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Using Molecular Tools to Dissect the Role of $G\alpha_s$ in Sensitization of AC1

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ABSTRACT

Short-term activation of $G\alpha_{i/o}$-coupled receptors inhibits adenylyl cyclase, whereas persistent activation of $G\alpha_{i/o}$-coupled receptors results in a compensatory sensitization of adenylyl cyclase activity after subsequent activation by $G\alpha_s$ or forskolin. Several indirect observations have suggested the involvement of increased $G\alpha_s$-adenylyl cyclase interactions in the expression of sensitization; however, evidence supporting a direct role for $G\alpha_s$ has not been well established. In the present report, we used two genetic approaches to further examine the role of $G\alpha_s$ in heterologous sensitization of Ca^{2+}-sensitive type 1 adenylyl cyclase (AC1). In the first approach, we constructed $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) that retained sensitivity to Ca^{2+} and forskolin activation. Persistent (2 h) activation of the D_2 dopamine receptor resulted in a significant augmentation of basal or Ca^{2+}- and forskolin-stimulated AC1 activity; however, sensitization of $G\alpha_s$-insensitive mutants of AC1 was markedly reduced compared with wild-type AC1. In the second strategy, we examined the requirement of an intact receptor-$G\alpha_s$ signaling pathway for the expression of sensitization using dominant-negative $G\alpha_s$ mutants ($\alpha3\beta5$ G226A/A366S or $\alpha3\beta5$ G226A/E268A/A366S) to disrupt D_1 dopamine receptor activation of recombinant AC1. D_1 dopamine receptor-$G\alpha_s$ signaling was attenuated in the presence of $\alpha3\beta5$ G226A/A366S or $\alpha3\beta5$ G226A/E268A/A366S, but D_2 agonist-induced sensitization of Ca^{2+}-stimulated AC1 activity was not altered. Together, the present findings directly support the hypothesis that the expression of sensitization of AC1 involves $G\alpha_s$-adenylyl cyclase interactions.

Short-term activation of $G\alpha_{i/o}$-coupled receptors inhibits adenylyl cyclase, whereas persistent activation of $G\alpha_{i/o}$-coupled receptors results in a compensatory increase in adenylyl cyclase activity after subsequent activation by $G\alpha_s$ or forskolin. This heterologous sensitization of adenylyl cyclase was described originally as a cellular model for opioid dependence and withdrawal (Sharma et al., 1975) and represents a common adaptive response to persistent activation of multiple $G\alpha_{i/o}$-coupled receptors, including the D_2 dopamine receptor (Watts, 2002). The molecular mechanisms involved in heterologous sensitization of adenylyl cyclase can be described by the early events (development) associated with activation of $G\alpha_{i/o}$-coupled receptors and the late events (expression) associated with the activation of adenylyl cyclase by $G\alpha_s$, forskolin, or isoform-selective activators such as Ca^{2+} for type 1 adenylyl cyclase (AC1) (Watts, 2002). The development of heterologous sensitization of adenylyl cyclase is prevented by pertussis toxin treatment, implicating a role for $G\alpha_{i/o}$ proteins (Watts and Neve, 1996; Rhee et al., 2000). In addition, sequestration of free $G\beta\gamma$ subunits, liberated from activated $G\alpha_{i/o}$-coupled receptors, can also block the development of heterologous sensitization of select adenylyl cyclase isoforms (i.e., AC5 and AC6) (Thomas and Hoffman, 1996; Rhee et al., 2000).

Although studies have focused on the development of heterologous sensitization, the mechanisms leading to the expression of sensitization remain to be determined. Several indirect observations have suggested a role for $G\alpha_s$ in the expression of heterologous sensitization (Watts, 2002). These observations are consistent with increased $G\alpha_s$-adenylyl cyclase interactions after persistent activation of $G\alpha_{i/o}$-coupled receptors. The requirement of $G\alpha_s$-adenylyl cyclase interactions in heterologous sensitization of adenylyl cyclase has also been examined directly using $G\alpha_s$-insensitive mutants of AC5 (Zimmermann et al., 1998; Watts et al., 2001). Persistent activation of the D_2 dopamine receptor resulted in heterologous sensitization of basal and forskolin-stimulated

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ABBREVIATIONS: AC, adenylyl cyclase; HEK, human embryonic kidney; A23187, calcimycin; EBSS, Earle's balanced salt solution; ANOVA, analysis of variance; SKF 38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; $G\alpha_s$, stimulatory G protein; $G\alpha_{i/o}$, inhibitory G protein; D_{2L} dopamine receptor, D_2 long isoform.

AC5 activity, whereas $G\alpha_s$-insensitive mutants of AC5 failed to exhibit sensitization (Watts et al., 2001). This observation provided the first direct evidence for the requirement of $G\alpha_s$ in heterologous sensitization of adenylyl cyclase. However, forskolin activation of the $G\alpha_s$-insensitive mutants of AC5 was markedly reduced, which may have contributed to the loss of sensitization (Watts et al., 2001). From these observations, we used two additional genetic approaches to further examine the hypothesis that persistent activation of the D_2 dopamine receptor enhances $G\alpha_s$-adenylyl cyclase interactions, resulting in the expression of heterologous sensitization.

In the first approach, we constructed $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) that retained sensitivity to Ca^{2+} and forskolin activation, providing a significant advantage to the $G\alpha_s$-insensitive mutants of AC5 described previously. The F293L mutation in the C_1 catalytic domain is designed to disrupt hydrophobic interactions with the $\alpha 3$ helix of $G\alpha_s$, whereas the Y973S mutation in the C_2 catalytic domain disrupts interactions with the switch II region of $G\alpha_s$ (Tesmer et al., 1997; Yan et al., 1997). D_2 agonist-induced sensitization of $G\alpha_s$-insensitive mutants of AC1 was observed; however, the magnitude of sensitization was markedly attenuated compared with wild-type AC1. The second strategy examined sensitization of D_1 dopamine receptor signaling in the presence of recently described dominant-negative $G\alpha_s$ mutants ($\alpha 3\beta 5$ G226A/A366S and $\alpha 3\beta 5$ G226A/E268A/A366S) (Berlot, 2002). These $G\alpha_s$ mutants contain substitutions designed to stabilize the receptor-$G\alpha_s$-$G\beta\gamma$ complex and inhibit receptor-mediated activation of adenylyl cyclase (Berlot and Bourne, 1992; Iiri et al., 1999; Grishina and Berlot, 2000). Our studies revealed that D_1 dopamine receptor-stimulated AC1 activity was attenuated in the presence of $\alpha 3\beta 5$ G226A/A366S or $\alpha 3\beta 5$ G226A/E268A/A366S. However, these dominant-negative $G\alpha_s$ mutants failed to alter D_2 agonist-induced sensitization of Ca^{2+}-stimulated AC1 activity. These findings suggest a role for $G\alpha_s$-dependent mechanisms in heterologous sensitization of AC1, although receptor activation of $G\alpha_s$ does not seem to be required.

