Phosphorylation of Gα11 Protein Contributes to Agonist-Induced Desensitization of 5-HT_{2A} Receptor Signaling

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Non-standard Abbreviations:
Ca2+-calmodulin kinase (CaMK)
DOI ((-)-1-(2,5-dimethoxy-4-lodophenyl)-2-aminopropane HCl)
integrated optical densities (IOD)
inositol phosphate (IP)
phosphoinositol (PI)
phospholipase C (PLC)
protein kinase C (PKC)
serotonin 2A (5-HT2A)
serotonin (5-HT)
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 Gαq/11 proteins in agonist-induced desensitization of serotonin 2A (5-HT2A) receptors. In an embryonic rat cortical cell line (A1A1v), 24 hour treatment with 100 nM of a 5-HT2A/2C receptor agonist DOI ((-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl) decreased DOI-stimulated inositol phosphate accumulation and increased the phosphorylation of Gαq/11 proteins as demonstrated by immunoprecipitation of Gαq/11 and both incorporation of 32P phosphate and labeling with a S/T/Y phosphorylation-dependent antibody. 30 min treatment with DOI induced desensitization but did not increase phosphorylation of Gαq/11 proteins suggesting that different mechanisms are involved in desensitization following acute and chronic treatments. Mutation of S154A in a protein kinase C (PKC) and calcium/calmodulin dependent kinase (CaMK) consensus site in Gαq11 significantly reduced DOI-stimulated phosphorylation of Gαq and DOI-induced desensitization of 5-HT2A receptor signaling. Inhibition of PKC and CaMK attenuated phosphorylation of Gαq/11 proteins and DOI-induced desensitization of 5-HT2A receptors. Expression of Gαq 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 IP accumulation. These data suggest that phosphorylation of Gαq11 protein by PKC and CaMK contributes to agonist-induced homologous desensitization of 5-HT2A receptor signaling as well as heterologous desensitization. The phosphorylation of Gα protein represents a novel mechanism involved in regulation of receptor signaling and agonist-induced desensitization of G protein-coupled receptors.
INTRODUCTION

Alterations in serotonin 2A (5-HT\textsubscript{2A}) receptor signaling have been implicated in the etiology of a number of psychiatric disorders such as schizophrenia, depression, and obsessive compulsive disorder (Baxter et al., 1995; Naughton et al., 2000; Roth, 1994). Currently several drugs used to treat psychiatric disorders target 5-HT\textsubscript{2A} receptors. However, regulation of 5-HT\textsubscript{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\textsubscript{2A} receptors. Internalization of 5-HT\textsubscript{2A} receptors was induced by agonist stimulation \textit{in vivo} and in cell culture models, although the mechanisms involved in internalization of 5-HT\textsubscript{2A} receptors are cell type specific (Gray et al., 2001; Grotewiel and Sanders-Bush, 1994; Hanley and Hensler, 2002). Blockade of receptor internalization prevented agonist-induced desensitization of endogenous 5-HT\textsubscript{2A} receptors in C6 glioma cells but not in HEK 293 cells transfected with 5-HT\textsubscript{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 following sustained agonist treatment (Anji et al., 2000; Buckholtz et al., 1988; McKenna et al., 1989; Smith et al., 1999; Valdez et al., 2002). McKenna et al. (McKenna et al., 1989) found a greater reduction in the B\textsubscript{max} of the high affinity DOI ((\pm)\textsubscript{2}-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl)-labeled receptors than the reduction in B\textsubscript{max} of ketanserin-labeled 5-HT\textsubscript{2A} receptors after chronic agonist treatment. The greater decrease in the DOI-labeled receptors would reflect a reduction in G proteins coupling to 5-HT\textsubscript{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 4-7 days of DOI treatment (Damjanoska et al., 2004). However, sustained DOI treatment had no effect on GTPγS-stimulated PLC activity, suggesting that an alteration at the receptor or an alteration in receptor-G protein interaction mediates the desensitization of 5-HT2A receptors. Additionally, this desensitization cannot fully be explained by an alteration in the levels of Gαq and Gα11 proteins (Damjanoska et al., 2004).

Previous studies suggested that second messenger-dependent kinases, such as protein kinase C (PKC) and Ca2+-calmodulin dependent kinase (CaMK) are important in desensitization of 5-HT2A receptors in cell culture models. Acute studies demonstrated that PKC is important in 5-HT2A receptor desensitization in Chinese hamster ovary (CHO) cell lines that stably express human 5-HT2A receptors (Berg et al., 2001) and HEK293 cells (Bhattacharyya et al., 2002). PKC inhibitors and CaMK inhibitors prevent agonist-induced 5-HT2A 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-HT2A receptor signaling, the target for phosphorylation is not known. Among the many potential targets, phosphorylation of 5-HT2A receptors and Gαq/11 proteins could lead to desensitization. Mutation of two serine residues to alanine residues on 5-HT2A receptors attenuated agonist-induced desensitization of 5-HT2A 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-HT2A receptors are not phosphorylated after PKC-mediated 5-HT2A receptor desensitization (Vouret-Craviari et al., 1995), suggesting that PKC-catalyzed phosphorylation of another protein such as Gαq or Gα11 proteins could be involved. Additionally, 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 since each contains several consensus sites for phosphorylation by either CaMK or PKC subtypes ($\alpha, \beta, \gamma, \mu, \zeta$).

