rvrvpttgii eypfdlenii frmvdvggqr serrkwihcf envtsimflv alseydqvlv
241 esdnenrmee skalfrtiit ypwfqhsSvi lflnkkdlle dkilhshlvd yfpefdgpqr
301 daqaarefil kmfvdlnpds dkiiyshftc atdtenirfv faavkdtilq lnlkeynlv
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$G_{\alpha q}$ -amino acid sequence

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1   mtlesimacc lseeakearr indeierqlr rdkrdarrel kllllgtges gksTfikqmr
61  iihgsgysde dkrgfTklvy qniftamqam vramdtlkip ykyehnkaha qlvrevdvek
121 vsafenpyvd aikslwndpg iqecydrrre yqlSdstkyy lndldrvadp sylptqqdvl
181 rvrvpttgii eypfdlqsvi frmvdvggqr serrkwihcf envtsimflv alseydqvlv
241 esdnenrmee skalfrtiit ypwfqnsSvi lflnkkdlle ekimyshlvd yfpeydgpqr
301 daqaarefil kmfvdlnpds dkiiyshftc atdtenirfv faavkdtilq lnlkeynlv
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Fig. 1. Possible phosphorylation sites on $G\alpha_q$ and $G\alpha_{11}$ proteins. Consensus sites for possible phosphorylation of $G\alpha_q$ and $G\alpha_{11}$proteins were identified using programs available on http://scansite.mit.edu. Possible Ser/Thr phosphorylation sites are underlined, *, possible PKC (α, β, μ, ζ) site; †, possible CaMK phosphorylation site.

nM DOI resulted in a significant decrease in the maximum IP accumulation in cells overexpressing wild-type and mutant Gα_{11} proteins compared with the respective vehicle-treated control group. There was no significant interaction between DOI treatment and Gα_{11} protein mutation ($F_{6,30}$ = 2.16, p = 0.08). Furthermore, as shown in Fig. 3C, there was a main effect of Gα_{11} protein mutation for the DOI-induced decrease in IP accumulation ($F_{6,15}$ = 4.85, $p < 0.01$). Overexpression of Gα_{11} S154A attenuated 5-HT$_{2A}$ receptor desensitization induced by 24-h pretreatment with DOI. The reduction in DOI-mediated IP accumulation in cells overexpressing Gα_{11} S154A was significantly attenuated by 40.9% ($p < 0.05$) compared with wild-type Gα_{11} (Fig. 3C).

Role of Serine and Threonine Residues of Gα_q on DOI-induced 5-HT$_{2A}$ Receptor Desensitization. Four consensus PKC and/or CaMK phosphorylation sites were found in Gα_q subunit: Ser154, Ser268, Thr54, and Thr76 (Fig. 1). Each of these residues was mutated, and an expression vector was constructed. Forty-eight hours after transfection, the overexpression of wild-type or mutant Gα_q protein subunit was verified by Western blot (Fig. 4A). Increases in Gα_q subunit levels of approximately 10-fold were found in cells transfected with wild-type and mutant Gα_q compared with vector control.

To investigate the effect of PKC and CaMK consensus site mutations in Gα_q on DOI-mediated desensitization of 5-HT$_{2A}$ receptor signaling, A1A1v cells were first transfected with either vector, wild-type Gα_q, or mutated Gα_q. After 24 h, transfected cells were treated with either vehicle or 100 nM DOI for another 24 h. Finally, the DOI-stimulated IP accumulation was used to estimate the effect of the mutation and DOI treatment. The two-way ANOVA for mutation and DOI treatment indicated significant main effects for Gα_q protein mutation ($F_{5,24}$ = 4.41, $p < 0.01$) and DOI treatment ($F_{1,24}$ = 144.63, $p < 0.001$). The interaction effect between mutation and DOI treatment was not statistically significant. There was no difference in the agonist-stimulated IP accumulation between wild-type Gα_q and vector-transfected cells. Overex-