Materials and Methods

Materials. [^{3}H]Cyclic AMP (25 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Quinpirole, spiperone, forskolin, A23187 (calcimycin), SKF 38393, 3-isobutyl-1-methylxanthine, growth media, and most other reagents were purchased from Sigma-Aldrich (St. Louis, MO). $G\alpha_s$ polyclonal antisera (number A-0715) was purchased from STI-Signal Transduction Products (San Clemente, CA).

Production and Maintenance of Cell Lines. HEK293 cells stably expressing the D_{2L} dopamine receptor (HEK-D_{2L}) were constructed with pcDNA1-D_{2L} (15 μg) and pBabe Puro (2 μg) using electroporation as described previously (Watts and Neve, 1996). HEK293 cells stably expressing AC1 were obtained from Dr. Daniel Storm (University of Washington, Seattle, WA) and were transfected with the D_{2L} dopamine receptor using standard LipofectAMINE (Invitrogen, Carlsbad, CA) transfection protocol. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% bovine calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 μg/ml puromycin and/or 440 units/ml hygromycin. Cells were grown in a humidified incubator in the presence of 5% CO_2 at 37°C. Transient transfection (48 h) of the D_1 dopamine receptor (Guthrie Research Institute, Sayre, PA) (pBudCE4-D_1), $G\alpha_s$ mutants (pBudCE4-D_1-$G\alpha_s$ $\alpha 3\beta 5$ G226A/A366S or pBudCE4-D_1-$G\alpha_s$ $\alpha 3\beta 5$ G226A/E268A/A366S), bovine AC1 (pcDNA3-AC1), and $G\alpha_s$-insensitive mutants of AC1 (pcDNA3-AC1 F293L or Y973S) (1 μg/well) was carried out in 24-well tissue-culture plates using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. $G\alpha_s$-insensitive mutants of AC1 were constructed using QuikChange site-directed mutagenesis with primers purchased from Integrated DNA Technologies, Inc. (Coralville, IA) (F293L: forward, 5′-CCC CCT GAG AGG ATT TTA CAC AAG ATT TAC ATC CAG-3′; reverse, 5′-CTG GAT GTA AAT CTT GTG TAA AAT CCT CTC AGG GGG-3′; Y973S: forward, 5′-GAG ATC AAC TAC CAG TCT TCT AAC GAC TTT GTG CTC C-3′; reverse, 5′-GGA GCA CAA AGT CGT TAG AAG ACT GGT AGT TGA TCT C-3′).

Cyclic AMP Accumulation Assay. Cells were seeded at concentrations between 100,000 and 200,000 cells/well in 24-well cluster plates. For short-term experiments, cells were washed in 400 μl of Earle's balanced salt solution (EBSS) assay buffer (EBSS containing 2% calf bovine serum, 0.025% ascorbic acid, and 15 mM HEPES, pH 7.4) for 10 min at room temperature. The media were then decanted, and the cells were placed on ice. Cyclic AMP accumulation was stimulated in the presence of forskolin, A23187, or SKF 38393 as indicated. Where indicated, short-term activation of adenylyl cyclase was performed in the presence of the D_2 agonist quinpirole. In addition, short-term experiments were performed in the presence of 3-isobutyl-1-methylxanthine (500 μM). Incubations were carried out for 15 min at 37°C, and then the assay buffer was decanted. The culture plates were placed on ice, and cells were lysed with 100 to 200 μl of 3% trichloroacetic acid. The plates were stored at 4°C overnight before quantification. For sensitization experiments, cells were preincubated for 2 h in the presence of vehicle or quinpirole (1 μM) as indicated at 37°C in a humidified incubator in the presence of 5% CO_2. After drug pretreatment, the cells were washed three times for 3 to 4 min with 400 μl of EBSS assay buffer, placed on ice, and cyclic AMP accumulation was stimulated as described above. Sensitization experiments were performed in the presence of spiperone (1 μM) to block the activation of D_{2L} receptors by residual agonist (Watts and Neve, 1996).

Quantification of Cyclic AMP. Cyclic AMP was quantified using a competitive binding assay as described previously (Watts and Neve, 1996). In brief, duplicate samples of the cell lysate (10–15 μl) were added to reaction tubes. [^{3}H]Cyclic AMP (~1 nM final concentration) and cyclic AMP binding protein (approximately 100–150 μg) were diluted in cyclic AMP binding buffer (100 mM Tris/HCl, pH 7.4, 100 mM NaCl, and 5 mM EDTA) and then added to each reaction tube. The reaction was incubated on ice at 4°C for 2 h and then harvested by filtration (Whatman GF/C filters) using a 96-well Packard Filtermate cell harvester (PerkinElmer Life and Analytical Sciences). Filter plates were dried overnight at room temperature, and 40 μl of Packard Microscint O scintillation fluid was added to each well. Radioactivity was determined using a Packard TopCount scintillation counter. Cyclic AMP concentrations in each sample were determined in duplicate from a standard curve ranging from 0.01 to 300 pmol of cyclic AMP. Dose-response curves for cyclic AMP accumulation were analyzed by nonlinear regression using Prism software (GraphPad Software Inc., San Diego, CA). All values for cyclic AMP accumulation are expressed as picomoles of cyclic AMP per well unless otherwise indicated.

Radioligand Binding Assay. Cells were transiently transfected (5 μg/well) as described above in six-well tissue-culture plates. To harvest, cells were lysed with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, and 2 mM EDTA). After swelling for 10 to 15 min, the cells were scraped from the plate and spun at 30,000*g* for 20 min. The resulting crude membrane fraction was resuspended in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, with 155 mM NaCl) with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 10 s and used for radioligand binding assays. The binding of [^{3}H]SCH23390 was assessed by adding aliquots of the membrane preparation (10–20 μg of protein) to duplicate assay tubes containing the following: Tris-buffered saline, 0.001%

bovine serum albumin, and increasing concentrations of radioligand. (+)-Butaclamol (5 μM) was used to define nonspecific binding. Incubations were carried out at 37°C for 60 min in a volume of 0.5 ml and terminated by filtration as described for the cAMP competition binding assay.

Western Blot Analysis. For $G\alpha_s$ blots, the media were aspirated, and cells were put on ice. Lysis buffer (1 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride) was added to each well of a six-well plate for 5 min. Cells were scraped into centrifuge tubes, homogenized briefly, and centrifuged at 30,000*g* for 20 min. The supernatant was removed, and the pellet was resuspended with resuspension buffer (15 mM HEPES, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride, pH 7.5). Protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL). Western blots were performed with 25 μg protein/lane using a primary polyclonal antibody (1:1000) directed against the carboxyl terminus of $G\alpha_s$. Immunoreactivity was detected with the ECF kit according to the manufacturer's protocols (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

Data Analysis. Statistical comparisons were made using repeated-measures ANOVA followed by Dunnett's or Bonferroni's post hoc analysis for comparison of multiple stimulation, pretreatment, or transfection conditions where indicated. Statistical comparisons and nonlinear regression analysis were performed using GraphPad Prism.

