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Short- and Long-Term Regulation of Adenylyl Cyclase Activity by δ-Opioid Receptor Are Mediated by $G\alpha_{i2}$ in Neuroblastoma N_2A Cells

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ABSTRACT

Activation of the opioid receptor results in short-term inhibition of intracellular cAMP levels followed by receptor desensitization and subsequent increase of cAMP above the control level (adenylyl cyclase superactivation). Using adenovirus to deliver pertussis toxin-insensitive mutants of the α-subunits of $G_{i/o}$ that are expressed in neuroblastoma Neuro2A cells ($G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$), we examined the identities of the G proteins involved in the short- and long-term action of the δ-opioid receptor (DOR). Pertussis toxin pretreatment completely abolished the ability of [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) to inhibit forskolin-stimulated intracellular cAMP production. Expression of the C352L mutant of $G\alpha_{i2}$, and not the C351L mutants of $G\alpha_{i3}$ or $G\alpha_o$, rescued the short-term effect of DPDPE after pertussis toxin treatment. The ability of $G\alpha_{i2}$ in mediating DOR inhibition of adenylyl cyclase activity was also reflected in the ability of $G\alpha_{i2}$, not $G\alpha_{i3}$ or $G\alpha_o$, to coimmunoprecipitate with DOR. Coincidently, after long-term DPDPE treatment, pertussis toxin treatment eliminated the antagonist naloxone-induced superactivation of adenylyl cyclase activity. Again, only the C352L mutant of $G\alpha_{i2}$ restored the adenylyl cyclase superactivation after pertussis toxin treatment. More importantly, the C352L mutant of $G\alpha_{i2}$ remained associated with DOR after long-term agonist and pertussis toxin treatment whereas the wild-type $G\alpha_{i2}$ did not. These data suggest that $G\alpha_{i2}$ serves as the signaling molecule in both DOR-mediated short- and long-term regulation of adenylyl cyclase activity.

Opioid receptors belong to the family of seven transmembrane domain receptors that transduce their signals via $G_{i/o}$ proteins (Law and Loh, 1999; Law et al., 2000). Short-term activation of opioid receptors results in myriad responses, including inhibition of adenylyl cyclase (AC), inhibition of voltage-gated Ca^{2+} channels, and activation of G-protein activated inwardly rectifying K^+ channels, leading to reduced excitability and inhibition of neurotransmitter release (Childers, 1991; Breivogel et al., 1997; Varga et al., 2003). However, long-term drug treatment results in tolerance and dependence development, the molecular mechanism of which may involve desensitization to $G_{i/o}$ protein-mediated responses, coupled with sensitization to excitatory opioid actions. One such excitatory action is the compensatory increase in intracellular cAMP accumulation after long-term agonist treatment, or AC superactivation, which is particularly significant upon the withdrawal of opioid agonist. This AC superactivation phenomenon has been postulated to be responsible for the development of drug tolerance and dependence (Koob and Bloom, 1988; Nestler and Aghajanian, 1997; Charles and Hales, 2004).

The heterotrimeric G proteins serve as central signaling molecules connecting cellular signals transduced from opioid receptors. Involvement of $G_{i/o}$ protein α subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$) in AC superactivation is clearly indicated by the ability of pertussis toxin (PTX) to block this response (Avidor-Reiss et al., 1995; Fields and Casey, 1997; Connor and Christie, 1999; Nevo et al., 2000). However, the specific Gα subunit(s) responsible and by what means it regulates AC superactivation are still unresolved. Tso and Wong (2000a,b, 2001) reported that in HEK293 cells stably expressing μ-opioid receptors (MOR), AC superactivation induced by long-term μ-agonist treatment cannot be supported by either $G\alpha_z$ (Tso and Wong, 2000b), $G\alpha_{i2}$ (Tso and Wong, 2000a), $G\alpha_{i1}$, or $G\alpha_{i3}$ (Tso and Wong, 2001) individually. On the other hand,

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ABBREVIATIONS: AC, adenylyl cyclase; PTX, pertussis toxin; HEK, human embryonic kidney; MOR, μ-opioid receptor; DOR, δ-opioid receptor; N_2A, Neuro2A; PCR, polymerase chain reaction; RT, reverse transcription; DMEM, Dulbecco's modified Eagle's medium; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; RGS, regulator of G protein signaling; GFP, green fluorescent protein; HA, hemagglutinin; N_2A, neuroblastoma Neuro2A cells.

$G\alpha_o$ has been suggested to be responsible for MOR-induced AC superactivation in C6 glioma cells (Clark et al., 2004). However, in these studies, systems were pretreated with PTX before long-term agonist exposure. The blunting of the long-term response could be the result of the absence of initial receptor signals being transduced.

To address these questions on the identity of G proteins involved in both short-term inhibition and superactivation of AC activity, we use adenovirus to deliver the individual PTX-insensitive $G_{i/o}$ α-subunit mutant to neuroblastoma Neuro2A cells (N_2A) stably expressing δ-opioid receptor (DOR). It can be demonstrated that only the $G\alpha_{i2}$ and not $G\alpha_{i3}$ or $G\alpha_o$ mutant could rescue the DPDPE induced inhibition of adenylyl cyclase activity after PTX pretreatment. Coincidentally, after long-term DPDPE treatment, only the $G\alpha_{i2}$ mutant could restore AC superactivation after PTX treatment. More importantly, only $G\alpha_{i2}$ and not $G\alpha_{i3}$ or $G\alpha_o$ coimmunoprecipated with DOR. Therefore, the same $G\alpha_{i2}$ mediates both short-term inhibition and long-term superactivation of adenylyl cyclase activity.

Materials and Methods

Mutagenesis of PTX-Resistant $G\alpha_{i/o}$. Point mutations were accomplished using QuikChange site-directed mutagenesis methods as outlined by Stratagene (La Jolla, CA). Previous studies indicated that substitution of the cysteine (Cys) residue within the CAXX motif of the $G_{i/o}$ α-subunit with leucine (Leu) resulted in full efficacy of the mutant to transduce receptor signal (Bahia et al., 1998). Hence, the Cys^{351} of $G\alpha_{i3}$ and $G\alpha_o$ or Cys^{352} of $G\alpha_{i2}$ was mutated to Leu. Point mutation primers were designed as follows: for $G\alpha_{i2}$, 5′-GAACAACCTGAAGGACCTAGGCCTCTTCTGAGGG-3′; for $G\alpha_{i3}$, 5′-CAACTTAAAGGAGCTCGGGCTTTACTGAGAG-3′; and for $G\alpha_o$, 5′-CAACAATCTCCGGGGCCTAGGCTTGTACTGACC-3′.

Adenovirus Construction. $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$ mutant was cloned into pShuttle-CMV vector (Stratagene) following the manufacturer's protocol. In brief, the recombinant plasmids were cotransformed with pAdEasy-1 into BJ 5183 cells by electroporation. The positive adeno-$G\alpha_{i2}$-Leu, adeno-$G\alpha_{i3}$-Leu, or adeno-$G\alpha_o$-Leu plasmid was transfected into HEK293 cells. The virus was harvested and amplified by repeatedly infecting HEK293 cells. The titer of virus was determined using the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA). The viruses used in current studies have titers from 3.0 to 8.0 $\times$ 10^8 plaque-forming units/ml.