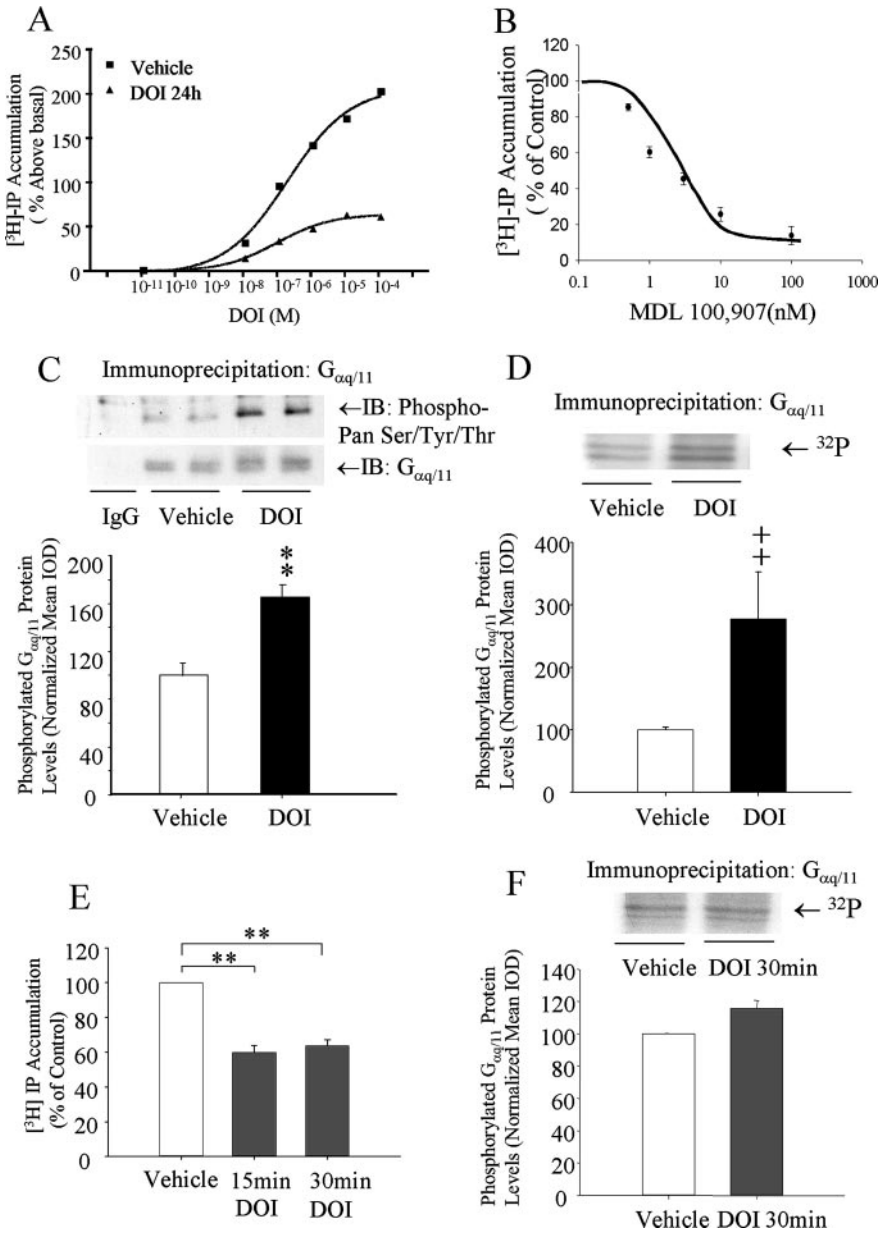

Fig. 2. Effect of sustained DOI treatment (100 nM, 24 h) on IP accumulation and levels of phosphorylated G$\alpha_{q/11}$ proteins in A1A1v cells. A, treatment of A1A1v cells with 100 nM DOI for 24 h resulted in a significant attenuation in IP accumulation stimulated by 100 μM DOI compared with vehicle group. Data shown are the mean of triplicate determinations of a single representative experiment. Basal IP accumulation was 37.7 ± 2.1 and 39.5 ± 3.6 dpm for vehicle- and DOI-treated A1A1v cells, respectively. B, 5-HT$_{2A}$ receptor antagonist MDL 100,907 caused a dose-dependent (0.5–100 nM) inhibition of DOI-stimulated IP accumulation in A1A1v cells. The concentration required for 50% inhibition is 5.35 ± 0.86 nM. C, treatment with 100 nM DOI for 24 h significantly increased phosphorylation of G$\alpha_{q/11}$ protein compared with vehicle treatment. A representative blot showing increased phosphorylation of G$\alpha_{q/11}$ proteins after DOI treatment. G$\alpha_{q/11}$ proteins were immunoprecipitated and then separated on an SDS-PAGE gel. The blot was probed with a phosphorylation-dependent antibody and then with an antibody for G$\alpha_{q/11}$ proteins. D, increased phosphorylation of G$\alpha_{q/11}$ proteins after DOI treatment was probed by [^{32}P]phosphate labeling. Phosphorylation data represent the mean ± S.E.M. of five experiments. E, treatment of cells with 100 nM DOI for 15 or 30 min resulted in a significant attenuation in IP accumulation stimulated by 100 μM DOI compared with the vehicle-treated group. Data shown are the mean ± S.E.M. of three experiments. F, levels of phosphorylation of G$\alpha_{q/11}$ proteins after DOI treatment for 30 min were probed by [^{32}P] phosphate labeling. Phosphorylation data represent the mean ± S.E.M. of three experiments. **, $p < 0.01$ using Student's t test; +, $p < 0.01$ using Wilcoxon Mann-Whitney U test (used because of unequal variance).

pression of $G\alpha_q$ T54A, T76A, and S268A did not cause a difference in IP accumulation after agonist stimulation compared with wild-type $G\alpha_q$ (Fig. 4B). It is noteworthy that overexpression of $G\alpha_q$ S154A caused an increase in IP accumulation compared with wild-type $G\alpha_q$ protein overexpression ($p < 0.01$).

We also examined the effect of these consensus site mutants on DOI-induced 5-HT_{2A} receptor desensitization. As shown in Fig. 4B, 24 h of 100 nM DOI treatment resulted in a significant decrease in the IP accumulation in cells overexpressing wild-type proteins and each of the mutant $G\alpha_q$ proteins compared with respective vehicle-treated control group ($F_{1,24} = 144.63$, $p < 0.001$). There was no significant interaction between DOI treatment and mutation ($F_{5,24} = 1.22$, $p = 0.33$). In the one-way ANOVA, there was no main effect of $G\alpha_q$ protein mutation on the 24 h DOI-induced decrease in IP accumulation ($F_{5,12} = 0.22$, $p = 0.95$). As seen in Fig. 4C, none of the mutant $G\alpha_q$ proteins caused change in the extent of IP accumulation produced by 24 h DOI treatment.