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$_{\alpha m}$ 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 $[^{32}\text{P}]$ 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 (Ellisville, MI). $[^3\text{H}]$ myo-inositol was supplied by PerkinElmer Life 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 CMV promoter was purchased from Invitrogen (Carlsbad, CA). The pcDNA3.1(+) clones for human wild-type $\alpha_{11}$ and $\alpha_{q}$ were obtained from the UMR cDNA Resource Center (www.cdna.org). The sequence similarity between rat $\alpha_{11}$ and human $\alpha_{11}$ is 96.1%. The sequence similarity between rat $\alpha_q$ and human $\alpha_{11}$ is 99.4%. $\alpha_{11}$ contains five consensus PKC and/or CaMK phosphorylation sites (serines 154, 156, 268 and threonine 54, 76). $\alpha_q$ contains four consensus PKC and/or CaMK phosphorylation sites (serines 154, 268 and threonine 54, 76). These consensus sites are identical in rat and human proteins. The codons in $\alpha_q$ and $\alpha_{11}$ genes that encode these serine or threonine residues were mutated to codons that encode alanine residues by using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutation of a serine or threonine to alanine is a commonly used approach to examine the role of phosphorylated residues. Codon 154 in $\alpha_{11}$ was mutated to aspartic acid to mimic serine phosphorylation. DNA constructs used for transfection were purified from TOP10 *E. coli* (Invitrogen life technologies, Carlsbad, CA) 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-HT2A 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% CO2. 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 \(2 \times 10^4\) 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, Carlsbad, CA) according to the manufacturer’s recommendations. A total of 4 \(\mu\)g/dish or 0.15 \(\mu\)g/well of DNA was used in each transfection. Over-expression of proteins was verified 48 hours after transfection by Western blots. Samples containing 10 \(\mu\)g of protein were separated by SDS-PAGE and immunodetection was performed with either anti- G\(_\alpha\)11 (1:500, Santa Cruz Biotechnology, CA, USA) or anti- G\(_\alpha\)q (1:500, Santa Cruz Biotechnology, CA, USA).

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). Cells were transfected with G\(_\alpha\)11-EE tagged and
48 h later used for immunocytochemistry to determine transfection efficiency. Expression of 
Gα11-EE was detected by FITC labeled Glu-Glu monoclonal antibody (1:500; Covance Research Products, Berkeley, CA). Vectashield mounting medium with DAPI (Vector Laboratories, Inc, Burlingame, CA) was used to label cell nuclei and the transfection ratio was determined by comparing FITC or GFP labeled cells to the total number of DAPI labeled cells.

**Immunoprecipitation of Gαq/11 Proteins**

After treatment, cells grown in 100mm dish 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, St Louis, MO) containing AEBSF 104 µM, Aprotinin 0.08 µM, Leupeptin 2 µM, Bestatin 4 µM, Pepstatin A 1.5 µM and E-64 1.4 µM, pH 7.4). After sonication and 30 min rotation at 4°C, the homogenate was centrifuged for 15 minutes at 20,000×g. The supernatant was saved from each sample and stored at -80°C prior to use in the immunoprecipitation assay. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

For immunoprecipitation of Gαq/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 hour. The samples were centrifuged for 10 minutes at 10,600×g and the supernatant was incubated overnight at 4°C with either 2 µg of Gαq/11 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or 2 µg of normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as a control for non-specific
binding. The immuno-complexes were precipitated using 30 µl of the rProtein G agarose at 4°C for 1 hour. The agarose-immuno complexes were washed three times using the Tris assay buffer and centrifuged after each wash at 1100×g for 3 minutes. After the last wash, the agarose-immuno complex was resuspended in 2×electrophoresis sample buffer containing bromophenol blue and heated for 10 minutes at 100°C. The samples were centrifuged at 15,300×g for 5 minutes 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 a sodium dodecyl sulfate (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 1hr 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 hr 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 Inc., Santa Cruz, CA).

Films were analyzed densitometrically using the Scion Image program (Frederick, Maryland, U.S.A.). For normalization, the integrated optical density (IOD) of phosphorylated G\(_{\alpha q/11}\) protein bands on each film were divided by the mean IOD of saline treated-animals and by
the IOD of the respective G\textsubscript{q/11} protein bands (independent of their phosphorylation state). The G\textsubscript{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 were included in the measurement of the G\textsubscript{q/11} protein bands.