RT-PCR. Total RNA of N_2A cells was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA samples were then treated with DNase I (Ambion, Austin, TX). Reverse transcription reaction (RT) was performed using First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations and 0.5 μg of DNase I-treated total RNA was added to each RT reaction. For each PCR reaction, 2 μl of RT reaction solution and 2 units of *Taq* DNA Polymerase (Roche Applied Science) was added to a final 50-μl scale. The PCR conditions were as follows: 2 min at 94°C and then 15 s at 94°C, 30 s at 60°C (53°C for $G\alpha_{i1}$ and $G\alpha_{oA}$), 30 s at 72°C for 25 cycles (35 cycles for $G\alpha_{i1}$ and $G\alpha_{oA}$), and 10 min at 72°C. The PCR primers for each specific $G\alpha$ subunit were designed as follows: $G\alpha_{i1}$: forward, ATGAACCGAATGCATGAGAGCA; reverse, GTCCTT CCTTTTATTGAGGTCT; $G\alpha_{i2}$: forward, GCCAACAAGTACGACGAGGCA; reverse, GTATCTCTCACGCTTCTTGTGCT; $G\alpha_{i3}$: forward, ATGAACCGAATGCATGAGAGCA; reverse, TTTGGTGTCAGTG GCACAGGTA; $G\alpha_{oA}$: forward, CCCGTAGATTTTTGGCGATGA; reverse, CCGCATGCACGAGTCTCTCAT; $G\alpha_{oB}$: forward, CCCGTAGGT TTTTGGCGATGA; reverse, CATGCACGAATCCCTGAAGC.

Cell Culture and Virus Infection. N_2A cells stably expressing mouse DOR with hemagglutinin (HA) tagged at the N terminus were used in this study. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 15 mM glucose, 0.43 M $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and 1 mg/ml G418 (Geneticin; Invitrogen) in a 10% CO_2 incubator. Each T-75 cm^2 flask of cells at 70% confluence was infected with adeno-GFP (Gene Transfer Vector Core, The University of Iowa), adeno-$G\alpha_{i2}$-Leu, adeno-$G\alpha_{i3}$-Leu, or adeno-$G\alpha_o$-Leu viruses using Superfect transfection reagent (QIAGEN) according to the manufacturer's instructions, with a cell-to-virus ratio of 1:100. After 48 h, cells were harvested for Western blotting and immunoprecipitation experiments. For the DOR-mediated regulation of intracellular cAMP level, cells were seeded to 96-well plates 30 h after virus infection and cultured for another 18 h before cAMP assay.

cAMP Assay. For short-term DPDPE inhibition assay, cells were seeded on a 96-well plate for 18 h, culture medium was removed, and the cells were placed on ice. Then, 100 μl of incubation buffer with or without DPDPE was added to each well. The incubation buffer consisted of 0.5 mM 3-isobutyl-1-methylxanthine and 10 μM forskolin in Krebs-Ringer-HEPES buffer (110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4). The assays were initiated by incubating the cells at 37°C for 15 min and then terminated by placing the plates in a water bath at 85°C for 6 min to lyse the cells and release the intracellular cAMP. The cAMP level was measured by using the AlphaScreen cAMP Detection Kit (BioSignal, Montreal, QC, Canada) and Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA) as described previously (Qiu et al., 2003). For the experiments in which pertussis toxin (List Biological Laboratories Inc., Campbell, CA) was used, 100 ng/ml PTX was added to the culture medium, and cells were incubated 12 h before the cAMP assay. For long-term DPDPE treatment assay, 1 μM DPDPE was added to the culture medium for 18 h to completely desensitize the short-term response to DOR activation. Six hours before cAMP assays, or 12 h after the initiation of DPDPE treatment, PTX was added to the designated 96-well plates. Culture medium was aspirated, cells were washed with DMEM at 37°C once, and then 100 μl of treatment buffer with or without naloxone was added. After incubation at 37°C for 15 min, reactions were terminated by incubating at 85°C for 6 min and cAMP level in each well was measured as described previously.

Cell Membrane Purification, Immunoprecipitation, and Western Blotting. Cells from one T-75 cm^2 flask were harvested and the pellet was frozen at −80°C for at least 30 min. Five milliliters of ice-cold TEP buffer (50 mM Tris, pH 7.0, 2.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) was added and the mixture was allowed to incubate on ice for 15 min. The cell suspension was homogenized with a Dounce homogenizer for 10 strokes with pestle A and was centrifuged at 2500 rpm for 10 min. The resulting supernatant was transferred to a new tube and the pellet was resuspended in 2.5 ml of TEP buffer. The homogenization and centrifugation processes were repeated. The two supernatants were combined and centrifuged at 34,000 rpm for 1 h (Beckman rotor Ti80). The final supernatant was discarded, and the pellet was used for immunoprecipitation or Western blotting experiments. For immunoprecipitation, cells from one T-75 cm^2 flask of N_2A cells were treated either with or without 100 ng/ml PTX for 6 h, with 1 μM DPDPE for 18 h, or with 1 μM DPDPE for 12 h, then 100 ng/ml PTX was added during the last 6 h of DPDPE treatment. Cells were then collected and membranes were purified as described above. Membrane pellet was suspended in 1 ml of extraction buffer [100 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1% digitonin (Sigma), 1 $\times$ Complete Protease Inhibitor Cocktail (Roche Applied Science)] and rotated slowly at 4°C overnight. Then the extract was centrifuged at 15,000 rpm for 20 min, and the resulting supernatant was diluted with 15 ml of buffer A (100 mM NaCl and 10 mM Tris, pH 7.4). The diluted supernatant

was concentrated to 2 ml with Centriplus YM-30 (Millipore Corporation, Billerica, MA). To the concentrated solution, mouse monoclonal anti-HA antibody (1:200; Covance, Princeton, NJ) was added, and the mixture was incubated at 4°C overnight with slow rotation. Sixty microliters of protein G agarose beads (Invitrogen) was added, and the mixture was incubated at 4°C for 3 h with slow rotation. The protein G agarose beads were then pelleted by centrifuging at 15,000 rpm for 15 min at 4°C and were washed 5 times with buffer A. Protein samples were eluted from the beads with 1× SDS sample buffer (75 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol), and the samples were heated at 65°C for 5 min. Proteins in the samples were resolved by 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (0.45 μm; Millipore). The membrane was blocked overnight at 4°C in a blocking solution (10% powdered nonfat milk in 0.1% Tris-buffered saline/Tween 20 (0.1% Tween 20, 25 mM Tris, and 150 mM NaCl, pH 7.6) and was then probed by anti-Gα_{i2}, anti-Gα_{i3} (Roerig et al., 1992), or anti-Gα_o (New England Biolabs) antibodies (1:1000) for 1 h at room temperature in the blocking solution. After washing three times of 15 min each with 0.1% Tris-buffered saline/Tween 20, the membrane was probed with goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:5000; Bio-Rad) for 1 h in the same blocking solution. The Gα_{i2}, Gα_{i3}, or Gα_o bands were detected by ECF substrate (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and scanned by the Storm 860 imaging system (GE Healthcare). The band intensities were quantified and analyzed with the ImageQuant software (GE Healthcare).

Results

Expression Pattern of Different G$\alpha_{i/o}$ Subunits in N_2A Cells. Before our investigation on the identities of G proteins involved in the short- and long-term action of DOR, the expression patterns of the G$\alpha_{i/o}$ subunits in N_2A cells were determined (Fig. 1). Both RT-PCR and Western blotting analysis were used to determine the expression patterns. As shown in Fig. 1, RT-PCR studies indicated that Gα_{i2}, Gα_{i3}, and Gα_{oB} were detected in N_2A cells. Even under much less stringent conditions (53°C annealing temperature and 35 cycles, compared with 60°C annealing temperature and 25 cycles for Gα_{i2}, Gα_{i3}, and Gα_{oB}), Gα_{i1} and Gα_{oA} were not detected. The inability to detect these α-subunits in N_2A was not due to the primer sequences, because both Gα_{i1} and Gα_{oA} were detected with mouse brain RNAs using the same PCR conditions and primers (data not shown). Quantitation of the RT-PCR results suggested that Gα_{i2}, Gα_{i3} were the most abundant subunits and that Gα_{oB} was expressed in a much lower level. Similar expression patterns for Gα_{i2}, Gα_{i3}, and Gα_o proteins could be detected by Western blotting analysis (Fig. 2B, control). Thus, in subsequent studies, only the PTX-insensitive mutants of Gα_{i2}, Gα_{i3}, and Gα_o were used to investigate their abilities to restore DOR functions after PTX treatment.