Expression of Phosphorylation State Mimic $G\alpha_{11}$S154D in A1A1v Cells Decreased the 5-HT_{2A} Receptor Signaling. To confirm the key role played by $G\alpha_{11}$

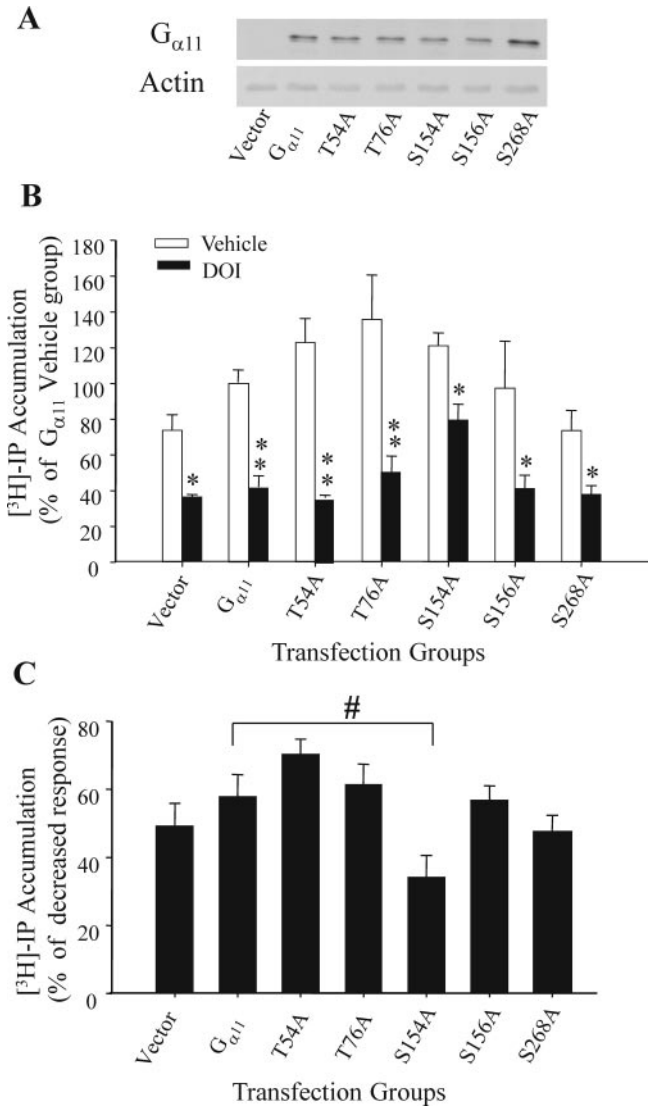

Fig. 3. Effect of PKC and CaMK consensus site mutations in $G\alpha_{11}$ on DOI-induced desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. A1A1v cells were transiently transfected with vector, wild-type $G\alpha_{11}$, or one of five $G\alpha_{11}$ mutants. A, a representative blot demonstrates overexpression of wild-type $G\alpha_{11}$ and five $G\alpha_{11}$ mutants 48 h after transfection. The blot was probed with $G\alpha_{11}$ antibody. B, treatment with 100 nM for 24 h significantly decreased IP accumulation in cells overexpressing of wild-type and mutant $G\alpha_{11}$. **, $p < 0.01$; *, $p < 0.05$ compared with cells transfected with the same construct but treated with vehicle. C, overexpression of $G\alpha_{11}$ S154A attenuated desensitization induced by DOI treatment. #, $p < 0.05$ compared with cells transfected with wild-type $G\alpha_{11}$. The data are presented as a percentage of the cells transfected with wild-type $G\alpha_{11}$ and treated with vehicle and are the mean ± S.E.M. of three experiments assayed in triplicate.

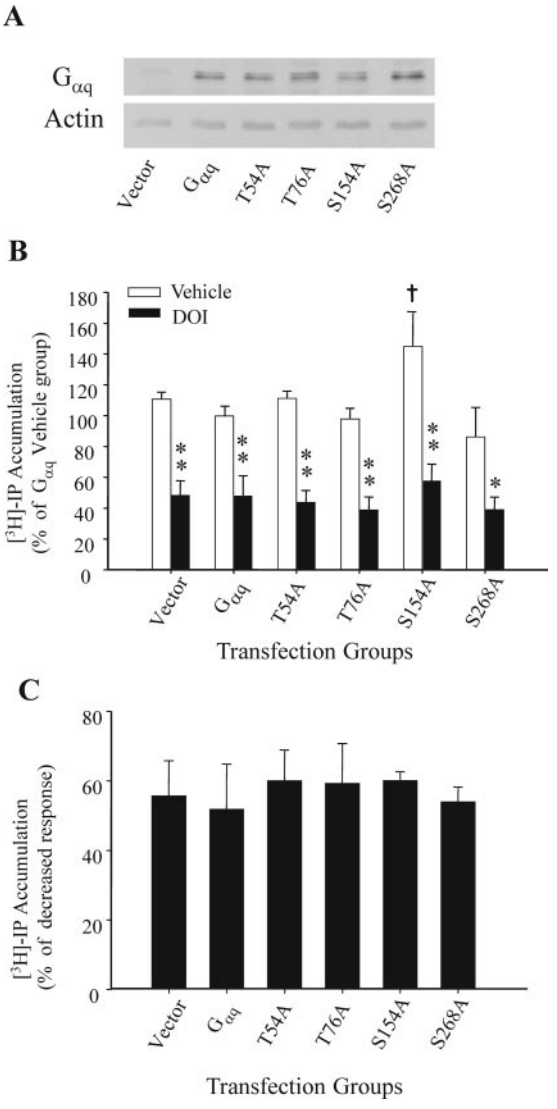

Fig. 4. Effect of PKC and CaMK consensus site mutation in $G\alpha_q$ on DOI-induced desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. A, a representative blot demonstrating overexpression of wild-type $G\alpha_q$ and four $G\alpha_q$ mutants 48 h after transfection. The blot was probed with $G\alpha_q$ antibody. B, treatment with 100 nM for 24 h resulted in a significant decrease in IP accumulation in cells overexpressing wild-type and $G\alpha_q$ mutant cells. **, $p < 0.01$; *, $p < 0.05$ compared with cells transfected with the same construct but treated with vehicle. Overexpression of $G\alpha_q$ T54A, T76A, and S268A did not cause a difference in IP accumulation after agonist stimulation compared with wild-type $G\alpha_q$. Overexpression of $G\alpha_q$ S154A caused an increase in IP accumulation. †, $p < 0.05$ compared with wild-type $G\alpha_q$. The data are presented as a percentage of cells transfected with wild-type $G\alpha_q$ and treated with vehicle and are the mean ± S.E.M. of three experiments assayed in triplicate. C, none of the mutant $G\alpha_q$ proteins caused difference in the extent of DOI-induced desensitization measured using IP accumulation.