**In Vivo Labeling with [\textsuperscript{32}P]phosphate**

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

**Phosphoinositol (PI) Hydrolysis.**

Cells were plated into 24-well plates with DMEM and 10% dialyzed fetal bovine serum. 18–24h before the assay, cells were labeled with 1 \textmu Ci/ml [\textsuperscript{3}H]myo-inositol in serum-free and inositol-free medium. PI hydrolysis assays were performed as described by Berg et al. (Berg et al., 1994). In brief, cells were washed with Hanks’ balanced salt solution containing 20 mM LiCl\textsubscript{2} and 20 mM HEPES, pH 7.4. After a 15-min preincubation in Hanks’s balanced salt solution, the stimulation of PI hydrolysis was initiated by addition of DOI or bradykinin at 37\textdegree C. Reaction was stopped after 30 min by the addition of ice-cold 10 mM formic acid. The
accumulation of total $[^3H]$ labeled inositol phosphate (IP) (inositol monophosphate, inositol bisphosphate and inositol triphosphate) was determined by ion exchange chromatography.

**Statistical Analyses**

All data are presented as group mean ± SEM. The data were analyzed using a one-way 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 $[^32P]$ phosphate labeling resulted in unequal variances between groups so the Wilcoxon Mann-Whitney U-test was used as a non-parametric test in place of a Students t-test. Inhibition data was analyzed using GraphPad Prism (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 $G_{\alpha q}$ and $G_{\alpha 11}$ Proteins**

Possible phosphorylation sites on $G_{\alpha q}$ and $G_{\alpha 11}$ proteins were searched for based on consensus sequences for various protein kinases and the amino acid sequences of $G_{\alpha q}$ and $G_{\alpha 11}$ proteins in the Swiss-Pro database using programs available on http://scansite.mit.edu. We found that $G_{\alpha q}$ and $G_{\alpha 11}$ proteins contain several consensus sites for phosphorylation by CaMK and for a number of PKC subtypes ($\alpha$, $\beta$, $\gamma$, $\mu$, $\zeta$) (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 $159.53 \pm 7.39$ fmol/mg 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 Emax 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 if DOI-induced IP accumulation in A1A1v cells is mediated by 5-HT$_{2A}$ receptors, we used the 5-HT$_{2A}$ selective antagonist MDL 100,907 ((±)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol)(pKi = 9.07) which has a much lower affinity for 5-HT$_{2C}$ receptors (pKi = 7.06) (Kehne et al., 1996). As shown in figure 2B, MDL 100,907 caused a concentration-dependent (0.5 to 100 nM) inhibition of DOI-induced IP accumulation. This inhibition data fit a one-site binding model as determined using GraphPad Prism4 (GraphPad Software, Inc. San Diego, CA). The concentration required for 50% inhibition (IC$_{50}$) is 5.35 ± 0.86 nM.

Treatment of A1A1v cells with 100 nM DOI for 24h resulted in a significant attenuation in 5-HT$_{2A}$ receptor signaling. The Emax 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 24h (Fig 2A). Using immunoprecipitation and western blotting, we found that 24h of treatment with 100 nM DOI increased the levels of phosphorylated G$_{aq/11}$ proteins in the A1A1v cell line probed by phospho-Ser/Thr/Tyr antibody (Fig. 2C). The levels of phosphorylation of G$_{aq/11}$ proteins after 24h 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). 24h of DOI treatment increased levels of phosphorylation of G\textsubscript{\alpha q/11} proteins to 278% of control levels as measured by \[^{32}P\] phosphate incorporation. Furthermore, 24h of sustained DOI treatment did not alter the total levels of G\textsubscript{\alpha q/11} proteins (data not shown).

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

\textbf{Role of Serine and Threonine Residues of G\textsubscript{\alpha 11} on DOI-induced 5-HT\textsubscript{2A} Receptor Desensitization}

G\textsubscript{\alpha 11} contains five consensus PKC and/or CaMK phosphorylation sites: serines 154, 156, 268 and threonine 54 and 76 (Fig. 1). To explore the functional consequence of phosphorylation at each of these serine or threonine residues on DOI-induced desensitization of 5-HT\textsubscript{2A} receptors in A1A1v cells, we individually mutated each of these serine and threonine residues in the G\textsubscript{\alpha 11} subunit to alanine. Expression vectors encoding full-length wild-type or mutant G\textsubscript{\alpha 11} were constructed. The overexpression of wild-type or mutated G\textsubscript{\alpha 11} was verified in cell homogenates 48h after transfection by Western blots analysis (Fig. 3A). Western blot analysis revealed that approximately 10~20 fold increase of level of G\textsubscript{\alpha 11} subunit in wild-type or mutant G\textsubscript{\alpha 11} in comparison of control vector group.
In order to investigate the effects of the PKC and CaMK consensus site mutations in \( \alpha_{11} \) on DOI-mediated desensitization of 5-HT\(_{2A} \) receptors, A1A1v cells were first transfected with either vector, wild-type \( \alpha_{11} \) or mutated \( \alpha_{11} \). After 24 hours, transfected cells were treated with either vehicle or 100 nM DOI for another 24 hours. 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 following agonist stimulation was not significantly different between cells transfected with wild-type \( \alpha_{11} \) or vector. Furthermore, IP accumulation following agonist stimulation was not significantly different in cells overexpressing wild-type \( \alpha_{11} \) compared to cells overexpressing each of the mutant \( \alpha_{11} \) (Fig. 3B). However, some of the mutations in \( \alpha_{11} \) protein had an effect on agonist stimulated IP accumulation compared with the vector-transfected group. Overexpression of \( \alpha_{11} \) 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 figure 3B, 24 hours of treatment with 100 nM DOI resulted in a significant decrease in the maximum IP accumulation in cells overexpressing wild-type and mutant \( \alpha_{11} \) proteins compared with the respective vehicle-treated control group. There was no significant interaction between DOI treatment and \( \alpha_{11} \) protein mutation \((F(6,30) = 2.16, p=0.08)\). Furthermore, as shown in figure 3C, there was a main effect of \( \alpha_{11} \) protein mutation for the DOI-induced decrease in IP accumulation \((F(6,15)= 4.85, p<0.01)\).
Overexpression of $G_{\alpha_{11}}$ S154A attenuated 5-HT$_2A$ receptor desensitization induced by 24h pre-treatment with DOI. The reduction in DOI-mediated IP accumulation in cells overexpressing $G_{\alpha_{11}}$ S154A was significantly attenuated by 40.9% ($p<0.05$) compared with wild-type $G_{\alpha_{11}}$ (Fig. 3C).