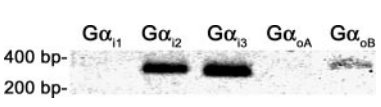

Fig. 1. RT-PCR analysis showing the expression of Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_{oA}, and Gα_{oB} in N_2A cells. The relative transcript levels of various G$_{i/o}$ α-subunits were determined by RT-PCR as described under *Materials and Methods*. Ten microliters of each PCR reaction (50 μl) was resolved on 1.5% NuSieve CTG agarose gel (Intermountain Scientific/BioExpress, Kaysville, UT) run in Tris-borate/EDTA buffer. The positions of molecular markers are indicated on the left side.

Expression of PTX-Resistant Adeno-Gα_{i2}-Leu, Adeno-Gα_{i3}-Leu, and Adeno-Gα_o-Leu Mutants in N_2A Cells. To determine the efficiency of adenovirus infection of N_2A cells, various cell–to–adeno-GFP virus ratios were used to infect the N_2A cells. After 2 days of infection, at 1:100, the highest cell-to-virus ratio used, 32% of cells expressing GFP were observed (Fig. 2A, top). To increase the infection rate, several transfection reagents were used during adenovirus

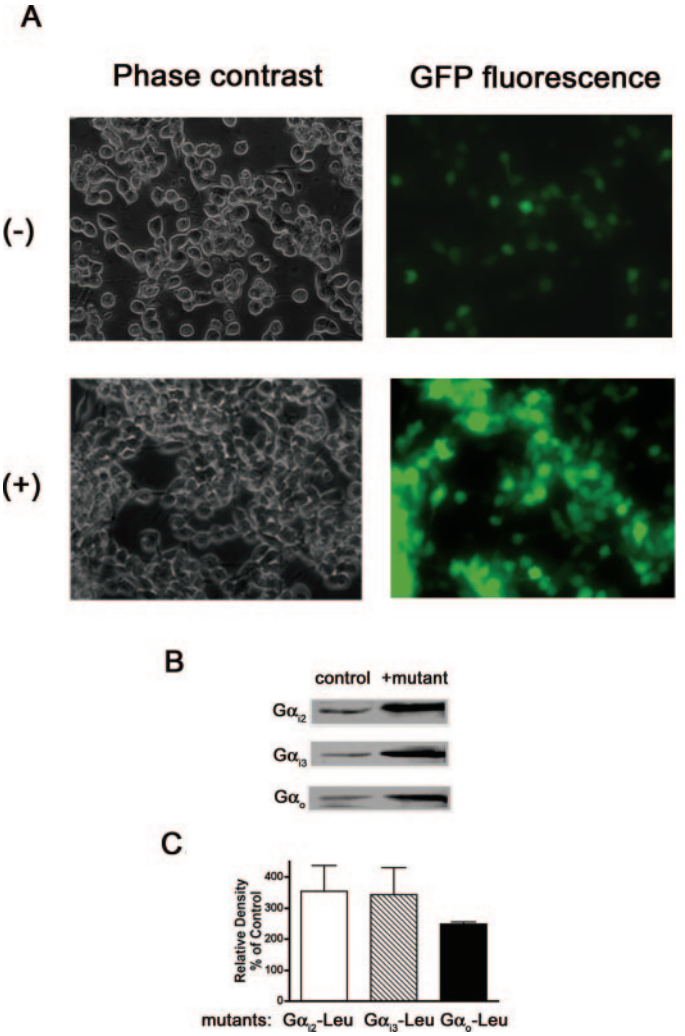

Fig. 2. Expression of PTX-resistant adeno-Gα_{i2}-leu, adeno-Gα_{i3}-leu, and adeno-Gα_o-leu mutants in N_2A cells. A, adenovirus infection efficiency of N_2A cells. N_2A cells were cultured on six-well plates to 70% confluence and were infected with adeno-GFP in the absence (−) or in the presence (+) of transfection reagent Superfect (QIAGEN) following the manufacturer's recommendations. A cell-to-virus ratio of 1:100 was used in the studies. Left, phase contrast views; right, fluorescence images of GFP. Images were taken after 48 h of virus infection using a Leica DMIRE2 microscope system (Leica Co.) with a 40× objective. B, Western analysis of adeno-Gα_{i2}-leu, adeno-Gα_{i3}-leu, and adeno-Gα_o-leu mutants expressed in N_2A cells. Similar to the studies summarized in A, N_2A cells were infected with a cell-to-virus ratio of 1:100. On the left, cells were infected with adeno-GFP; on the right, cells were infected with individual mutants as indicated. In each sample, 50 μg of cell membrane extracts was loaded. The Gα bands were probed by specific anti-Gα_{i2}, Gα_{i3}, or Gα_o antibodies (1:1000). C, the relative density of each Gα mutant subunits expressed in N_2A cells. The relative density of the Gα in N_2A cells infected with respective mutant was compared with that in cells infected with adeno-GFP (control). The density of control bands was designated as 100%. The white, shaded, and black bars represent the relative densities of adeno-Gα_{i2}-leu, adeno-Gα_{i3}-leu, and adeno-Gα_o-leu mutants, respectively, compared with the control (n = 2).

infection. Among the reagents tested, Superfect (QIAGEN) can dramatically increase the infection rate to 82% in a cell-to-virus ratio of 1:100 (Fig. 2A, bottom). Therefore, in all the following studies, Superfect was added to virus-infection-related experiments. Using such a paradigm, the PTX-resistant mutants of $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ were overexpressed in N_2A cells. The Cys^{351} of $G\alpha_{i3}$ and $G\alpha_o$ and the Cys^{352} of $G\alpha_{i2}$ were mutated to Leu because previous reports suggested that such mutation could retain the function of G protein α-subunits (Bahia et al., 1998). The expression of adenovirus-delivered $G\alpha_{i/o}$ mutants were confirmed by Western blotting using specific antibodies for $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ (Fig. 2B). Cells infected with the adeno-$G\alpha_{i2}$-leu mutant showed a 353.0 ± 82.9% increase in $G\alpha_{i2}$ protein compared with control cells that were infected with adeno-GFP (Fig. 2C). Likewise, cells infected with adeno-$G\alpha_{i3}$-leu or adeno-$G\alpha_o$-leu mutant had a 342.8 ± 86.4% and 247.3 ± 6.4% increase in protein level, respectively, compared with the control (Fig. 2C).