Ser154 in mediating the effect of DOI-induced 5-HT$_{2A}$ receptor desensitization, we mutated Gα_{11} Ser154 to aspartic acid (Gα_{11}S154D), which is a commonly used approach for approximating a phosphorylated residue. A1A1v cells were transfected with Gα_{11}S154D and 24 h later were treated with vehicle or DOI 100 nM for 24 h. We also overexpressed Gα_{11}S154A as a control. Expression of Gα_{11}S154D was confirmed by Western blotting 48 h after transfection (Fig. 5A). Overexpression of Gα_{11}S154D resulted in decreased DOI-induced PI accumulation compared with wild-type Gα_{11} ($p < 0.05$) and Gα_{11}S154A ($p < 0.05$) (Fig. 5B). We also examined the effect of this mutant on DOI-induced 5-HT$_{2A}$ receptor desensitization. As shown in Fig. 5B, 24 h of 100 nM DOI treatment resulted in a significant decrease in the IP accumulation in cells overexpressing wild-type Gα_{11} proteins and each of the mutant Gα_{11} proteins compared with respective vehicle-treated control group ($F_{1,31} = 116.24$, $p < 0.001$). There was no significant interaction between DOI treatment and mutation ($F_{3,24} = 2.9$, $p = 0.051$). Furthermore, after 24-h pretreatment with DOI, the DOI-induced IP accumulation in cells overexpressing Gα_{11} S154D was not significantly different from wild-type Gα_{11} (Fig. 5B).

Effect of Expression of Gα_{11}S154A on DOI-Induced Phosphorylation of G$\alpha_{q/11}$ Proteins in A1A1v Cells. To determine whether 24-h DOI treatment altered phosphorylation of G$\alpha_{q/11}$ at sites other than Gα_{11} Ser154, we compared phosphorylation in cells transfected with wild-type Gα_{11} or Gα_{11}S154A and treated with DOI or vehicle for 24 h. To normalize phosphorylation levels to the levels of G$\alpha_{q/11}$ (i.e., to normalize for equal loading of protein), we also examined the G$\alpha_{q/11}$ protein levels on Western blots. As shown in Fig. 6A, 24 h of treatment with 100 nM DOI significantly increased phosphorylated G$\alpha_{q/11}$ proteins levels to 190% of vehicle treated cells transfected with wild-type Gα_{11} proteins as measured by [^{32}P]phosphate incorporation. Levels of phosphorylation of G$\alpha_{q/11}$ proteins were not increased in the cells transfected with Gα_{11}S154A after DOI treatment (Fig. 6A).

Effect of Kinase Inhibitor on DOI Induced Desensitization. To determine whether second messenger-dependent kinases, such as PKC or CaMK, play a role in DOI-induced desensitization of 5-HT$_{2A}$ receptors, cells were pretreated with the selective CaMK inhibitor KN-93 or PKC activator PMA. Overnight treatment with PMA causes the down-regulation of most PKC isoforms (Dempsey et al., 2000). Therefore, in our experiment, we used it as a PKC inhibitor. Different concentrations of inhibitors were tested to determine the concentration that produced inhibition of phosphorylation without causing cell death (data not shown). As shown in Fig. 7, pretreatment of cells with KN-93 (Fig. 7A) or PMA (Fig. 7B) for 24 h attenuated DOI-induced attenuation of DOI-stimulated IP accumulation by 36.9% ($p < 0.01$) and 23.52% ($p < 0.01$), respectively. Next, we measured the effects of KN-93 and PMA on the phosphorylation of G$_{\alpha q/11}$ using [^{32}P] phosphate incorporation. Pretreatment of cells with KN-93 or PMA for 24 h attenuated the DOI-induced increase in phosphorylation of G$\alpha_{q/11}$ proteins by 41.0% ($p < 0.05$) and 28.3% ($p < 0.05$), respectively (Fig. 7C).

Effect of DOI Treatment on Another G$\alpha_{q/11}$-Coupled Receptor. Our data suggest that DOI-induced desensitization of 5-HT$_{2A}$ receptors resulted in part from phosphorylation at Gα_{11} Ser154. Next, we wanted to determine whether cross-desensitization occurred in other native receptors ex-

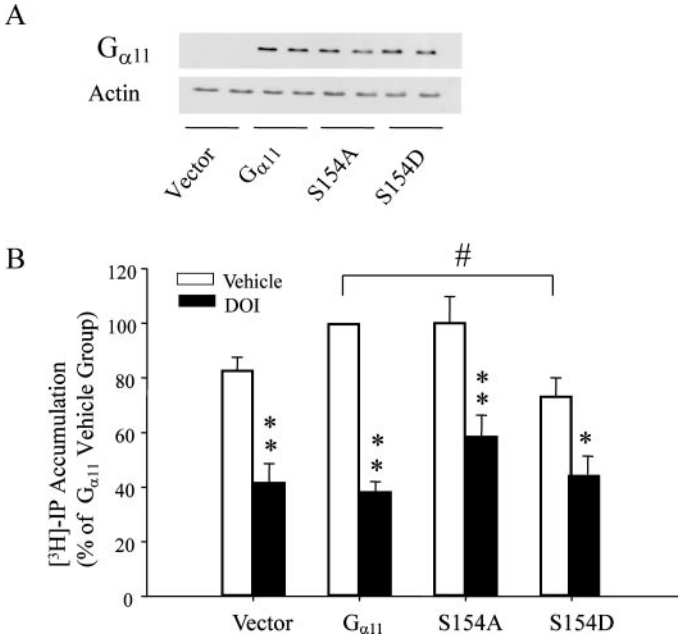

Fig. 5. Expression of Gα_{11}S154D in A1A1v cells decreased 5-HT$_{2A}$ receptor signaling. A, a representative blot demonstrates overexpression of wild-type Gα_{11}, Gα_{11}S154A, or Gα_{11}S154D mutant 48 h after transfection. B, overexpression of Gα_{11}S154D resulted in decreased DOI-induced IP accumulation compared with cells transfected with wild-type Gα_{11} and Gα_{11}S154A and treated with vehicle. #, $p < 0.05$; **, $p < 0.01$ compared with cells transfected with the same construct but treated with vehicle. The data are presented as a percentage of the cells transfected with wild-type Gα_{11} and treated with vehicle and are the mean ± S.E.M. of three experiments assayed in triplicate.