**Role of Serine and Threonine Residues of $G_{\alpha q}$ on DOI-induced 5-HT$_2A$ Receptor Desensitization**

Four consensus PKC and/or CaMK phosphorylation sites were found in $G_{\alpha q}$ subunit: serine 154, 268 and threonine 54, 76 (Fig. 1). Each of these serine or threonine residues was mutated and an expression vector was constructed. 48h after transfection, the overexpression of wild-type or mutant $G_{\alpha q}$ protein subunit was verified by Western blot (Fig. 4A). Approximately 10 fold increases of $G_{\alpha q}$ subunit levels were found in cells transfected with wild-type and mutant $G_{\alpha q}$ compared to vector control.

In order to investigate the effect of PKC and CaMK consensus site mutations in $G_{\alpha q}$ on DOI-mediated desensitization of 5-HT$_2A$ receptor signaling, A1A1v cells were first transfected with either vector, wild-type $G_{\alpha q}$ or mutated $G_{\alpha q}$. After 24 hours, transfected cells were treated with either vehicle or 100 nM DOI for another 24 hours. 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 $G_{\alpha q}$ 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 $G_{\alpha q}$ and vector transfected cells. Overexpression of $G_{\alpha q}$ T54A, T76A and S268A did not cause a difference in IP
accumulation following agonist stimulation compared with wild-type $G_{\alpha q}$ (Fig. 4B).

Interestingly, overexpression of $G_{\alpha q}$ S154A caused an increase in IP accumulation in comparison to 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 figure 4B, 24 hours 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 hour DOI-induced decrease in IP accumulation ($F(5,12) = 0.22, p = 0.95$). As seen in figure 4C, none of the mutant $G_{\alpha q}$ proteins caused change in the extent of IP accumulation produced by 24 hour 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 $G_{\alpha 11}$S154 in mediating the effect of DOI-induced 5-HT$_{2A}$ receptor desensitization, we mutated $G_{\alpha 11}$ serine 154 to aspartic acid ($G_{\alpha 11}$S154D), which is a commonly used approach for approximating a phosphorylated residue. A1A1v cells were transfected with $G_{\alpha 11}$S154D and 24 hours later were treated with vehicle or DOI 100nM for 24 hours. We also overexpressed $G_{\alpha 11}$S154A as a control. Expression of $G_{\alpha 11}$S154D was confirmed by western blotting 48 hours after transfection (Figure 5A). Overexpression of $G_{\alpha 11}$S154D resulted in decreased DOI-induced PI accumulation compared to wild-type $G_{\alpha 11}$ ($p<0.05$) and $G_{\alpha 11}$S154A ($p<0.05$) (Figure 5B). We also examined the effect of this mutant on DOI-induced 5-
HT2A receptor desensitization. As shown in figure 5B, 24 hours of 100 nM DOI treatment resulted in a significant decrease in the IP accumulation in cells overexpressing wild-type Gα11 proteins and each of the mutant Gα11 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 24h pre-treatment with DOI, the DOI-induced IP accumulation in cells overexpressing Gα11 S154D was not significantly different than wild-type Gα11 (Figure 5B).