The Identity of the G Protein Involved in DOR Short-Term Inhibition of Adenylyl Cyclase Activity. To determine the identity of the G protein involved in DOR regulation of AC activity in N_2A cells, the ability of the PTX-insensitive mutants to restore DPDPE inhibition was investigated. As summarized in Fig. 3 and Table 1, overexpression of the PTX-insensitive $G\alpha$ mutants affected neither the potency nor the maximal inhibition level of DPDPE. From the DPDPE concentration-dependent inhibition of forskolin-stimulated cAMP production in N_2A cell studies, the maximal inhibition levels were calculated to be 63 ± 2.7% (n = 4) for cells infected with GFP-adenovirus, and in $G\alpha_{i/o}$ mutant-expressing cells, 61 ± 2.4% (n = 4) for cells infected with adeno-$G\alpha_{i2}$-leu, 62 ± 2.6% (n = 4) for cells infected with adeno-$G\alpha_{i3}$-leu, and 64 ± 2.5% (n = 4) for cells infected with adeno-$G\alpha_o$-leu. PTX-pretreatment totally abolished the DPDPE-induced inhibition effect on cAMP accumulation in control cells, adeno-$G\alpha_{i3}$-leu–, and adeno-$G\alpha_o$-leu– expressing cells. It is noteworthy that the inhibition effect of cAMP accumulation can still be observed after PTX-pretreatment only in adeno-$G\alpha_{i2}$-leu mutant expressing cells (41 ± 3.1%, n = 4; Fig. 3B). The potency of DPDPE in N_2A cells overexpressing the adeno-$G\alpha_{i2}$-leu mutant after PTX pretreatment was not significantly different from that of control cells without PTX treatment (Table 1). The inability of other PTX-insensitive $G\alpha$ mutants to restore the DPDPE inhibition of AC activity in N_2A cells could not be caused by low expression of these $G\alpha$ mutants, because a similar level of increase in the adeno-$G\alpha_{i3}$-leu, compared with that of adeno-$G\alpha_{i2}$-leu, was observed 48 h after virus infection as determined by Western analysis (Fig. 2). Similar results were observed in cells that express DOR endogenously, such as neuroblastoma x glioma hybrid NG108-15 and neuroblastoma NIE115 cells. The maximal inhibition levels were calculated to be 60 ± 3.5% and 58 ± 2.5% (n = 2) for NG108-15 and NIE115 cells infected with GFP-adenovirus and, in $G\alpha_{i/o}$ mutant-expressing cells, 53 ± 2.8% and 58 ± 3.0% (n = 2), respectively, for cells infected with adeno-$G\alpha_{i2}$-leu, 55 ± 1.4% and 55 ± 6.0% (n = 2), respectively, for cells infected with adeno-$G\alpha_{i3}$-leu, and 59 ± 1.6% and 55 ± 3.7% (n = 2), respectively, for cells infected with adeno-$G\alpha_o$-leu, in NG108-15 (Fig. 4A) and NIE115 cells (Fig. 4B). PTX pretreatment totally abolished the DPDPE-induced inhibition effect on cAMP accumulation in control cells, adeno-$G\alpha_{i3}$-leu–, and adeno-$G\alpha_o$-leu–expressing cells. But the DPDPE inhibition effect of cAMP accumulation can still be observed after PTX pretreatment only in adeno-$G\alpha_{i2}$-leu mutant expressing cells (55 ± 3.0% and 61 ± 4.8%, n = 2, in NG108-15 and NIE115 cells, respectively). The potencies of DPDPE in these cells over-expressing the

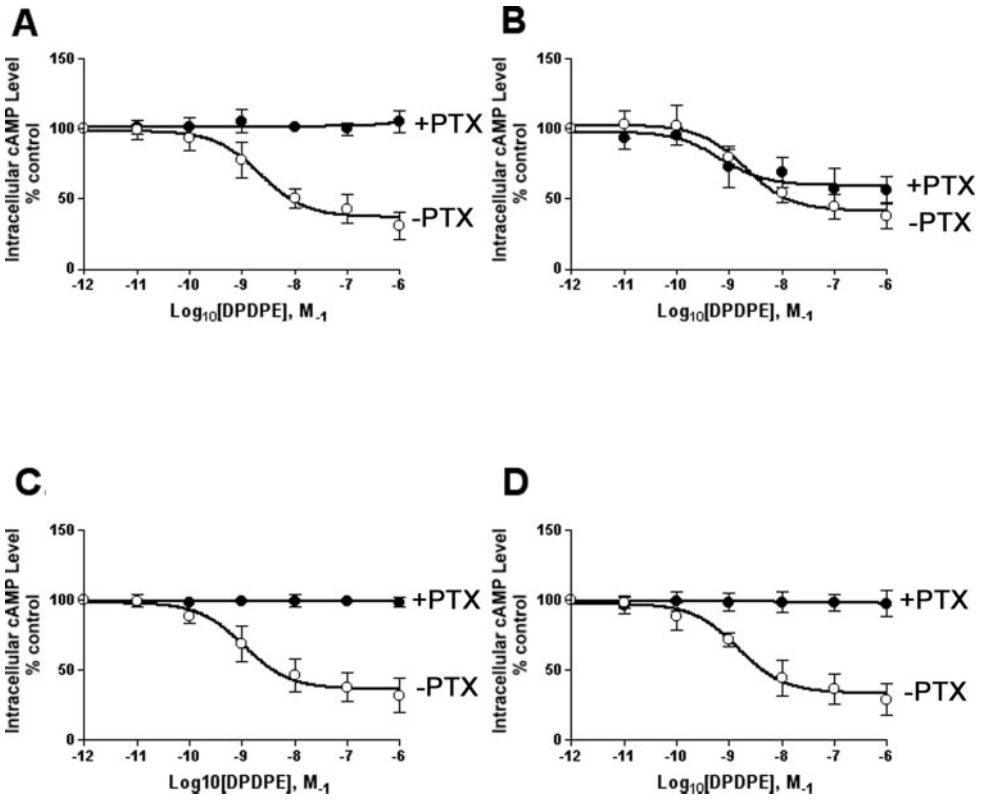

Fig. 3. $G\alpha_{i2}$ involves in short-term DPDPE inhibition of forskolin-stimulated intracellular cAMP production. N_2A cells were infected in a cell versus virus ratio of 1:100 with adeno-GFP (A), adeno-$G\alpha_{i2}$-leu (B), adeno-$G\alpha_{i3}$-leu (C), and adeno-$G\alpha_o$-leu (D) mutants and were pretreated with PTX (100 ng/ml, 12 h, ●) or without PTX (○). The abilities of various concentrations of DPDPE to inhibit the 10 μM forskolin-stimulated intracellular cAMP production were determined as described under *Materials and Methods*. The values represent averages of the concentration-dependent curves generated from four separate virus infection experiments with triplicate determinations for each agonist concentration.

adeno-Gα_{i2}-leu mutant after PTX pretreatment (1.7 ± 0.6 and 1.5 ± 0.3 nM for NG108-15 and NIE115 cells, respectively) was not significantly different from those of control cells without PTX treatment (0.7 ± 0.2 and 0.6 ± 0.1 nM for NG108-15 and NIE115 cells, respectively). Thus, similar to reports using G protein α-subunit selective antibodies (McKenzie and Milligan, 1990), DOR inhibition of AC activity is mediated via the Gα_{i2} subunit in N_2A cells heterologously expressing DOR or in NG108-15 and NIE115 cells endogenously expressing the receptor.