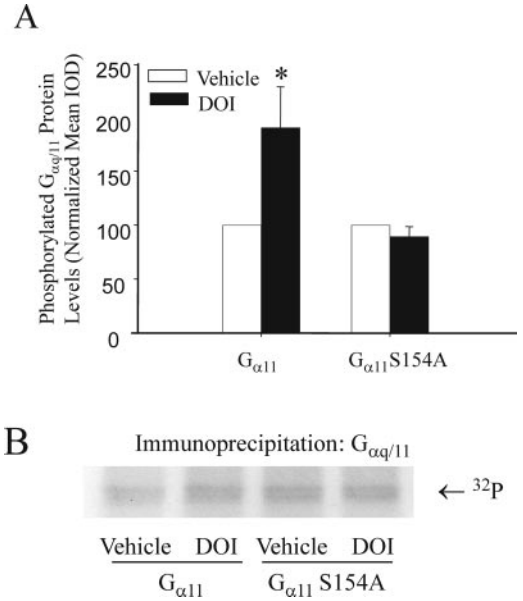

Fig. 6. Effect of expression of Gα_{11}S154A on DOI-induced phosphorylation of G$\alpha_{q/11}$ proteins. A, A1A1v cells were transfected with wild-type Gα_{11} or Gα_{11}S154A and treated with DOI or vehicle for 24 h. The level of phosphorylated G$\alpha_{q/11}$ protein was measured by [^{32}P] phosphate incorporation and normalized to the levels of G$\alpha_{q/11}$ (to verify the equal loading of protein). *, $p < 0.05$ compared with cells transfected with the same construct but treated with vehicle. B, a representative blot demonstrating the change of the phosphorylated G$\alpha_{q/11}$ protein probed with [^{32}P] phosphate.

pressed in the A1A1v cells and functionally coupled with $G\alpha_{q/11}$. Bradykinin receptors are reported to couple to $G\alpha_{q/11}$ proteins, and activation of endogenous bradykinin receptor in PC-12 cells induces IP accumulation (Moskvina et al., 2003). To verify that the A1A1v cells are capable of synthesizing IP in response to bradykinin, A1A1v cells were labeled with [*myo*-^{3}H]inositol and preincubated in 10 mM LiCl for 15 min to block inositol phosphatases. Thereafter, the cells were incubated in various concentrations of bradykinin (10^{-10}~10^{-5} M) for 30 min. As shown in Fig. 8, bradykinin caused concentration-dependent increases in IP accumulation; maximal stimulation occurred at 10^{-5} M and 50% of maximal stimulation at approximately 10^{-8} M in A1A1v cells. To determine whether DOI treatment could desensitize bradykinin receptor-mediated signaling, A1A1v cells were stimulated by bradykinin after treatment with DOI for 24 h. As shown in Fig. 8B, DOI treatment for 24 h significantly decreased the bradykinin-stimulated IP accumulation at the EC_{50} concentration of 10^{-8} M compared with vehicle-treated cells ($p < 0.05$), but not at the E_{max} concentration of 10^{-5} M. To further determine whether DOI-induced increases in phosphorylation of $G\alpha_{q/11}$ proteins are responsible for decreased bradykinin-stimulated IP accumulation, cells were transfected either wild-type $G\alpha_{11}$ or the phosphorylation mimic $G\alpha_{11}$ S154D mutant. Forty-eight hours after transfection, the bradykinin-stimulated IP accumulation was measured. IP accumulation stimulated with both 10^{-8} M (Fig. 8C) and 10^{-5} M (Fig. 8D) bradykinin was lower in cells transfected with $G\alpha_{11}$ S154D compared with cells transfected with wild-type $G_{\alpha 11}$ ($p < 0.05$).

Discussion

To our knowledge, this is the first report to investigate the phosphorylation of $G\alpha$ proteins in agonist-induced regulation of receptor signaling. Compared with the saline-treated control group, DOI-stimulated IP accumulation was decreased by 51% and phosphorylation of $G\alpha_{q/11}$ proteins was increased by 65% after 24-h treatment with 100 nM DOI. DOI treatment for 24 h also induced heterologous desensitization, as indicated by a decrease in bradykinin-stimulated IP accumulation. To test the hypothesis that phosphorylation of $G\alpha$ proteins contributes to sustained agonist-induced desensitization of 5-HT_{2A} receptor signaling, we mutated potential phosphorylation sites in the $G_{\alpha 11}$ and $G\alpha_q$ subunits. We found that mutation of Ser154 to alanine in the $G\alpha_{11}$ subunit significantly attenuated the DOI-induced increase of phosphorylation of $G\alpha_{q/11}$ proteins and desensitization of 5-HT_{2A} receptor signaling in A1A1v cells. Consistent with these results, expression of S154D $G\alpha_{11}$, a phosphorylation mimic, reduced DOI-stimulated IP accumulation.