Results

Short-Term Regulation of Wild-Type and $G\alpha_s$-Insensitive Mutants of AC1 in HEK-D_{2L} Cells. Our previous studies suggested that $G\alpha_s$-insensitive mutants of adenylyl cyclase may provide useful pharmacological tools to directly examine the role of $G\alpha_s$ in heterologous sensitization of individual adenylyl cyclase isoforms (Watts et al., 2001). In the present study, we constructed $G\alpha_s$-insensitive mutants of Ca^{2+}-sensitive AC1 in the C_1 (F293L) and C_2 (Y973S) catalytic domains. Wild-type and $G\alpha_s$-insensitive mutants of AC1 were transiently transfected into HEK-D_{2L} cells. After transient transfection, A23187 robustly increased cyclic AMP accumulation above basal levels in HEK-D_{2L} cells expressing wild-type or $G\alpha_s$-insensitive mutants of AC1 (Fig. 1A), indicating that F293L and Y973S retained the Ca^{2+} sensitivity observed for wild-type AC1. In contrast, A23187 failed to increase cyclic AMP accumulation to greater than basal levels in vector-transfected cells (Fig. 1A). The magnitude of A23187-stimulated cyclic AMP accumulation was comparable for wild-type and mutant AC1 constructs, suggesting similar functional expression under the transfection conditions used in the present study.

To confirm the $G\alpha_s$-insensitive phenotype of F293L and Y973S AC1 mutants, we examined the ability of constitutively active $G\alpha_s$, Q227L, to augment A23187-stimulated cyclic AMP accumulation. HEK-D_{2L} cells were transfected with a dual expression vector containing either wild-type or $G\alpha_s$-insensitive mutants of AC1 and $G\alpha_s$ Q227L. Experiments were also performed under control transfection conditions in the absence of $G\alpha_s$ Q227L. Constitutively active $G\alpha_s$ significantly augmented A23187-stimulated cyclic AMP accumulation in HEK-D_{2L} cells expressing wild-type AC1 (Fig. 1B). In contrast, the presence of $G\alpha_s$ Q227L produced only a small, nonstatistically significant increase in Ca^{2+} activation of AC1 F293L and Y973S compared with vector-transfected HEK-D_{2L} cells (Fig. 1B). Western blot analysis confirmed that the expression of $G\alpha_s$ Q227L was comparable in the presence of wild-type or $G\alpha_s$-insensitive mutants of AC1 (Fig. 1C). These observations confirm that $G\alpha_s$ stimulation of AC1 F293L and Y973S is markedly reduced compared with wild-type AC1.

We further examined additional regulatory properties of these $G\alpha_s$-insensitive mutants of AC1. Forskolin (1 μM) stimulation alone failed to activate either wild type or $G\alpha_s$-insensitive mutants of AC1 greater than vector control (data not shown). The lack of forskolin-stimulated AC1 activity in the absence of Ca^{2+} may reflect protein expression levels or the experimental conditions (e.g., intact mammalian cells and 1 μM forskolin) used in the present studies. However, subse-

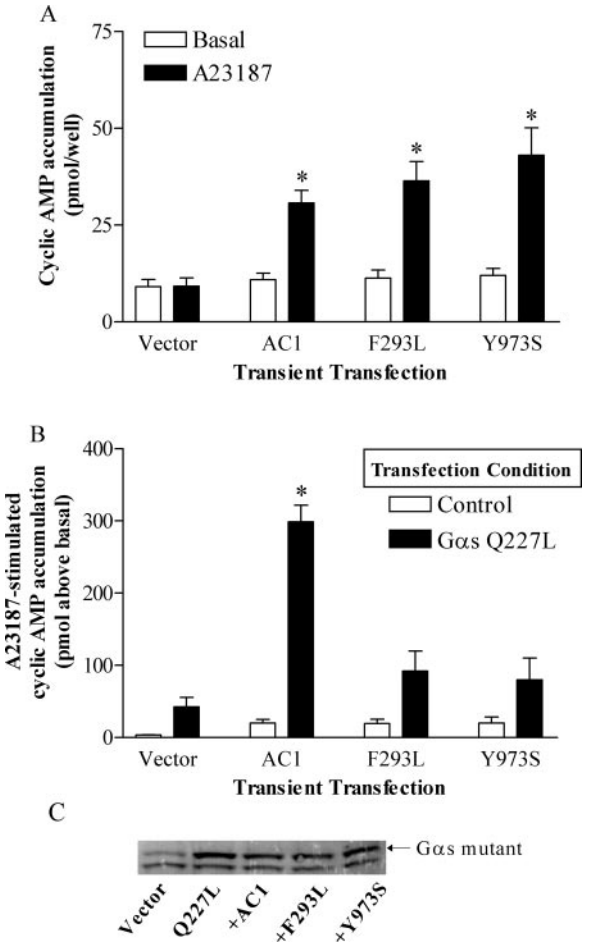

Fig. 1. Ca^{2+} and $G\alpha_s$ stimulation of wild-type and $G\alpha_s$-insensitive mutants of AC1. A, HEK-D_{2L} cells were transfected with pcDNA3 (vector) or plasmid encoding wild-type (AC1) or $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) as indicated. B, for $G\alpha_s$ regulation experiments, cells were transfected with pBudCE4 (vector) or plasmid encoding either wild-type (AC1) or $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) and constitutively active $G\alpha_s$ Q227L as indicated. Experiments were also performed under control transfection conditions with plasmid encoding either wild-type or $G\alpha_s$-insensitive mutants of AC1 in the absence of $G\alpha_s$ Q227L. After transient transfection, cells were washed, and cyclic AMP accumulation was measured under basal conditions or in the presence of 3 μM A23187 for 15 min. Data shown are the mean ± S.E.M. of three to four independent experiments assayed in duplicate. A, *, $p < 0.05$ compared with basal cyclic AMP accumulation (Bonferroni's post hoc repeated-measures ANOVA); B, *, $p < 0.05$ compared with the control condition for the indicated transfection (Bonferroni's post hoc one-way ANOVA). C, Western blot analysis of HEK-D_{2L} cells transfected with pBudCE4 (vector), $G\alpha_s$ Q227L (Q227L), or $G\alpha_s$ Q227L in combination with wild-type AC1 (+AC1) or $G\alpha_s$-insensitive mutants of AC1 (+F293L or +Y973S) as indicated. Polyclonal $G\alpha_s$ antibody was used to detect immunoreactivity of endogenous and transiently expressed $G\alpha_s$ Q227L. Data shown are representative of two independent experiments.