The Superactivation of Adenylyl Cyclase after Long-Term Treatment with DPDPE. Long-term treatment (18 h) of N_2A cells with DPDPE resulted in a complete loss of DOR-mediated inhibition of AC activity or receptor desensitization. The complete receptor desensitization was also accompanied by a dramatic rapid increase of forskolin-induced cAMP accumulation after agonist washout and/or addition of opioid antagonist such as naloxone, generally known as AC superactivation (Sharma et al., 1975, 1977; Thomas and Hoffman, 1987). Because after complete receptor desensitization, the intracellular cAMP level in agonist-treated cells in the presence of agonist was identical to that in the control cells in the absence of agonist (Sharma et al., 1975, 1977), AC superactivation has been attributed to a mechanism such as an increase in receptor constitutive activities caused by coupling of the receptor to G proteins other than $G_{i/o}$ (Wong et al., 1992; Lai et al., 1995; Tsu et al., 1995). However, our earlier DOR high-affinity states binding studies in NG108-15 cells indicated reduction but not abolition of the agonist high-affinity states after long-term agonist treatment (Law et al., 1991). Such results suggested DOR remained coupled to $G_{i/o}$ proteins after long-term agonist treatment. Hence, the PTX-insensitive $G_{i/o}$ α-subunits were used to examine the role of these G proteins in AC superactivation after long-term agonist treatment. To distinguish the effects of these mutants on receptor desensitization and AC superactivation, N_2A cells were infected with the Gα mutants first, and were subjected to long-term treatment with 1 μM DPDPE for 12 h to elicit complete receptor desensitization before treatment with 100 ng/ml of PTX during the last 6 h of agonist treatment so as to uncouple any $G_{i/o}$ proteins from the receptor. Treating the N_2A cells according to this paradigm has resulted in a complete uncoupling of $G_{i/o}$ proteins from DOR as reflected in the absence of agonist high-affinity binding states (Law et al., 1991; Chakrabarti et al., 1997).

When N_2A cells were treated with 1 μM DPDPE for 18 h, complete receptor desensitization was observed. After long-term treatment, 1 μM DPDPE elicited 4.8 ± 2.2% inhibition of forskolin-stimulated cAMP production compared with 64.3 ± 2.3% in cells not subjected to long-term treatment with the agonist. Similar levels of receptor desensitization were observed in N_2A cells infected either with adeno-Gα_{i2}, adeno-Gα_{i3}, or adeno-Gα_o. When naloxone was used to displace the DPDPE bound to DOR during long-term treatment, there was a naloxone concentration-dependent increase in AC activity (Fig. 5). Such a naloxone concentration-dependent increase in AC activity was also observed in N_2A cells infected with various adenoviruses and treated with DPDPE. There was no statistically significant difference in the maximal level of AC superactivation in N_2A cells infected either with the adeno-GFP

TABLE 1

The ability of various $G_{i/o}$ α-subunit mutants to rescue the DPDPE inhibition of forskolin-stimulated intracellular cAMP production in N_2A cells after PTX treatment

N_2A cells were infected with respective adenoviruses as described under *Materials and Methods*. Thirty-six hours later, the cells were then treated with 100 ng/ml of PTX for 12 h before cAMP measurement. Then the ability of various concentrations of DPDPE to inhibit 10 μM forskolin-stimulated intracellular cAMP production was determined. The IC_{50} values represent the average and S.D. from four separate experiments. Values in parentheses represent the maximal DPDPE-induced inhibition of cAMP production.

	IC_{50}	
	−PTX	+PTX
	nM	
+ Adeno-GFP (Control)	2.2 ± 0.1 (63 ± 2.7%)	N.I.
+ Adeno-Gα_{i2}-Leu	2.1 ± 0.1 (61 ± 2.4%)	0.7 ± 0.2 (41 ± 3.1%)
+ Adeno-Gα_{i3}-Leu	1.1 ± 0.1 (62 ± 2.6%)	N.I.
+ Adeno-Gα_o-Leu	1.50 ± 0.1 (64 ± 2.5%)	N.I.

N.I., no inhibition when DPDPE ≤ 10^{-6} M.

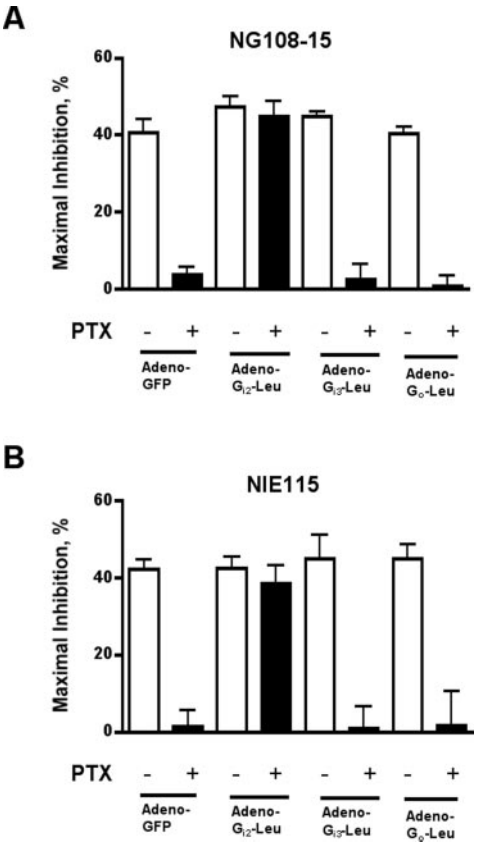

Fig. 4. Gα_{i2} is also involved in short-term DPDPE inhibition of forskolin-stimulated intracellular cAMP production in NG108-15 (A) and NIE115 (B) cells. Cells were infected in a cell-to-virus ratio of 1:100 with adeno-GFP, adeno-Gα_{i2}-leu, adeno-Gα_{i3}-leu, and adeno-Gα_o-leu mutants and were pretreated with PTX (100 ng/ml, 12 h, ■) or without PTX (□). The abilities of various concentrations of DPDPE to inhibit the 10 μM forskolin-stimulated intracellular cAMP production were determined as described under *Materials and Methods*. The values represent averages of the maximal inhibition of forskolin-induced cAMP accumulation from DPDPE concentration-dependent curves generated from two separate virus infection experiments with triplicate determinations for each agonist concentration.

virus (215 ± 12%, $n = 4$), adeno-$G\alpha_{i2}$-leu virus (204 ± 14% $n = 4$), adeno-$G\alpha_{i3}$-leu virus (200 ± 7%, $n = 4$), or adeno-$G\alpha_o$-leu virus (230 ± 11%, $n = 4$). There was also no dramatic difference in the concentration of naloxone to induce 50% of maximal level (EC_{50}) of AC superactivation in the N_2A cells infected with these viruses (Table 2). When the N_2A cells infected either with adeno-GFP, adeno-$G\alpha_{i3}$-leu, or adeno-$G\alpha_o$-leu were treated with PTX after long-term DPDPE treatment, AC superactivation observed in the presence of antagonist was completely eliminated (Fig. 5. A, C, and D). Coincident with the short-term treatment experiment results, only the cells expressing with adeno-$G\alpha_{i2}$-leu showed superactivation after PTX-treatment. In these N_2A cells, the maximal level of adenylyl cyclase superactivation was observed to be 155 ± 6% ($n = 4$, Fig. 5B). Likewise, such a naloxone concentration-dependent increase in AC activity was observed in NG108-15 (Fig. 6A) and NIE115 (Fig. 6B) cells infected with various adenoviruses and treated with DPDPE. There was no statistically significant difference in the maximal level of AC superactivation in these cells infected either with the adeno-GFP virus (327 ± 55% and 284 ± 8%, for NG108-15 and NIE115 cells, respectively, $n = 2$), adeno-$G\alpha_{i2}$-leu virus (316 ± 28% and 296 ± 2%, respectively, $n = 2$), adeno-$G\alpha_{i3}$-leu virus (293 ± 16% and 252 ± 41%, respectively, $n = 2$), and adeno-$G\alpha_o$-leu virus (299 ± 8% and 257 ± 29%, $n = 2$). There was also no dramatic difference in the concentration of naloxone to induce 50% of maximal level (EC_{50}) of AC superactivation in these cells infected with these viruses (2.8 ± 0.1, 2.7 ± 1.2, 2.5 ± 0.8, and 2.4 ± 1.6 nM for NG108-15 cells and 2.4 ± 0.4, 2.9 ± 0.6, 1.4 ± 1.0, and 2.3 ± 0.7 nM for NIE115 cells infected either with the adeno-GFP, adeno-$G\alpha_{i2}$-leu, adeno-$G\alpha_{i3}$-leu, or adeno-$G\alpha_o$-leu virus, respectively). Coincident with the result from N_2A cells, only the cells expressing with adeno-$G\alpha_{i2}$-leu showed superactivation after PTX-treatment (304 ± 19% and 271 ± 40%, for NG108-15 and NIE115 cells, respectively, $n = 2$). Thus, although DOR could not inhibit the forskolin-stimulated intracellular cAMP production via the $G\alpha_{i2}$ after long-term DPDPE treatment, the same $G\alpha_{i2}$ protein was responsible for the expression of AC superactivation.