Desensitization of the 5-HT_{2A} receptor was induced by both short-term (30-min) and prolonged (24-h) DOI exposure in our study. However, the increased phosphorylation of $G\alpha_{q/11}$ proteins occurred only with prolonged DOI exposure, not with short-term, 30-min DOI exposure. Consistent with previous reports, our results suggest that different mechanisms are involved in the production of desensitization of 5-HT_{2A} receptors after short-term and prolonged exposure to agonist (Roth et al., 1995; Hanley and Hensler, 2002).

To determine whether the phosphorylation of $G\alpha_{11}$ and

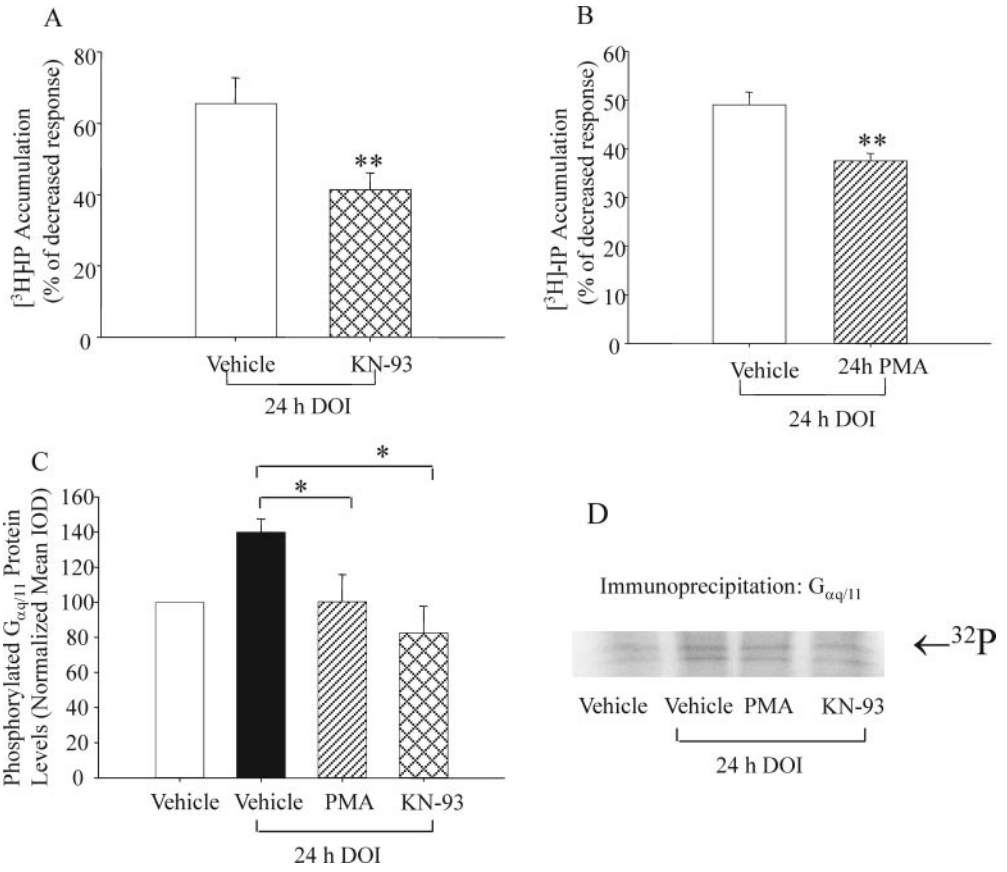

Fig. 7. Inhibitors of second messenger-dependent kinases attenuate DOI-induced desensitization of 5-HT_{2A} receptor functions and phosphorylation of $G\alpha_{q/11}$ protein. Cells were pretreated with 10 μM PMA for 24 h or 10 nM KN-93 for 30 min before the addition of 100 nM DOI for 24 h first, then incubated with kinase inhibitors during the DOI treatment. A and B, accumulation of [^{3}H]IP was measured in triplicate after 30-min incubation with DOI. Treatment of cells with PMA alone did not alter the E_{max} (vehicle, 253.5 ± 54.3% above basal; PMA-treated, 216.6 ± 35.5% above basal; $n = 4$). Treatment of cells with KN-93 alone did not alter the E_{max} (vehicle, 176.8 ± 17.5% above basal; KN-93-treated, 134.1 ± 12.3% above basal; $n = 5$). Data plotted are expressed as E_{max}, percentage of respective control. Each value represents the mean ± S.E.M. of four experiments. Data were analyzed by Student's t test; **, $p < 0.01$. C, the levels of phosphorylated $G\alpha_{q/11}$ protein were measured by [^{32}P] phosphate incorporation and normalized to the levels of $G\alpha_{q/11}$ (for the equal loading of $G\alpha_{q/11}$ protein). Data were analyzed by one-way ANOVA and Newman-Keuls post hoc [$F_{3,11} = 4.5$, $p = 0.039$]; *, $p < 0.05$ compared with cells treated with DOI. D, a representative blot of the phosphorylated $G\alpha_{q/11}$ protein probed with [^{32}P]phosphate.

$G\alpha_q$ is necessary for agonist-induced desensitization, we overexpressed the wild-type and mutant $G\alpha_{11}$ and/or $G\alpha_q$ in A1A1v cells. A recent study reported a large increase in maximal agonist-induced IP production after the overexpression of $G\alpha_q$ protein using an inducible system that produced a 38-fold increase compared with noninduced cells (Scragg et al., 2005). However, we found no difference in DOI-stimulated IP accumulation among cells transfected with vector, overexpression of wild-type $G\alpha_{11}$ or $G\alpha_q$, and mutant $G\alpha_{11}$ or $G\alpha_q$ except for the $G\alpha_q$ S154A mutant. The difference between the results of that report and the results reported herein may be due to the lower levels of $G\alpha_{11}$ or $G\alpha_q$ expression in our studies (10–20-fold). It is noteworthy that we found that DOI-induced desensitization was not completely suppressed in cells transiently transfected with $G\alpha_{11}$S154A. One simple explanation for this phenomenon is transfection efficiency, because only 50% of cells were transfected. Another possible explanation is that other mechanisms also contribute to the desensitization response produced by 24-h exposure to DOI. We were surprised to find that the increase in phosphorylation of $G\alpha_{q/11}$ protein was completely suppressed in cells transfected with $G\alpha_{11}$S154A and treated with DOI for 24 h. We would expect to see the same reduction in phosphorylation that occurred for the IP accumulation. We suspect that the sensitivity of the IP accumulation assay is greater than the sensitivity in the assay measuring phosphorylation; this difference could account for our inability to detect phosphorylation differences in cells transfected with $G\alpha_{11}$S154A compared with wild-type $G\alpha_{11}$.