To determine whether 24 hr DOI treatment altered phosphorylation of Gαq/11 at sites other than Gα11S154, we compared phosphorylation in cells transfected with wild-type Gα11 or Gα11S154A, and treated with DOI or vehicle for 24hr. In order to normalize phosphorylation levels to the levels of Gαq/11 (i.e., to normalize for equal loading of protein), we also examined the Gαq/11 protein levels on western blots. As shown in figure 6A, 24 hr of treatment with 100 nM DOI significantly increased phosphorylated Gαq/11 proteins levels to 190% of vehicle treated cells transfected with wild-type Gα11 proteins as measured by [32P]phosphate incorporation. Levels of phosphorylation of Gαq/11 proteins were not increased in the cells transfected with Gα11S154A 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-HT2A receptors, cells were pretreated
with the selective CaMK inhibitor KN-93 or PKC activator phorbol 12-myristate 13-acetate (PMA). Overnight treatment with PMA causes the down-regulation of most PKC isoforms (Dempsey et al., 2000). So in our experiment we used it as PKC inhibitor. Different concentrations of inhibitors were tested to determine the concentration which produces 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 24hr 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 $G_{aq/11}$ using [$^{32}$P] phosphate incorporation. Pretreatment of cells with KN-93 or PMA for 24 h attenuated the DOI-induced increase in phosphorylation of $G_{aq/11}$ proteins by 41.0% (p<0.05) and 28.3% (p<0.05) respectively (Fig. 7C).

**Effect of DOI-treatment on Another $G_{aq/11}$-coupled Receptor**

Our data suggests that DOI-induced desensitization of 5-HT$_2$A receptors resulted in part from phosphorylation at $G_{a11}$S154. Next, we wanted to determine if cross-desensitization occurred in other native receptors expressed in the A1A1v cells and functionally coupled with $G_{aq/11}$. Bradykinin receptors are reported to couple to $G_{aq/11}$ proteins and activation of endogenous bradykinin receptor in PC12 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 [$^3$H]myo-inositol and preincubated in 10mM LiCl for 15 min to block inositol phosphatases. Thereafter, the cells were incubated in various concentrations of bradykinin ($10^{-10}$ to $10^{-5}$M) for 30 min. As shown in Fig. 8, bradykinin caused concentration-dependent increases in IP accumulation with maximal stimulation at $10^{-5}$M and 50% of maximal
stimulation at around $10^{-8}$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 24h. 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 if DOI-induced increases in phosphorylation of G$_{aq/11}$ proteins are responsible
for decreased bradykinin-stimulated IP accumulation, cells were transfected either wild-type
G$_{a11}$ or the phosphorylation mimic G$_{a11}$ S154D mutant. 48h 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$_{a11}$ S154D compared with
cells transfected with wild-type G$_{a11}$ (p<0.05).
DISCUSSION

To our knowledge, this is the first report investigating 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. 24h DOI treatment 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 serine 154 residue 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 only occurred 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 following short-term and prolonged exposure to agonist (Hanley and Hensler, 2002; Roth et al., 1995).

In order to determine if the phosphorylation of $G_{\alpha m}$ and $G_{\alpha q}$ is necessary for agonist-induced desensitization, we overexpressed the wild-type and mutant $G_{\alpha 11}$ and/or $G_{\alpha q}$ in A1A1v cells. Although a recent study reported a large increase in maximal agonist-induced IP production following the overexpression of $G_{\alpha q}$ protein using an inducible system that produced...
a 38-fold increase compared with non-induced cells (Scragg et al., 2005), we did not find a 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 that report and the results herein may due to the lower levels of $G_{\alpha 11}$ or $G_{\alpha q}$ expression in our studies (10 to 20-fold). Interestingly, we found that DOI-induced desensitization was not completely suppressed in cells transiently transfected with $G_{\alpha 11}$S154A.

One simple explanation for this phenomena is the transfection efficiency, since 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. Surprisingly, we found 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 DOI. We would expect to see the same reduction in the phosphorylation as 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 to wild-type $G_{\alpha 11}$.

Previous studies have shown that PKC and CaMK are neccessary for acute desensitization of 5-HT$_{2A}$ receptor signaling but 5-HT$_{2A}$ receptors do not appear to be the neccessary substrate of these kinases (Anji et al., 2001;Berg et al., 2001;Gray et al., 2003;Vouret-Craviari et al., 1995). Since serine 154 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 we observed can 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-HT₂A receptor agonist treatment. As shown in this report and many others, 5-HT₂A receptor stimulation leads to an increase in PLC activity (Berg et al., 2001). Subsequently, PLC-catalyzed production of diacylglycerol stimulates PKC activity. Additionally, PLC-catalyzed production of inositoltriphosphate 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 Gαq/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 Gαq/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 GTPγS-mediated PLC activity. These differential effects suggest that desensitization of 5-HT₂A 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 Gαq/11 proteins may hinder receptor and G protein interaction. Indeed, phosphorylation of tyrosine residues in Gαq/11 proteins diminished the interaction of Gαq/11 proteins with M1 muscarinic receptors in mouse embryo fibroblast cells (Umemori et al., 1997). Other in vitro studies suggested that some but not all Gα subunits are substrates for phosphorylation by protein kinase C (Fields and Casey, 1995; Glick et al., 1998; Kozasa and Gilman, 1996). Phosphorylation of the Gα₁₂ and Gα₁₅ protein subunits inhibit the interaction of these subunits with the Gβγ heterodimer (Fields and Casey, 1995; Kozasa and Gilman, 1996), phosphorylation of Gα₁₂ inhibits interaction with the effector enzyme (Strassheim and Malbon, 1994) and phosphorylation of Gα₁₅ inhibits interaction with RGS proteins (Glick et al., 1998).
Phosphorylation of the $\text{G}_{\alpha 11}$ protein could prevent the formation of the $\text{G}_{\alpha 3\beta\gamma}$ 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 $\text{G}_{\alpha 11}$ could potentially interfere with the interaction of $\text{G}_{\alpha 11}$ 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 Emax 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 (Emax). A right-shift in the dose-response curve suggests a reduction in coupling of G protein to bradykinin receptors. Consistent with phosphorylation of $\text{G}_{\alpha 11}$ causing the heterologous desensitization of bradykinin receptor system in A1A1v cells, transfection of $\text{G}_{\alpha 11S154D}$, the phosphorylation state mimic also produced heterologous desensitization of bradykinin receptor signaling.