Coimmunoprecipitation of DOR and $G\alpha_{i2}$ Subunit. After long-term agonist treatment, the activation of the $G_{i/o}$ proteins by DOR as reflected by [^{35}S]GTPγS binding was completely abolished (Eisinger et al., 2002). Because the α-subunits of $G_{i/o}$ proteins are not known to activate AC activity, the observed AC superactivation in N_2A cells infected with adeno-$G\alpha_{i2}$-leu after PTX treatment could be attributed to the release of the $\beta\gamma$ subunits associated with this G protein. However, the observed AC superactivation with AC subtypes I and V excluded the absolute requirement for the G protein $\beta\gamma$ subunits in this cellular response (Nevo et al., 2000). The $G\alpha_{i2}$ could probably serve as a scaffold to recruit cellular proteins involved in AC superactivation. If this was the case, then $G\alpha_{i2}$ and not other G protein α-subunits would be coimmunoprecipitated with DOR.

When HA epitope tagged DOR was immunoprecipitated with the anti-HA monoclonal antibody, as shown in Fig. 7A, $G\alpha_{i2}$ was coimmunoprecipitated with DOR. Western blotting analyses of similar immunoprecipitates with $G\alpha_{i3}$ and $G\alpha_o$ selective antibodies did not reveal the presence of these G protein α-subunits (Fig. 7B). Alteration in the extraction procedure by using detergents other than digitonin did not result in coimmunoprecipitation of G protein α-subunits with DOR other than the $G\alpha_{i2}$. Thus, only $G\alpha_{i2}$ formed a tight complex with DOR that could be detected with the immunoprecipitation studies. PTX treatment lowered the amount of

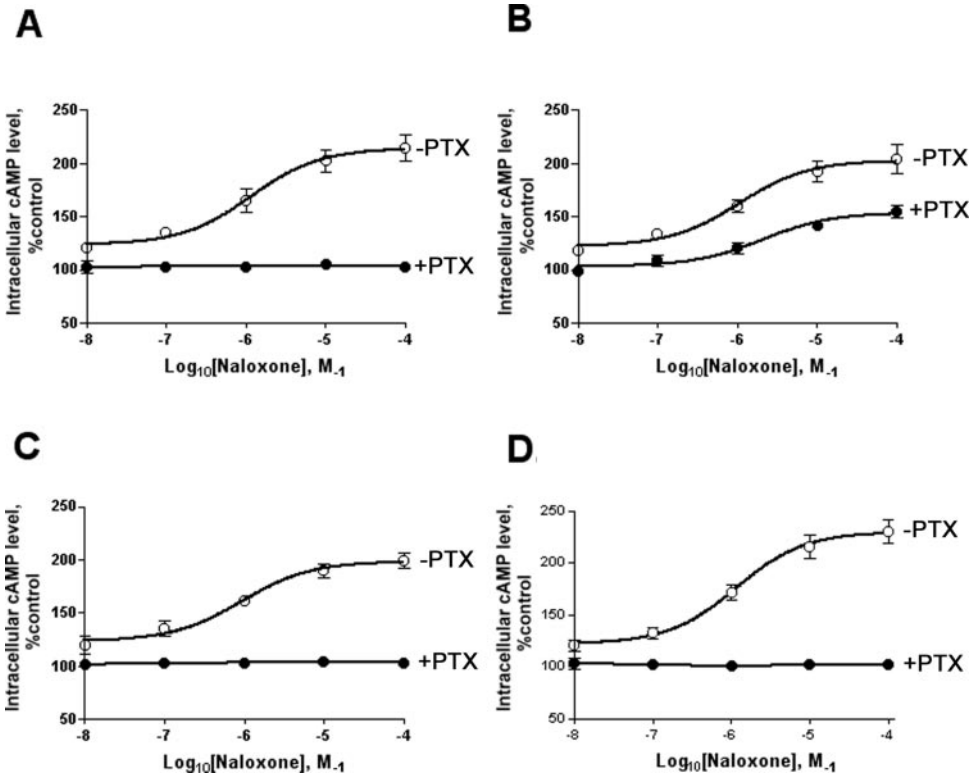

Fig. 5. $G\alpha_{i2}$ mutant restored the adenylyl cyclase superactivation after long-term treatment with DPDPE followed by PTX treatment. N_2A cells were infected with adeno-GFP (A), adeno-$G\alpha_{i2}$-leu (B), adeno-$G\alpha_{i3}$-leu (C), and adeno-$G\alpha_o$-leu (D) mutants in a cell-to-virus ratio of 1:100. These cells were treated with 1 μM DPDPE for 18 h (○) or with 1 μM DPDPE for 12 h followed by the addition of PTX (100 ng/ml) for 6 h (●). Afterward, incubation medium was removed and cells were washed with DMEM at 37°C once to remove the agonist DPDPE. The ability of forskolin to stimulate the intracellular cAMP production was determined at various concentrations of naloxone as described under *Materials and Methods*. The amount of cAMP produced in the presence of naloxone was compared with that observed in the presence of 1 μM DPDPE. The values represent the averages from four separate virus infection experiments with triplicate determinations for each agonist concentration.

precipitated $G\alpha_{i2}$ proteins to 22% ($n = 2$) compared with the control (designated as 100%), whereas the amount of precipitated $G\alpha_{i2}$ proteins after long-term 1 μM DPDPE treatment was 69% ($n = 2$). Treating the cells with 1 μM DPDPE and PTX also lowered the amount of $G\alpha_{i2}$ proteins coimmunoprecipitated with DOR to 24% ($n = 2$). Again, as in the case with control cells, $G\alpha_{i3}$ and $G\alpha_o$ could not be coimmunoprecipitated with DOR in the above drug and/or PTX treatments (Fig. 7B).

The overexpression of $G\alpha_{i2}$-leu in N_2A cells infected with adeno-$G\alpha_{i2}$-leu significantly increased the amount of $G\alpha_{i2}$ proteins coimmunoprecipitated with DOR (Fig. 7C). PTX pretreatment did not significantly reduce the amount of $G\alpha_{i2}$ coimmunoprecipitated in these cells. Although no attempt to distinguish between the wild-type and mutant $G\alpha_{i2}$ in the coimmunoprecipitated was made, because PTX treatment reduced the amount of wild-type $G\alpha_{i2}$ that was coimmunoprecipitated (Fig. 7A), it is reasonable to assume that most of the $G\alpha_{i2}$ coimmunoprecipitated with DOR reflects the PTX-insensitive mutant. Overexpression of $G\alpha_{i3}$-leu or $G\alpha_o$-leu with respective adenovirus infection did not result in the coimmunoprecipitation of these proteins with DOR in either control or PTX-treated cells (data not shown). Again, these data suggest that DOR in particular tightly interacts with $G\alpha_{i2}$.