quent experiments revealed that AC1 F293L and Y973S were synergistically activated by forskolin in the presence of A23187 compared with the summation of the cyclic AMP response to forskolin and A23187 alone (Fig. 2A). These observations indicate that $G\alpha_s$-insensitive mutants of AC1 retained synergistic activation by forskolin and Ca^{2+} similar to wild-type AC1. We also examined the sensitivity of wild-type and $G\alpha_s$-insensitive mutants of AC1 to inhibition by $G\alpha_{i/o}$ subunits. A23187-stimulated cyclic AMP accumulation was markedly inhibited after short-term quinpirole (1 μM) activation of the D_{2L} dopamine receptor in HEK-D_{2L} cells expressing wild-type or $G\alpha_s$-insensitive mutants of AC1 (Fig. 2B), demonstrating that AC1 F293L and Y973S are negatively regulated by $G\alpha_{i/o}$ in a manner similar to wild-type AC1. Taken together, our data suggest that AC1 F293L and Y973S retained the short-term regulatory properties of wild-type AC1 with the exception that they exhibit a marked loss of $G\alpha_s$ activation, confirming the postulated $G\alpha_s$-insensitive phenotype.

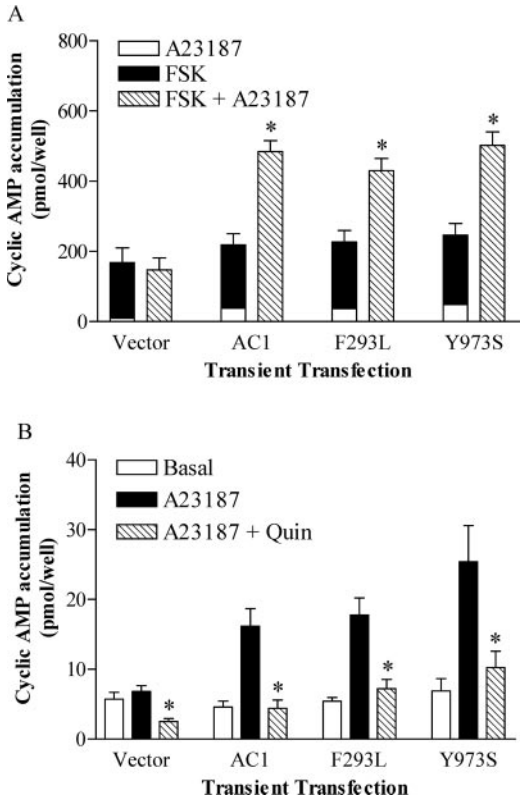

Fig. 2. Regulation of wild-type and $G\alpha_s$-insensitive mutants of AC1. HEK-D_{2L} cells were transfected with pcDNA3 (vector) or plasmid encoding wild-type (AC1) or $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) as indicated. A, cyclic AMP accumulation was measured in the presence of 3 μM A23187, 1 μM forskolin (FSK), or 1 μM forskolin and 3 μM A23187 (FSK + A23187) for 15 min. Data shown are the mean ± S.E.M. of four independent experiments assayed in duplicate. *, $p < 0.05$ compared with the summation of A23187- and forskolin-stimulated cyclic AMP accumulation alone, as represented by the stacked bars for the indicated transfection (Bonferroni's post hoc repeated-measures ANOVA). Basal cyclic AMP accumulation was 10 ± 2, 11 ± 1, 12 ± 1, and 11 ± 1 pmol/well for vector, AC1, F293L, and Y973S, respectively. B, cyclic AMP accumulation was measured under basal conditions or after stimulation with 3 μM A23187 in the absence (A23187) or presence of 1 μM quinpirole (A23187 + Quin) for 15 min. Data shown are the mean ± S.E.M. of four independent experiments assayed in duplicate. *, $p < 0.05$ compared with A23187-stimulated cyclic AMP accumulation for the indicated transfection (Bonferroni's post hoc repeated-measures ANOVA).

D_2 Dopamine Receptor-Induced Heterologous Sensitization of Wild-Type and $G\alpha_s$-Insensitive Mutants of AC1. On the basis of the short-term regulatory properties, $G\alpha_s$-insensitive mutants of AC1 seem to be useful pharmacological tools to directly test the hypothesis that $G\alpha_s$-adenylyl cyclase interactions are required for the expression of heterologous sensitization. Therefore, we examined the ability of persistent (2 h) activation of the D_{2L} dopamine receptor to induce heterologous sensitization of wild-type AC1 and $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S). Pretreatment with the D_2 agonist quinpirole for 2 h resulted in a significant enhancement of basal cyclic AMP accumulation in HEK-D_{2L} cells transfected with vector, wild-type AC1, as well as $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) (Fig. 3A). Further analysis revealed that the magnitude of this augmentation was greater in cells transfected with wild-type AC1 compared with the $G\alpha_s$-insensitive mutants of AC1 (2.0 ± 0.1-fold for AC1 versus 1.4 ± 0.1 and 1.5 ± 0.1-fold for F293L and Y973S, respectively; $p < 0.05$). Subsequent studies examining selective activation of AC1 revealed that persistent (2 h) activation of the D_{2L} dopamine receptor resulted in a robust enhancement of subsequent A23187- or forskolin/A23187-stimulated cyclic AMP accumulation in HEK-D_{2L} cells expressing wild-type AC1, as well as F293L and Y973S (Fig. 3, B and C). However, the magnitude of the quinpirole-induced augmentation of A23187-stimulated cyclic AMP accumulation was markedly reduced in HEK-D_{2L} cells expressing $G\alpha_s$-insensitive AC1 F293L (1.6 ± 0.1-fold) and Y973S (1.6 ± 0.1-fold) compared with wild-type AC1 (2.4 ± 0.1-fold) ($p < 0.01$). Likewise, D_{2L} dopamine receptor-induced sensitization to synergistic activation by forskolin and A23187 was also reduced in HEK-D_{2L} cells transiently transfected with F293L (1.5 ± 0.1-fold) and Y973S (1.5 ± 0.1-fold) compared with wild-type AC1 (1.9 ± 0.1-fold) ($p < 0.05$). Moreover, the reduced augmentation of cyclic AMP accumulation in cells transfected with F293L or Y973S seemed to result from alterations in the ability of quinpirole to induce sensitization, because both drug-stimulated and basal cyclic AMP levels were not significantly different in cells transfected with AC1 compared with F293L or Y973S ($p > 0.05$). These results support the hypothesis that $G\alpha_s$-adenylyl cyclase interactions are required for the maximal expression of D_2 dopamine receptor-induced sensitization of AC1. However, the observation of significant sensitization in HEK-D_{2L} cells expressing $G\alpha_s$-insensitive mutants of AC1 suggests that robust activation by $G\alpha_s$ is not required for heterologous sensitization.