Previous studies have shown that PKC and CaMK are necessary for short-term desensitization of 5-HT$_{2A}$ receptor signaling, but 5-HT$_{2A}$ receptors do not appear to be the necessary substrate of these kinases (Vouret-Craviari et al., 1995; Anji et al., 2001; Berg et al., 2001; Gray et al., 2003). Because Ser154 in $G\alpha_{11}$ is a PKC and CaMK consensus site, these previous studies are consistent with our data, suggesting that phosphorylation of $G\alpha_{11}$ protein is necessary for complete agonist-induced desensitization of 5-HT$_{2A}$ receptor signaling. The increase in phosphorylation of $G\alpha_{q/11}$ proteins that we observed could be due to a number of mechanisms. Agonist treatment could increase activity of kinases or decrease activity of phosphatases. Both PKC and CaMK could be activated by sustained 5-HT$_{2A}$ receptor agonist treatment. As shown in this report and many others, 5-HT$_{2A}$ receptor stimulation leads to an increase in PLC activity (Berg et al., 2001). Thereafter, PLC-catalyzed production of diacylglycerol stimulates PKC activity. In addition, PLC-catalyzed production of inositol triphosphate leads to increases in intracellular calcium, leading to an increase in CaMK activity. Our results suggest that both PKC and CaMK play a role in increased phosphorylation of $G\alpha_{q/11}$ proteins because treatment of cells with a CaMK inhibitor (KN-93) or PKC inhibitor (overnight treatment of PMA) attenuated DOI-induced attenuation of DOI-stimulated IP accumulation and phosphorylation of $G\alpha_{q/11}$ proteins.

The data from our lab have shown that 4 and 7 days of DOI treatment are sufficient to induce a desensitization of PLC activity stimulated 5-HT in rat frontal cortex (Damjanoska et al., 2004). This treatment does not, however, induce a desensitization of guanosine 5′-*O*-(3-thio)triphosphate-mediated PLC activity. These differential effects suggest that desensitization of 5-HT$_{2A}$ receptor signaling is due to a disruption between the receptor and the G protein and not between the G protein and the effector. Phosphorylation of $G\alpha_{q/11}$ proteins