Discussion

Long-term opioid treatment results in the development of tolerance and dependence, which decreases the therapeutic effects of these drugs and contributes to the development of drug addiction. Studies with PTX have implicated a critical role of $G\alpha_{i/o}$ in the mechanism of long-term opioid action (Lux and Schulz, 1986; Avidor-Reiss et al., 1995, 1996; Williams et al., 2001). However, a key question pertaining to the molecular basis of opioid-induced tolerance/dependence, the identity of the specific G-protein, which carries and transduces the signal, has never been answered clearly. Numerous attempts have been made to answer this question. For example, some studies suggested that opioid receptors could activate $G\alpha_z$, which is a PTX-insensitive G protein subunit (Wong et al., 1992; Lai et al., 1995; Tsu et al., 1995). However, these studies are controversial given that PTX can totally block the signals from activation of the opioid receptor to AC activity in both in vitro and in vivo models (Abood et al., 1985; Self and Stein, 1993; Fields and Casey, 1997). There is evidence to support the selectivity of G proteins involved in the activation of specific second-messenger systems. Studies with $G\alpha$-specific antibodies suggest that $G\alpha_{i2}$ mediates DOR inhibition of AC in NG108-15 cells (McKenzie and Milligan, 1990), although the cross activity between $G\alpha_{i1}$ and $G\alpha_{i2}$ of the antibodies used did not eliminate the role of $G\alpha_{i1}$ in this process. Using antisense oligodeoxynucleotides, it was shown that opioid-induced intracellular Ca^{2+} mobilization in ND8-47 neuroblastoma $\times$ DRG hybrid cells is mediated by $G\alpha_{i2}$ (Tang et al., 1995). Another study using antisense oligodeoxynucleotides showed that $G\alpha_{i2}$ and $G\alpha_{x/z}$ antisense probes blocked spinal μ-opioid analgesia (Standifer et al., 1996). However, the unstable and less effective nature of the antisense oligodeoxynucleotides reduces the accuracy of these studies. The presence of $G\alpha$ transcripts and partial

TABLE 2

The ability of various $G_{i/o}$ α-subunit mutants to restore the superactivation of adenylyl cyclase activity after chronic DPDPE and PTX treatment

N_2A cells were infected with various adenovirus and treated with DPDPE and PTX chronically as described under *Materials and Methods*. The superactivation of adenylyl cyclase in these cells was then measured in the presence of various concentrations of naloxone. The values represent the average naloxone concentrations needed to induce 50% maximal superactivation ± S.D. from four separate experiments. Values in parentheses represent the maximal agonist-induced increase in adenylyl cyclase activity after long-term DPDPE treatment.

	EC_{50}	
	−PTX	+PTX
	nM	
+ Adeno-GFP (Control)	2.0 ± 0.1 (215 ± 12)	N.E.
+ Adeno-$G\alpha_{i2}$-Leu	1.2 ± 0.2 (204 ± 14)	2.5 ± 0.2 (155 ± 6)
+ Adeno-$G\alpha_{i3}$-Leu	0.4 ± 0.1 (200 ± 7)	N.E.
+ Adeno-$G\alpha_o$-Leu	0.9 ± 0.1 (230 ± 11)	N.E.

N.E., no excitation when naloxone $\leq 10^{-4}$ M.

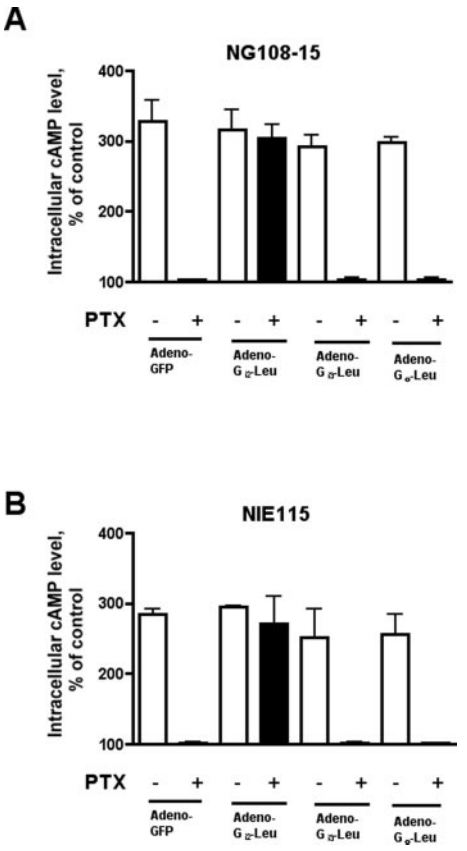

Fig. 6. In NG108-15 (A) and NIE115 (B) cells, $G\alpha_{i2}$ mutant restored the adenylyl cyclase superactivation after long-term treatment with DPDPE followed by PTX treatment. Cells were infected with adeno-GFP, adeno-$G\alpha_{i2}$-leu, adeno-$G\alpha_{i3}$-leu, and adeno-$G\alpha_o$-leu mutants in a cell-to-virus ratio of 1:100. These cells were treated with 1 μM DPDPE for 18 h (□) or with 1 μM DPDPE for 12 h followed by the addition of PTX (100 ng/ml) for 6 h (■). Afterward, incubation medium was removed and cells were washed with DMEM at 37°C once to remove the agonist DPDPE. The ability of forskolin to stimulate the intracellular cAMP production was determined at various concentrations of naloxone as described under *Materials and Methods*. The amount of cAMP produced in the presence of naloxone was compared with that observed in the presence of 1 μM DPDPE. The values represent averages of maximal intracellular cAMP level from two separate virus infection experiments with triplicate determinations for each agonist concentration.

reduction of the $G\alpha$ subunit content after antisense oligodeoxynucleotides treatment might not be able to alter the signaling of an effector system that is efficiently coupled to the receptor, as in the case of DOR inhibition of adenylyl cyclase. Our recent small interfering RNA studies to knock-down $G\alpha_{i2}$ subunit transcripts revealed 75% reduction in the protein level without altering the DOR mediated inhibition of adenylyl cyclase activity (L. Zhang, H. H. Loh, and P. Y. Law, unpublished observation). Some other studies support that more than one type of G protein is involved in the same opioid receptor signal. For example, a series of articles suggested that in HEK293 cells stably expressing MOR, long-term μ-agonist treatment induced AC superactivation was not due to the activation of either $G\alpha_z$ (Tso and Wong, 2000b), $G\alpha_{i2}$ (Tso and Wong, 2000a), $G\alpha_{i1}$, or $G\alpha_{i3}$ (Tso and Wong, 2001) individually. However, a methodological drawback in these studies is that the cell systems were pretreated with PTX before long-term agonist exposure, which caused blunting of initial receptor signals being transduced and resulted in the absence of the long-term response. To avoid this problem, in our current studies, N_2A cells were infected with individual genetically engineered PTX-resistant G proteins ($G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$) and were treated by DPDPE in the short or long term (18 h), then PTX was used to down-regulate all the endogenous $G\alpha$ subunits. We could demonstrate that under this paradigm, before the PTX treatment, the DOR receptors in N_2A cells were completely desensitized, and that upon addition of naloxone, AC super-activation was observed. Based on these strategies, it is demonstrated that the selective activation of $G\alpha_{i2}$ by DOR receptors mediated both inhibition and superactivation of adenylyl cyclase activity. Expression of the $G\alpha_{i3}$ or $G\alpha_o$ mutant did not rescue the inhibition or AC superactivation after PTX treatment. One could argue that the observed AC superactivation in the presence of the $G\alpha_{i2}$ mutant was the consequence of DOR resensitization during PTX treatment. However, this was not the case because DPDPE remained unable to lower the intracellular cAMP level after long-term agonist and PTX treatment. Moreover, only $G\alpha_{i2}$ and neither $G\alpha_{i3}$ nor $G\alpha_o$ directly interacts with DOR tightly. Recent studies on rhodopsin suggested that there is a direct and tight interaction between rhodopsin dimers and G_t (Fotiadis et al., 2003; Liang et al., 2003). Hence, the model that $G\alpha_{i2}$ plays a crucial role connecting the opioid receptors and downstream adenylyl cyclase activity could offer a plausible explanation for our current studies. Further investigation will clearly be needed to elucidate the relationship of DOR, $G\alpha_{i2}$, and downstream functional molecules.