Interestingly, overexpression of mutant $\text{G}_{\alpha qS154A}$ increased the DOI-stimulated IP accumulation compared to wild-type $\text{G}_{\alpha q}$. These results are consistent with the hypothesis that the phosphorylation of $\text{G}_{\alpha qS154}$ may occur constitutively in A1A1v cells and decrease 5-HT$_{2A}$ receptor-mediated signaling. Further studies are needed to determine if this site is constitutively phosphorylated as the data with the $\text{G}_{\alpha qS154A}$ mutant suggests. Mutation of the same site in $\text{G}_{\alpha 11}$ resulted in diminished agonist-mediated desensitization of 5-HT$_{2A}$ receptor signaling. Furthermore, expression of the phosphorylation state mimic $\text{G}_{\alpha 11S154D}$ decreased the 5-HT$_{2A}$ receptor signaling. All these data support the hypothesis that $\text{G}_{\alpha q/11}$ S154 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 represent 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 $G_{\alpha 11}$ S154 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 $G_{\alpha q/11}$ is 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 $G_{\alpha 11}$ is 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 post-synaptic proteins in receptor signaling pathways to fully delineate the mechanisms mediating desensitization phenomena. In this study, we only examined single-mutations in $G_{\alpha q/11}$ proteins. It would be of interest to determine the effects of mutants with multiple substitutions for serine and threonine residues in comparison with those single mutations in $G_{\alpha q/11}$ proteins, since previous studies have demonstrated functional co-operativity 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.
References


Footnotes

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Figure Legends

**Figure 1.** Possible phosphorylation sites on G\textsubscript{\alpha q} and G\textsubscript{\alpha 11} proteins. Consensus sites for possible phosphorylation of G\textsubscript{\alpha q} and G\textsubscript{\alpha 11} proteins were identified using programs available on http://scansite.mit.edu. Possible Ser/Thr phosphorylation sites are underlined, * denotes possible PKC (\alpha, \beta, \mu, \zeta) site, † denotes possible CaMK phosphorylation sites.

**Figure 2.** Effect of sustained DOI treatment (100 nM, 24 hours) on IP accumulation and levels of phosphorylated G\textsubscript{\alpha q/11} proteins in A1A1\textsubscript{v} cells. A) Treatment of A1A1\textsubscript{v} cells with 100 nM DOI for 24 hours resulted in a significant attenuation in IP accumulation stimulated by 100 \mu M DOI compared with vehicle group. Data shown are the mean of triplicate determinations of a single representative experiment. Basal IP accumulation was 37.7\pm2.1 and 39.5\pm3.6 dpm for vehicle and DOI-treated A1A1\textsubscript{v} cells, respectively. B) 5-HT\textsubscript{2A} receptor antagonist MDL 100,907 caused a dose-dependent (0.5 to 100 nM) inhibition of DOI-stimulated IP accumulation in A1A1\textsubscript{v} cells. The concentration required for 50% inhibition is 5.35\pm0.86 nM. C) 100 nM DOI treatment for 24h significantly increased phosphorylation of G\textsubscript{\alpha q/11} protein when compared to vehicle treatment. A representative blot showing increased phosphorylation of G\textsubscript{\alpha q/11} proteins after DOI treatment. G\textsubscript{\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\textsubscript{\alpha q/11} proteins. D) Increased phosphorylation of G\textsubscript{\alpha q/11} proteins after DOI treatment was probed by \[^{32}P\] phosphate labeling. Phosphorylation data represent the mean \pm SEM of 5 experiments. E) Treatment of cells with 100 nM DOI for 15 min or 30 min resulted in a significant attenuation in IP accumulation stimulated by 100 \mu M DOI compared with the
vehicle-treated group. Data shown are the mean ± SEM of three experiments. F) Levels of phosphorylation of Gαq/11 proteins after DOI treatment for 30 min were probed by [32P] phosphate labeling. Phosphorylation data represent the mean ± SEM of 3 experiments. ** indicates p<0.01 using Student t-test or † † Wilcoxon Mann-Whitney U-test (used because of unequal variance).