Effect of Dominant-Negative $G\alpha_s$ Mutants on the Short-Term Regulation of Adenylyl Cyclase. In this report, we have demonstrated that disrupting $G\alpha_s$-adenylyl cyclase interactions markedly attenuates sensitization of AC1. To investigate the requirement of an intact receptor-$G\alpha_s$ signaling pathway for the expression of sensitization, we used recently described dominant-negative $G\alpha_s$ mutants $\alpha3\beta5$ G226A/A366S ($\alpha3\beta5$ 2X) and $\alpha3\beta5$ G226A/E268A/A366S ($\alpha3\beta5$ 3X) (Berlot, 2002) to inhibit D_1 dopamine receptor signaling. Initial experiments examined the effects of dominant-negative $G\alpha_s$ mutants on the regulation of endogenous adenylyl cyclases. HEK293 cells stably expressing the D_{2L} dopamine receptor were transiently transfected with a dual expression vector containing the D_1 dopamine receptor and $\alpha3\beta5$ 2X or $\alpha3\beta5$ 3X. After transient transfection, cyclic

AMP accumulation was stimulated with increasing concentrations of the D_1 agonist SKF 38393. Short-term activation of the D_1 dopamine receptor resulted in a dose-dependent increase of SKF 38393-stimulated cyclic AMP accumulation in HEK-D_{2L} cells (Fig. 4A). At saturating concentrations of D_1 agonist, $G\alpha_s$-stimulated cyclic AMP accumulation was attenuated by greater than 70% in HEK-D_{2L} cells expressing either $\alpha3\beta5$ 2X or $\alpha3\beta5$ $\alpha3\beta5$ 3X (Fig. 4A). Subsequent experiments revealed the effects of dominant-negative $G\alpha_s$ mutants on D_1 dopamine receptor activation of recombinant AC1. HEK293 cells stably expressing AC1 (HEK-AC1) were transiently transfected with the D_1 dopamine receptor in the absence or presence of $\alpha3\beta5$ 2X or $\alpha3\beta5$ 3X. SKF 38393 (100 nM) activation of the D_1 dopamine receptor resulted in a significant increase in $G\alpha_s$-stimulated cyclic AMP accumulation to greater than basal levels. In contrast, SKF 38393-stimulated cyclic AMP accumulation was markedly reduced in HEK-AC1 cells coexpressing the D_1 dopamine receptor and either $\alpha3\beta5$ 2X or $\alpha3\beta5$ 3X (Fig. 4B). Western blot analysis confirmed robust expression levels for $\alpha3\beta5$ 2X and $\alpha3\beta5$ 3X compared with endogenous $G\alpha_s$ (Fig. 4C).

Saturation radioreceptor binding analysis revealed that D_1

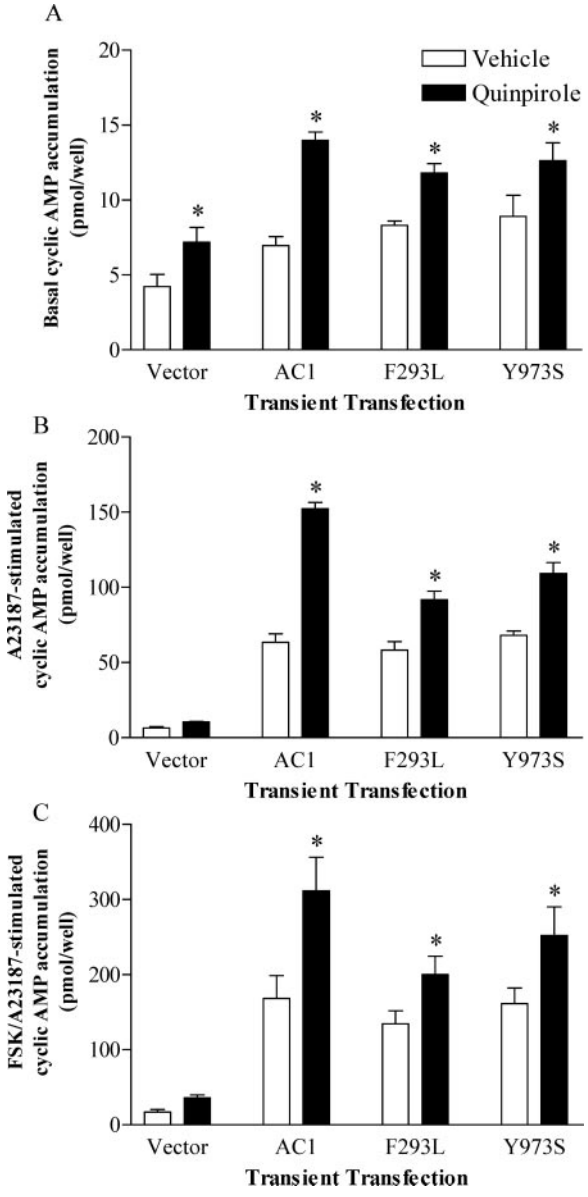

Fig. 3. D_{2L} dopamine receptor-induced heterologous sensitization of wild-type and $G\alpha_s$-insensitive mutants of AC1. HEK-D_{2L} cells were transfected with pcDNA3 (vector) or plasmid encoding wild-type (AC1) or $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S) as indicated. Transfected cells were pretreated for 2 h with vehicle or 1 μM quinpirole. After pretreatment, cells were washed extensively, and cyclic AMP accumulation was measured under basal conditions (A) or was stimulated with 3 μM A23187 (B) in the absence (A23187) or presence of 100 nM forskolin (FSK/A23187) for 15 min (C). Data shown are the mean ± S.E.M. of four to five independent experiments assayed in duplicate. *, $p < 0.05$ compared with vehicle-treated cells (Bonferroni's post hoc repeated-measures ANOVA).