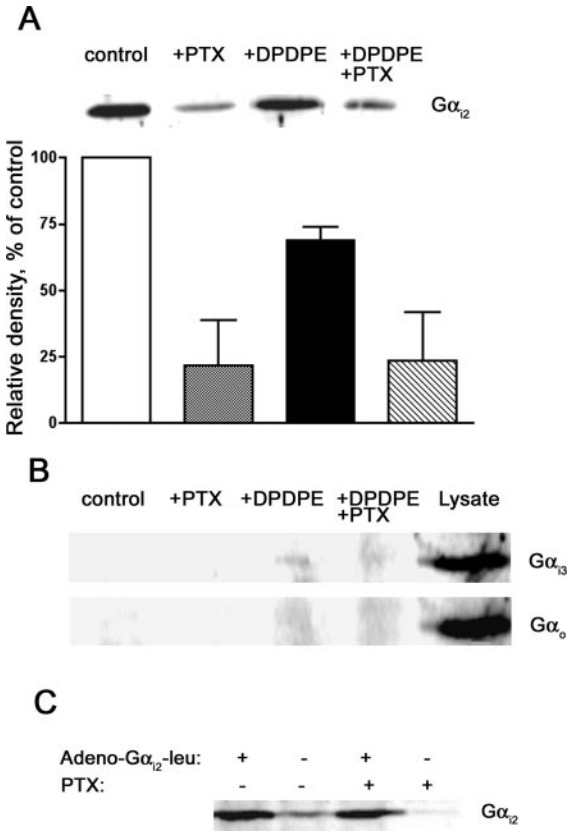

Fig. 7. Interaction of δ-opioid receptor and $G\alpha_{i2}$ subunit in vivo. N_2A cells were treated with saline (control), with 100 ng/ml PTX for 6 h (+PTX), with 1 μM DPDPE for 18 h (+DPDPE), or with DPDPE for 12 h and then PTX for another 6 h (+DPDPE+PTX). Afterward, cells from one T-75 cm^2 flask were harvested and immunoprecipitations were carried out as described under *Materials and Methods*. DOR was immunoprecipitated with the mouse monoclonal anti-HA antibody (Covance; 1:200), and the presence of $G\alpha_{i2}$ was determined with Western analysis using the polyclonal anti-$G\alpha_{i2}$ antibody (1:1000). A, top, $G\alpha_{i2}$ can be coimmunoprecipitated with DOR in control, PTX, DPDPE, and DPDPE+PTX treatment. Bottom, quantification (n = 2) of the relative band density shown in A, top, with the density of control bands designated as 100%. The white, dotted, black, and shaded bars represent the relative densities of control, PTX treatment, DPDPE treatment, and DPDPE with PTX treatment, respectively. B, $G\alpha_{i3}$ (top) and $G\alpha_o$ (bottom) cannot be coimmunoprecipitated with DOR in control, PTX, DPDPE, and DPDPE+PTX treatment. The cell lysates are loaded on the right lanes as the positive control. C, the effect of expression of adeno-$G\alpha_{i2}$-leu in the amount of $G\alpha_{i2}$ interacting with DOR. N_2A cells were infected with the adeno-GFP (−) or with adeno-$G\alpha_{i2}$-leu (+) virus in a cell-to-virus ratio of 1:100. Subsequently, these cells were treated with (+) or without (−) 100 ng/ml of PTX for 6 h. Afterward, the cells from 1 T-75 cm^2 flask were processed, DOR was immunoprecipitated, and $G\alpha_{i2}$ was detected on the Western blot as described in A.

Although the current studies suggest the important role of $G\alpha_{i2}$ in DOR short- and long-term actions, it does not mean to rule out the possibility of participation of other signal pathways. For example, after activation of G proteins, $G\beta\gamma$ subunits are released. An indirect action of $G\beta\gamma$ with possible feedback regulatory functions, such as ERK/MAPK is possible. In support of this, Raf-1, a protein kinase in the MAPK signaling cascade, has been shown to play a role in DOR-mediated superactivation (Varga et al., 2002). However, other studies using MAPK inhibitors could not block AC superactivation after long-term agonist treatment (Tso and Wong, 2001). In addition, $G\beta\gamma$ could activate specific isozymes of adenylyl cyclase in the presence of Gs α-subunit, such as type II (Weitmann et al., 2001) and IV (Debernardi et al., 1993). AC superactivation has been shown to be isozyme-specific, with the ability of long-term opioid treatment to superactivate the AC type I, V, VI, and VIII but not the type II, III, IV, and VII (Avidor-Reiss et al., 1996, 1997; Ammer and Christ, 2002). In N_2A cells, the expression of type II, III, IV, VI, and VII adenylyl cyclase is detected by RT-PCR (data not shown). These data, together with the observation that opioid agonist could not increase [^{35}S]GTPγS binding after long-term treatment (Eisinger et al., 2002), suggest that the superactivation of adenylyl cyclase could not be directly related to the release of $G\beta\gamma$ from $G\alpha$. It also indicates the possibility that either $G\alpha$ or $G\beta\gamma$ interacts with the cellular proteins, which can modulate the AC superactivation.

Instead of serving as a signaling molecule, $G\alpha_{i2}$ could serve as a scaffold molecule for proteins that could affect AC superactivation. Chakrabarti et al. (1998a,b) reported the phosphorylation of adenylyl cyclase during long-term morphine treatment. Whether AC superactivation is the result of such phosphorylation has not been demonstrated. Nevertheless, it is attractive to suggest that within the DOR-$G\alpha_{i2}$ complex, protein kinases mediate the observed AC superactivation. However, $G\alpha_{i2}$ could also serve as scaffold for other cellular proteins, such as the regulator of G protein signaling (RGS). RGS proteins act as GTPase-activating proteins to increase the rate of GTP hydrolysis by the $G\alpha$ subunit and decrease the lifetime of the active $G\alpha$-GTP and free $G\beta\gamma$ subunits (De Vries et al., 2000; Hepler, 2003). Multiple RGS proteins have been shown to negatively regulate G protein-mediated opioid signaling and facilitate opioid receptor desensitization and internalization (Potenza et al., 1999; Garzon et al., 2001; Clark et al., 2003). Recently, studies using RGS9 knockout mice suggested that RGS9 is a potential negative modulator of opiate action in vivo, and opiate-induced changes in RGS9 level contribute to the behavioral and neural plasticity associated with long-term opiate administration (Zachariou et al., 2003). Thus, the observed AC superactivation could be the consequence of $G\alpha_{i2}$ recruiting specific RGS [for example, RGS4 (Cavalli et al., 2000)] to the vicinity of DOR, or the DOR receptor and $G\alpha_{i2}$ together decide the selectivity of specific RGS (Hepler, 2003; Roy et al., 2003).

In conclusion, the present studies suggest that, in N_2A cells, only $G\alpha_{i2}$ is required for both DPDPE-induced short-term inhibition and superactivation of adenylyl cyclase after long-term agonist treatment. Because agonist could not activate G proteins after long-term agonist treatment, as indicated by GTP binding studies, $G\alpha_{i2}$ has a dual functional role in two different opioid receptor signaling events resulting in opposing changes in adenylyl cyclase activities.

Acknowledgments

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