**Figure 3.** Effect of PKC and CaMK consensus site mutations in Gα11 on DOI-induced desensitization of 5-HT2A receptor signaling in A1A1v cells. A1A1v cells were transiently transfected with vector, wild-type Gα11 or one of five Gα11 mutants. A) A representative blot demonstrates overexpression of wild-type Gα11 and five Gα11 mutants 48 hours after transfection. The blot was probed with Gα11 antibody. B) 24 hours of 100 nM DOI treatment significantly decreased IP accumulation in cells overexpressing of wild-type and mutant Gα11. ** indicates p<0.01 and * indicates p<0.05 compared with cells transfected with the same construct but treated with vehicle. C) Overexpression of Gα11 S154A attenuated desensitization induced by DOI treatment. # indicated p<0.05 compared with cells transfected with wild-type Gα11. The data are presented as a percent of the cells transfected with wild-type Gα11 and treated with vehicle and are the mean ± SEM of 3 experiments assayed in triplicate.

**Figure 4.** Effect of PKC and CaMK consensus site mutation in Gαq on DOI-induced desensitization of 5-HT2A receptor signaling in A1A1v cells. A) A representative blot demonstrating overexpression of wild-type Gαq and four Gαq mutants 48 hours after transfection. The blot was probed with Gαq antibody. B) 24 hours of 100 nM DOI treatment resulted in a significant decrease in IP accumulation in cells overexpressing wild-type and Gαq mutants cells.
** indicates p<0.01 and * indicates p<0.05 compared with cells transfected with the same construct but treated with vehicle. Overexpression of G\(_{\alpha_q}\) T54A, T54A and S268A did not cause a difference in IP accumulation following agonist stimulation compared with wild-type G\(_{\alpha_q}\). Overexpression of G\(_{\alpha_q}\) S154A caused an increase in IP accumulation. † indicates p<0.05 in comparison to wild-type G\(_{\alpha_q}\). The data are presented as a percent of cells transfected with wild-type G\(_{\alpha_q}\) and treated with vehicle and are the mean ± SEM of 3 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.

**Figure 5.** Expression of G\(_{\alpha_11}\)S154D in A1A1v cells decreased 5-HT\(_{2A}\) receptor signaling.

A) A representative blot demonstrates overexpression of wild-type G\(_{\alpha_11}\), G\(_{\alpha_11}\)S154A or G\(_{\alpha_11}\)S154D mutant 48 hours after transfection. B) Overexpression of G\(_{\alpha_11}\)S154D resulted in decreased DOI-induced IP accumulation compared with cells transfected with wild-type G\(_{\alpha_11}\) and G\(_{\alpha_11}\)S154A and treated with vehicle, # indicates p<0.05. ** indicates p<0.01 compared with cells transfected with the same construct but treated with vehicle. The data are presented as a percent of the cells transfected with wild-type G\(_{\alpha_11}\) and treated with vehicle and are the mean ± SEM of 3 experiments assayed in triplicate.

**Figure 6.** Effect of expression of G\(_{\alpha_11}\)S154A on DOI-induced phosphorylation of G\(_{\alpha_q/11}\) proteins. A) A1A1v cells were transfected with wild-type G\(_{\alpha_11}\) or G\(_{\alpha_11}\)S154A, and treated with DOI or vehicle for 24h. The levels of phosphorylated G\(_{\alpha_q/11}\) protein was measured by \([^{32}\text{P}]\) phosphate incorporation and normalized to the levels of G\(_{\alpha_q/11}\) (to verify the equal loading of
protein). * indicates 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 \([^{32}\text{P}]\) phosphate.

**Figure 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\(\mu\)M PMA for 24h or 10nM KN-93 for 30min before the addition of 100nM DOI for 24h first, then incubated with kinase inhibitors during the DOI treatment. A) and B) Accumulation of \([^{3}\text{H}]\)IP was measured in triplicate after 30min incubation with DOI. Treatment of cells with PMA alone did not alter the Emax (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 Emax (vehicle 176.8±17.5% above basal; KN-93-treated, 134.1±12.3% above basal n=5). Data plotted are expressed as Emax, percentage of respective control. Each value represents the mean ± S.E.M. of four experiments. Data were analyzed by Student’s t-test; ** indicated p<0.01. C) The levels of phosphorylated G\(_{\alpha q/11}\) protein were measured by \([^{32}\text{P}]\) 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 \([F(3,11)=4.5, p=0.039]\); * indicates p<0.05 compared with cells treated with DOI. D) A representative blot of the phosphorylated G\(_{\alpha q/11}\) protein probed with \([^{32}\text{P}]\) phosphate.

**Figure 8.** Effect of sustained DOI treatment (100 nM, 24 hours) on bradykinin receptor mediated signaling. A) The concentration-response curves represent IP accumulation after 30
min incubations with varying concentrations of bradykinin (10^{-10} to 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 hours resulted in a significant attenuation in IP accumulation stimulated by 10^{-8} M bradykinin compared with the vehicle-treated group. Data represent the mean ± SEM of 3 experiments. Basal IP accumulations were 45.6±5.5 and 48.5±2.5 dpm for vehicle- and DOI-treated A1A1v cells, respectively. * indicates significantly different at p<0.05 using Student t-test.