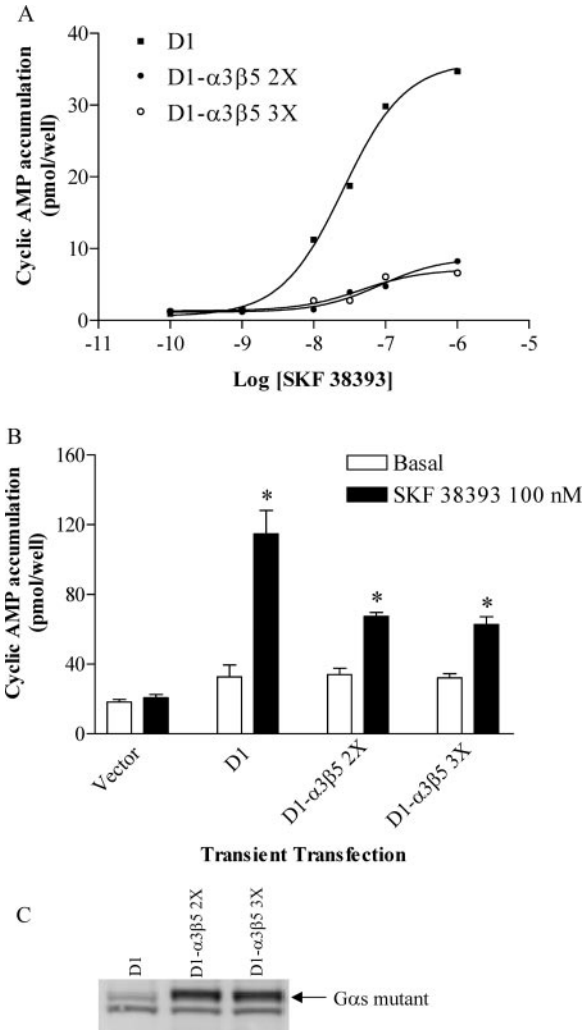

Fig. 4. Effect of dominant-negative $G\alpha_s$ mutants on D_1 dopamine receptor signaling. HEK-D_{2L} cells (A) or HEK-AC1 cells (B) were transfected with the dual expression plasmid (pBudCE4) encoding the D_1 dopamine receptor in the absence (D_1) or presence of dominant-negative $G\alpha_s$ mutants (D_1-$\alpha3\beta5$ 2X or D_1-$\alpha3\beta5$ 3X) as indicated. After transient transfection, cells were washed, and cyclic AMP accumulation was measured under basal conditions or in the presence of increasing concentrations of SKF 38393 for 15 min as indicated. Data shown in A are representative of two independent experiments assayed in duplicate with a constant hill slope of 1.0. Data shown in B are the mean ± S.E.M. of five independent experiments assayed in duplicate. *, $p < 0.05$ compared with basal cyclic AMP accumulation (Bonferroni's post hoc repeated-measures ANOVA). C, Western blot analysis of HEK-AC1/D_{2L} cell membranes transiently transfected with dominant-negative $G\alpha_s$ mutants as described above is representative of two independent experiments. Polyclonal $G\alpha_s$ antibody was used to detect immunoreactivity of endogenous and transiently expressed $G\alpha_s$.

dopamine receptor expression was robust in the absence or presence of each dominant-negative mutant. The B_{max} values for [^{3}H]SCH23390 binding were 3940 ± 610, 1620 ± 210, and 1680 ± 170 fmol/mg for D_1 alone, D_1-$\alpha3\beta5$ 2X, and D_1-$\alpha3\beta5$ 3X, respectively ($n = 4$). The K_d values were similar under each transfection condition (data not shown). Although there was reduced D_1 receptor expression in the presence of the dominant-negative mutants, additional studies revealed that the decrease was not sufficient to account for the decreased receptor-mediated activation of AC1. This was determined by transfecting cells with a reduced amount of D_1 cDNA (0.5×), resulting in the expression of D_1 receptors at a level similar to that observed in the presence of the dominant-negatives (1764 ± 178 fmol/mg, $n = 3$, compared with approximately 1700 fmol/mg in the presence of the mutants). Using these transfection conditions, subsequent functional assays revealed that SKF38393-stimulated cyclic AMP accumulation was indistinguishable from that observed when the D_1 receptor was expressed at B_{max} values approaching 4000 fmol/mg. The specific value for SKF3893-stimulated cyclic AMP after transfection with a 0.5× concentration (i.e., 0.5 μg) of D_1 cDNA/well was 114 ± 10 pmol/well ($n = 4$), which was similar to that observed when cells were transfected with 1.0 μg of D_1 cDNA/well (115 ± 14 pmol/well) (Fig. 4B). Basal cyclic AMP values were also similar under both transfection conditions (0.5 μg cDNA, 42 ± 9.0 pmol/well; 1.0 μg cDNA, 32 ± 7.0 pmol/well, $n = 4$–5). These data support the ability of the dominant-negative mutants to reduce D_1 dopamine receptor-stimulated cyclic AMP accumulation.

Effect of Dominant-Negative $G\alpha_s$ Mutants on Heterologous Sensitization of AC1. From the initial observation that dominant-negative $G\alpha_s$ mutants markedly decreased receptor-$G\alpha_s$ activation of AC1, we used $\alpha3\beta5$ 2X and $\alpha3\beta5$ 3X as pharmacological tools to test the hypothesis that an intact receptor-$G\alpha_s$ signaling pathway was required for the expression of heterologous sensitization of AC1. HEK-AC1/D_{2L} cells were transiently transfected with the D_1 dopamine receptor in the absence or presence of either $\alpha3\beta5$ 2X or $\alpha3\beta5$ $\alpha3\beta5$ 3X. After transient transfection, the effects of D_2 agonist pretreatment on subsequent drug-stimulated cyclic AMP accumulation were examined. Quinpirole pretreatment for 2 h resulted in a significant increase in SKF 38393-stimulated cyclic AMP accumulation in HEK-AC1/D_{2L} cells transiently transfected with the D_1 dopamine receptor (Fig. 5A). As expected, D_1 dopamine receptor-stimulated AC1 activity was significantly reduced by either $\alpha3\beta5$ 2X or $\alpha3\beta5$ 3X in the absence or presence of quinpirole pretreatment (Fig. 5A). However, transient expression of $\alpha3\beta5$ 2X and $\alpha3\beta5$ 3X failed to alter quinpirole-induced sensitization of A23187-stimulated cyclic AMP accumulation in HEK-AC1/D_{2L} cells (Fig. 5B). To eliminate the possibility that the inability of these dominant-negative $G\alpha_s$ mutants to attenuate sensitization of Ca^{2+}-stimulated AC1 activity may be caused by low transfection efficiency in HEK-AC1/D_{2L} cells, we coexpressed recombinant AC1 and $\alpha3\beta5$ 2X in HEK-D_{2L} cells using a dual expression vector. This approach was designed to ensure the expression of AC1 and the dominant-negative $G\alpha_s$ mutant in the same cell population, thereby eliminating the concern of transfection efficiency. Persistent (2 h) activation of the D_2 dopamine receptor resulted in a significant increase of A23187-stimulated cyclic AMP accumulation in HEK-D_{2L} cells expressing AC1 in the absence or presence of $\alpha3\beta5$ 2X (Fig. 6). Taken together, these observations suggest that heterologous sensitization of AC1 does not require receptor activation of $G\alpha_s$.