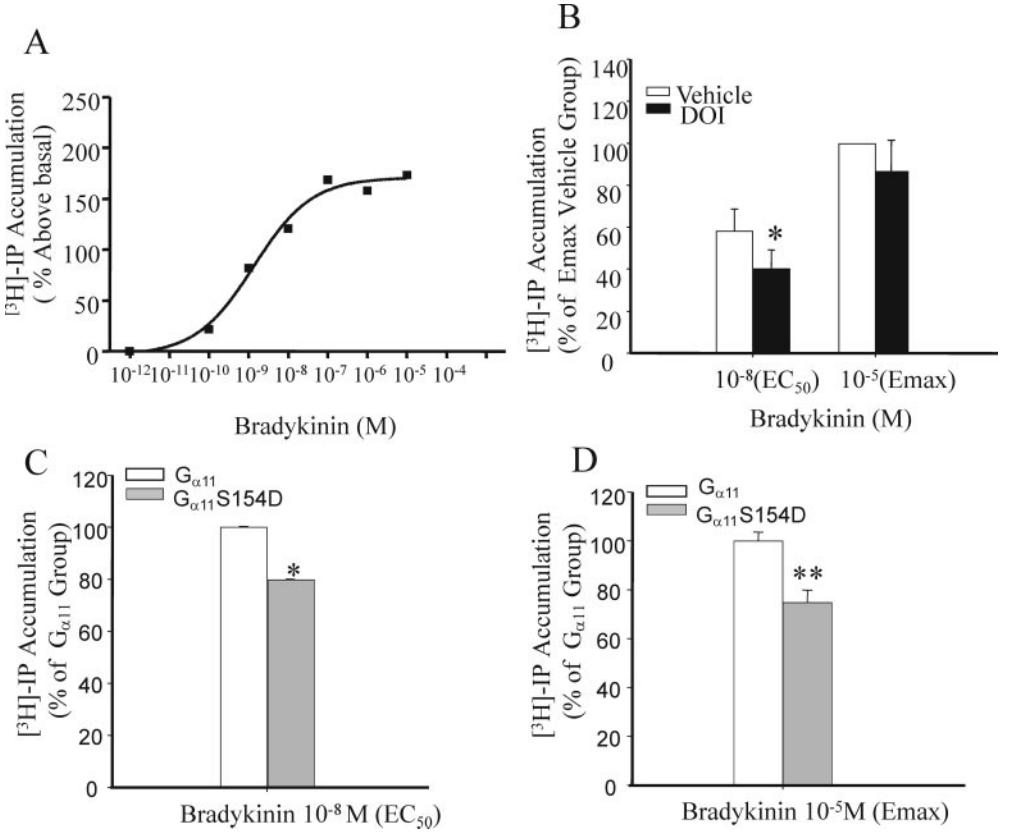

Fig. 8. Effect of sustained DOI treatment (100 nM, 24 h) on bradykinin receptor-mediated signaling. A, the concentration-response curves represent IP accumulation after 30-min incubations with various concentrations of bradykinin (10^{-10}~10^{-5} M) induced. We found concentration-dependent increases of IP accumulation with an EC_{50} at 10^{-8} M and an E_{max} at 10^{-5} M. Data shown are the mean of triplicate determinations in a single representative experiment. B, treatment of A1A1v cells with 100 nM DOI for 24 h resulted in a significant attenuation in IP accumulation stimulated by 10^{-8} M bradykinin compared with the vehicle-treated group. Data represent the mean ± S.E.M. of three experiments. Basal IP accumulations were 45.6 ± 5.5 and 48.5 ± 2.5 dpm for vehicle- and DOI-treated A1A1v cells, respectively. *, significantly different at $p < 0.05$ using Student's t test. C and D, cells transfected with $G\alpha_{11}$S154D (4 μg/plate) for 48 h had a lower IP accumulation after stimulation with 10^{-8} M (C) and 10^{-5} M (D) bradykinin compared with cells transfected with wild-type $G\alpha_{11}$. Data represent the mean ± S.E.M. of 3 experiments. *, significantly different at $p < 0.05$ using Student's t test; **, significantly different at $p < 0.01$ using Student's t test.

may hinder receptor and G protein interaction. Indeed, phosphorylation of tyrosine residues in $G\alpha_{q/11}$ proteins diminished the interaction of $G\alpha_{q/11}$ proteins with M1 muscarinic receptors in mouse embryo fibroblast cells (Umemori et al., 1997). Other in vitro studies suggest that some but not all $G\alpha$ subunits are substrates for phosphorylation by protein kinase C (Fields and Casey, 1995; Kozasa and Gilman, 1996; Glick et al., 1998). Phosphorylation of the $G\alpha_{12}$ and $G\alpha_z$ protein subunits inhibit the interaction of these subunits with the $G\beta\gamma$ heterodimer (Fields and Casey, 1995; Kozasa and Gilman, 1996); phosphorylation of $G\alpha_{i2}$ inhibits interaction with the effector enzyme (Strassheim and Malbon, 1994), and phosphorylation of $G\alpha_z$ inhibits interaction with RGS proteins (Glick et al., 1998). Phosphorylation of the $G\alpha_{11}$ protein could prevent the formation of the $G_{\alpha\beta\gamma}$ protein trimer or prevent the G protein trimer from associating with the receptor, or both. Caveolin-1 has also been shown to be important in the coupling of 5-HT_{2A} receptors to G proteins, indicating that phosphorylation of $G\alpha_{11}$ could potentially interfere with the interaction of $G\alpha_{11}$ protein with caveolin-1 (Bhatnagar et al., 2001).

In our current study, we found that 24-h DOI treatment significantly decreased bradykinin-stimulated IP accumulation at the ED_{50} concentration compared with vehicle-treated cells ($p < 0.05$), but not at the E_{max} concentration. This DOI-mediated heterologous desensitization of bradykinin stimulated IP accumulation is pharmacologically characterized as a right-shift in the dose-response curve with no change in the maximal response (E_{max}). A right-shift in the dose-response curve suggests a reduction in coupling of G protein to bradykinin receptors. Consistent with phosphorylation of $G\alpha_{11}$ causing the heterologous desensitization of bradykinin receptor system in A1A1v cells, transfection of $G\alpha_{11}$S154D, the phosphorylation state mimic also produced heterologous desensitization of bradykinin receptor signaling.

It is noteworthy that overexpression of mutant $G\alpha_q$S154A increased the DOI-stimulated IP accumulation compared with wild-type $G\alpha_q$. These results are consistent with the hypothesis that the phosphorylation of $G\alpha_q$ Ser154 may occur constitutively in A1A1v cells and decrease 5-HT_{2A} receptor-mediated signaling. Further studies are needed to determine whether this site is constitutively phosphorylated, as the data with the $G\alpha_q$S154A mutant suggest. Mutation of the same site in $G\alpha_{11}$ resulted in diminished agonist-mediated desensitization of 5-HT_{2A} receptor signaling. Furthermore, expression of the phosphorylation state mimic $G\alpha_{11}$S154D decreased the 5-HT_{2A} receptor signaling. All these data support the hypothesis that the $G\alpha_{q/11}$ Ser154 site is important in the function of $G\alpha_{q/11}$ proteins and that phosphorylation of this site plays a regulatory role in signaling.

In conclusion, these studies are the first to show that agonist-induced desensitization of 5-HT_{2A} receptor signaling correlates with increased phosphorylation of $G\alpha_{q/11}$ proteins and that phosphorylation of $G\alpha_{11}$ Ser154 is necessary for the full desensitization response of the 5-HT_{2A} receptor system in a cell culture model. Although our assay to detect phosphorylation of $G\alpha_{q/11}$ was not able to differentiate phosphorylation of $G\alpha_q$ and $G\alpha_{11}$, our results with mutant $G\alpha_{q/11}$ proteins suggest that phosphorylation of only $G\alpha_{11}$ was involved in the desensitization response induced by DOI. The contribution of phosphorylation of $G\alpha_{11}$ in the desensitization of 5-HT_{2A} receptor signaling underscores the importance of studying the involvement of various postsynaptic proteins in receptor signaling pathways to fully delineate the mechanisms mediating desensitization phenomena. In this study, we examined only single mutations in $G\alpha_{q/11}$ proteins. It would be interesting to determine the effects of mutants with multiple substitutions for serine and threonine residues compared with single mutations in $G\alpha_{q/11}$ proteins, because previous studies have demonstrated functional cooperativity in phosphorylation. Further studies are also needed to determine whether phosphorylation of $G\alpha_{11}$ is involved in antagonist-induced desensitization of 5-HT_{2A} receptors or in agonist-induced desensitization of 5-HT_{2A} receptors in animal models.

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