C & D) Cells transfected with G_{\alpha 11}S154D (4 \mu g/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 ± SEM of 3 experiments. * indicates significantly different at p<0.05 and ** indicates p<0.01 using Student t-test.
**G\textsubscript{\(a1\)} amino acid sequence**

1  mtlesmiacc lsdevkeskr inaeiekqlr rdkrdarrel kllllgtges gks\textsuperscript{t}fikqmr
61  iihgagysse dkrgef\textsuperscript{t}klvy qniftamqav vramdtkir ykyeqnkana llirevdvek
121  vttfehqynv aiktlwsdpq vqecydrrre fql\textsuperscript{s}sakyy ltdvdrotav gylptqvdvl
181  rvrvpttgiu eypfdlenii frmvdvgqqr serrkwihcfn envtsimfvlv alseydqvvlv
241  esdnenrmee skalfrtiit ypwfqhs\textsuperscript{s}vi lflnkkdlle dkilhsdlvd yfpefdgpqrr
301  daqaarefil kmfvdlnpds dkiiyshftc atdtenirfv faavkdtilq ln1keynvlv

**G\textsubscript{\(a2\)} amino acid sequence**

1  mtlesimacc lseeakearr indeierqlr rdkrdarrel kllllgtges gks\textsuperscript{t}fikqmr
61  iihgsgysde dkrgef\textsuperscript{t}klvy qniftamqam vramdtkkip ykyehnkaha qlvrevdvek
121  vsafenpyvd aikslwndpg iqecydrrre yql\textsuperscript{s}dstkyy lndldrvadp sylptqqdvl
181  rvrvpttgiu eypfdlqs\textsuperscript{s}vi frmvdvgqqr serrkwihcfn envtsimfvlv alseydqvvlv
241  esdnenrmee skalfrtiit ypwfqns\textsuperscript{s}vi lflnkkdlle ekimyshlvd yfpeydgpqrr
301  daqaarefil kmfvdlnpds dkiiyshftc atdtenirfv faavkdtilq ln1keynvlv
Fig. 2

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I. 

J. 

K. 

L. 

M. 

N. 

O. 

P. 

Q. 

R. 

S. 

T. 

U. 

V. 

W. 

X. 

Y. 

Z.
Fig. 3

A

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This article has not been copyedited and formatted. The final version may differ from this version.

G\(_{\alpha_{11}}\)

Actin

Vector, G\(_{\alpha_{11}}\), T54A, T76A, S154A, S156A, S268A

B

![Graph showing [H]-IP Accumulation (% of G\(_{\alpha_{11}}\) Vehicle group)]

- **Vehicle**
- **DOI**

Transfection Groups

C

![Graph showing [H]-IP Accumulation (% of decreased response)]

Transfection Groups

#
Fig. 5

A

G<sub>α11</sub>  
Actin

Vector  G<sub>α11</sub>  S154A  S154D

B

[<sup>3</sup>H]-IP Accumulation (% of G<sub>α11</sub> Vehicle Group)

Vector  G<sub>α11</sub>  S154A  S154D

Vehicle  DOI

*  **  #
**A**

Phosphorylated $G_{\alpha q/11}$ Protein Levels (Normalized Mean IOD)

- **Vehicle**
- **DOI**

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Vehicle</th>
<th>DOI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\alpha 11}$</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>$G_{\alpha 11} S154A$</td>
<td>100</td>
<td>120</td>
</tr>
</tbody>
</table>

*Significant difference between Vehicle and DOI.

**B**

Immunoprecipitation: $G_{\alpha q/11}$

Vehicle | DOI | Vehicle | DOI
--- | --- | --- | ---
$G_{\alpha 11}$ |  | $G_{\alpha 11} S154A$ |  | 32P
Fig. 7

A

[^3]HP Accumulation (% of decreased response)

Vehicle

KN-93

24 h DOI

B


Vehicle

24h PMA

24 h DOI

C

Phosphorylated G_{αq/11} Protein Levels (Normalized Mean IOD)

Vehicle

Vehicle

PMA

KN-93

24 h DOI

D

Immunoprecipitation: G_{αq/11}

Vehicle Vehicle PMA KN-93

24 h DOI

←^{32}P
Fig. 8

A. 

[3H]-IP Accumulation (% of Emax Vehicle Group) vs Bradykinin (M)

B. 

[3H]-IP Accumulation (% of Emax Vehicle Group) for Vehicle and DOI at 10^-8 (EC50) and 10^-5 (Emax) Bradykinin (M)

C. 

[3H]-IP Accumulation (% of Gα11 Group) for Gα11 and Gα11S154D at Bradykinin 10^-8 M (EC50)

D. 

[3H]-IP Accumulation (% of Gα11 Group) for Gα11 and Gα11S154D at Bradykinin 10^-5 M (Emax)