Discussion

In the present study, we demonstrated the involvement of $G\alpha_s$-adenylyl cyclase interactions in D_2 dopamine receptor-induced sensitization of recombinant AC1 using $G\alpha_s$-insensitive mutants of AC1 (F293L and Y973S). The AC1 F293L mutation in the amino terminus of the C_1 catalytic domain is designed to impair $G\alpha_s$-adenylyl cyclase interactions at the C_1-C_2 domain interface. In particular, AC1 F293L disrupts hydrophobic interactions with Tyr281 on the $\alpha3$ helix of $G\alpha_s$, thereby disturbing the major contact site between the C_1 catalytic domain of adenylyl cyclase and $G\alpha_s$ that results in decreased $G\alpha_s$-stimulated AC1 activity (Tesmer et al., 1997; Yan et al., 1997). A majority of $G\alpha_s$-adenylyl cyclase interactions are localized to the groove formed by the $\alpha2$ helix and the $\alpha3$-$\beta4$ loop of adenylyl cyclase (Tesmer et al., 1997). The AC1 Y973S mutation in the $\alpha3$-$\beta4$ loop of the C_2 catalytic domain disrupts adenylyl cyclase interactions with the

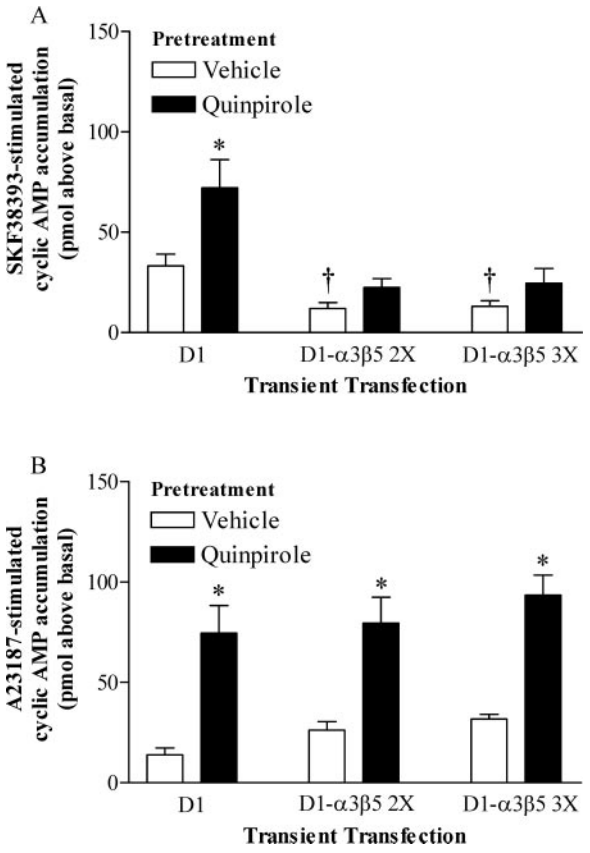

Fig. 5. Effect of dominant-negative $G\alpha_s$ mutants on heterologous sensitization of AC1. HEK-AC1/D_{2L} cells were transiently transfected with the dual expression plasmid (pBudCE4) encoding the D_1 dopamine receptor in the absence (D_1) or presence of dominant-negative $G\alpha_s$ mutants (D_1-$\alpha3\beta5$ 2X or D_1-$\alpha3\beta5$ 3X) as indicated. Transfected cells were pretreated for 2 h with vehicle or 1 μM quinpirole. After pretreatment, cells were washed extensively, and cyclic AMP accumulation was measured in the presence of 100 nM SKF 38393 (A) or 1 μM A23187 (B). Data shown are the mean ± S.E.M. of four to six independent experiments assayed in duplicate. *, $p < 0.05$ compared with vehicle-treated cells for the indicated transfection; †, $p < 0.05$ compared with vehicle-treated cells for the D_1 dopamine receptor transfection (Bonferroni's post hoc repeated-measures ANOVA).

switch II region (α2 helix) of $G\alpha_s$, significantly reducing $G\alpha_s$ stimulation without altering responsiveness to forskolin (Yan et al., 1997). In the present study, $G\alpha_s$-insensitive mutants of AC1 also retained sensitivity to Ca^{2+} activation comparable with wild-type AC1, whereas short-term studies revealed that AC1 F293L and Y973S exhibited a loss of significant $G\alpha_s$ stimulation consistent with the postulated $G\alpha_s$-insensitive phenotype.

The magnitude of sensitization for $G\alpha_s$-insensitive mutants of AC1 was markedly attenuated compared with wild-type AC1, suggesting the requirement of $G\alpha_s$-AC1 interactions for the maximal expression of sensitization. There are two potential explanations for the reduced but significant heterologous sensitization of the $G\alpha_s$-insensitive mutants of AC1. In light of the slight and nonstatistically significant trend toward a $G\alpha_s$ Q227L-induced increase in adenylyl cyclase activity with both AC1 recombinants (Fig. 1B), one possibility is that a very low level of activation of these mutants by $G\alpha_s$ may be sufficient to permit a small degree of heterologous sensitization. On the other hand, the residual sensitization of the $G\alpha_s$-insensitive mutants of AC1 may involve a $G\alpha_s$-independent pathway. This hypothesis is supported by recent studies in a $G\alpha_s$-deficient ($Gnas^{E2-/E2-}$) cell model (Bastepe et al., 2002). These studies revealed that persistent activation of the D_2 dopamine receptor resulted in sensitization to Ca^{2+}- and forskolin-stimulated AC1 activity in the absence of $G\alpha_s$ (Vortherms et al., submitted).

The present study examining $G\alpha_s$-insensitive mutants of AC1 is consistent with previous studies examining $G\alpha_s$-insensitive mutants of AC5 that were first identified using a yeast genetic screen (Zimmermann et al., 1998). $G\alpha_s$-insensitive mutants of AC5 in the C_1 (F379L) and C_2 (R1021Q and F1093S) catalytic domains exhibited decreased $G\alpha_s$ binding and stimulation compared with wild-type AC5 (Zimmermann et al., 1998; Watts et al., 2001). Persistent activation of the D_2 dopamine receptor resulted in heterologous sensitization of basal and forskolin-stimulated AC5 activity. In contrast, D_2 agonist pretreatment failed to induce sensitization of $G\alpha_s$-insensitive mutants of AC5, suggesting a requirement of $G\alpha_s$-AC5 interactions for the expression of heterologous sensitization (Watts et al., 2001). On the other hand, the lack of sensitization for $G\alpha_s$-insensitive mutants of AC5 may be caused, in part, by a significantly reduced response to forskolin activation (Watts et al., 2001). This reduced forskolin responsiveness is consistent with a synergistic interaction between $G\alpha_s$ and forskolin in the activation of AC5, whereas coactivation of AC1 by forskolin and $G\alpha_s$ seems to be additive (Sutkowski et al., 1994). In fact, AC1 F293L and Y973S retained forskolin-responsiveness comparable with wild-type AC1 in the presence of Ca^{2+} stimulation. That the $G\alpha_s$-insensitive mutants of AC1 retain many regulatory features of wild-type AC1 further supports their usefulness as pharmacological tools to examine the involvement of $G\alpha_s$-adenylyl cyclase interactions in sensitization of adenylyl cyclase.

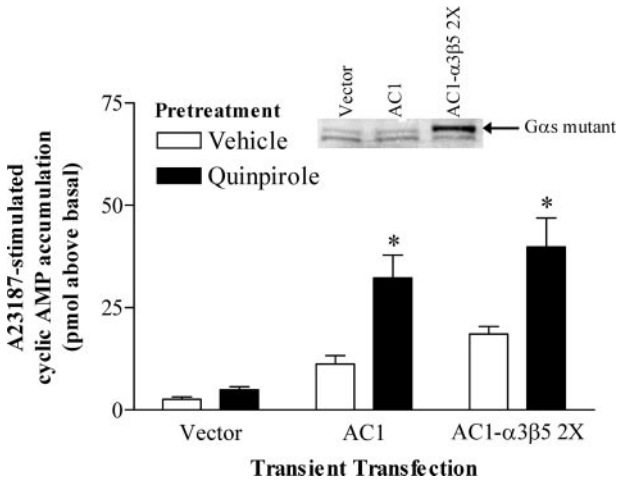

Fig. 6. Effect of $G\alpha_s$ $\alpha3\beta5$ 2X on D_2 dopamine receptor-induced sensitization of AC1. HEK-D_{2L} cells were transiently transfected with pBudCE4 (vector) or plasmid encoding recombinant AC1 in the absence (AC1) or presence of a dominant-negative $G\alpha_s$ mutant (AC1-$\alpha3\beta5$ 2X) as indicated. Transfected cells were pretreated for 2 h with vehicle or 1 μM quinpirole. After pretreatment, cells were washed extensively, and cyclic AMP accumulation was measured in the presence of 3 μM A23187. Data shown are the mean ± S.E.M. of three independent experiments assayed in duplicate. *, $p < 0.05$ compared with vehicle-treated cells for the indicated transfection (Bonferroni's post hoc repeated-measures ANOVA). Inset, Western blot analysis for $G\alpha_s$ expression in HEK-D_{2L} cell membranes transiently transfected as described above.

The molecular basis for enhanced $G\alpha_s$-adenylyl cyclase interactions during sensitization remains to be determined. Recent studies have suggested that localization of cyclic AMP signaling components to cholesterol-enriched environments may play an important role in the regulation of G protein-coupled receptor signaling. In fact, colocalization of the β-adrenergic receptor and AC6 to caveolin-rich membranes in cardiomyocytes selectively enhances $G\alpha_s$-stimulated cyclic AMP accumulation (Ostrom et al., 2000). In contrast, disruption of subcellular microdomains through cholesterol depletion markedly augments β-adrenergic receptor-stimulated cyclic AMP accumulation in HEK293 cells, suggesting that cholesterol-rich microdomains may also function to negatively regulate $G\alpha_s$-coupled receptor signaling (Rybin et al., 2000). Long-term antidepressant treatment in C6 glioma cells also decreased the localization of $G\alpha_s$ in caveolin-rich membrane fractions, resulting in increased adenylyl cyclase activity (Toki et al., 1999), presumably through enhanced $G\alpha_s$-adenylyl cyclase interactions (Chen and Rasenick, 1995). These observations suggest that subcellular localization of $G\alpha_s$ has significant effects on cyclic AMP signaling; therefore, understanding the regulation of receptor-$G\alpha_s$ interactions may be critical for unraveling the molecular mechanisms leading to heterologous sensitization.

Dominant-negative $G\alpha_s$ mutants ($\alpha3\beta5$ G226A/A366S and $\alpha3\beta5$ G226A/E268A/A366S) (Berlot, 2002) markedly reduced D_1 dopamine receptor-stimulated cyclic AMP accumulation. These mutants serve to stabilize the receptor–$G\alpha_s$-$G\beta\gamma$ complex and the guanine nucleotide-free state of $G\alpha_s$ to effectively attenuate $G\alpha_s$-coupled receptor signaling. To this end, dominant-negative $G\alpha_s$ mutants increase the affinity of $G\alpha_s$ for $G\beta\gamma$ (G226A) (Lee et al., 1992) and decrease $G\alpha_s$ affinity for GTP (E268A) (Iiri et al., 1997, 1999) and GDP (A366S) (Iiri et al., 1994). $G\alpha_s$ mutations in the $\alpha3\beta5$ loop region (N271K, K274D, R280K, T284D, and I285T) decrease the activation of adenylyl cyclase (Berlot and Bourne, 1992) and increase the affinity of $G\alpha_s$ for the β-adrenergic receptor (Grishina and Berlot, 2000). In the present study, dominant-negative $G\alpha_s$ mutants significantly reduced SKF 38393-stimulated cyclic AMP accumulation; however, disruption of the D_1 dopamine receptor-$G\alpha_s$ signaling pathway failed to alter heterologous sensitization of Ca^{2+}-stimulated AC1 activity. These observations suggest that receptor activation of $G\alpha_s$ is

not required for the development of the sensitization response.

In the present study, we demonstrated that persistent activation of the D_2 dopamine receptor results in heterologous sensitization of D_1 dopamine receptor-stimulated cyclic AMP accumulation. Studies have revealed that $G\alpha_{i/o}$-coupled D_2 dopamine receptors are colocalized with $G\alpha_s$-coupled D_1 dopamine receptors in several brain regions, including the striatum and nucleus accumbens, supporting the physiological relevance of this adaptive response (Aizman et al., 2000; Chao et al., 2002). For example, it has been proposed that colocalization and the dual inputs created between inhibitory D_2-like and stimulatory D_1-like dopamine receptors in the nucleus accumbens may mediate relapse to drug-seeking behaviors subsequent to addiction to various drugs of abuse (Self et al., 1998). Furthermore, $G\beta\gamma$ dimers released from $G\alpha_{i/o}$-coupled receptors may be involved in response to drugs of abuse (Yao et al., 2003). This $G\beta\gamma$ effect is dependent on concomitant activation of $G\alpha_s$-coupled A_{2A} adenosine receptors, which are colocalized with D_2 dopamine receptors on striatopallidal GABAergic neurons in the striatum (Fink et al., 1992; Yao et al., 2003). Functional interactions between D_2 dopamine and A_{2A} adenosine receptors have also been implicated in the pharmacotherapy of basal ganglia disorders, including Parkinson's disease and schizophrenia (Ferre et al., 1997). In fact, recent studies have shown that persistent activation of the D_2 dopamine receptor results in sensitization of A_{2A} adenosine receptor-stimulated cyclic AMP accumulation (Vortherms and Watts, 2004).

In summary, we have demonstrated that $G\alpha_s$-insensitive mutants of AC1 and dominant-negative $G\alpha_s$ mutants provide useful pharmacological tools to examine $G\alpha_s$ signaling pathways, including heterologous sensitization of adenylyl cyclase. D_2 agonist-induced sensitization of $G\alpha_s$-insensitive mutants of AC1 was markedly attenuated compared with wild-type AC1, directly supporting the hypothesis that the expression of sensitization involves $G\alpha_s$-adenylyl cyclase interactions. In addition, studies with dominant-negative $G\alpha_s$ mutants suggested that receptor activation of $G\alpha_s$ is not a requisite step in the molecular mechanisms leading to heterologous sensitization of AC1.

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