Short- and Long-Term Regulation of Adenylyl Cyclase Activity by δ-Opioid Receptor Are Mediated by Gαi2 in Neuroblastoma N2A Cells

Lei Zhang, Joan Tetrault, Wei Wang, Horace H. Loh, and Ping-Yee Law

Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, Minnesota

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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 Gα0 that are expressed in neuroblastoma Neuro2A cells (Gαi2, Gαi3, and Gαo), we examined the identities of the G proteins involved in the short- and long-term action of the δ-opioid receptor (DOR). Pertussis toxin treatment completely abolished the ability of [d-Pen2,d-Pen5]-enkephalin (DPDPE) to inhibit forskolin-stimulated intracellular cAMP production. Expression of the C352L mutant of Gαi2, and not the C351L mutants of Gαi3 or Gαo, rescued the short-term effect of DPDPE after pertussis toxin treatment. The ability of Gαi2 in mediating DOR inhibition of adenylyl cyclase activity was also reflected in the ability of Gαi2, not Gαi3 or Gαo, 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 Gαi2 restored the adenylyl cyclase superactivation after pertussis toxin treatment. More importantly, the C352L mutant of Gαi2 remained associated with DOR after long-term agonist and pertussis toxin treatment whereas the wild-type Gαi2 did not. These data suggest that Gαi2 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 Gα0 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 Ca2+ 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 Gα0 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 Gα0 protein α subunits (Gαi1, Gαi2, Gαo,i3, and Gαo) 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 Gαi2 (Tso and Wong, 2000b), Gαo,i2 (Tso and Wong, 2000a), Gαi3, or Gαo,i3 (Tso and Wong, 2001) individually. On the other hand,
G_{oA} 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_{i2}, G_{i3} subunit mutant to neuroblastoma Neuro2A cells (N2A) stably expressing 8- opioid receptor (DOR). It can be demonstrated that only the G_{i2} and not G_{i3} or G_{oA} mutant could rescue the DPDPE induced inhibition of adenyllylcyclase activity after PTX pretreatment. Coincidentally, after long-term DPDPE treatment, only the G_{i2} mutant could restore AC superactivation after PTX treatment. More importantly, only G_{i2} and not G_{i3} or G_{oA} communoprecipitated with DOR. Therefore, the same G_{i2} mediates both short-term inhibition and long-term superactivation of adenyllylcyclase activity.

**Materials and Methods**

**Mutagenesis of PTX-Resistant G_{oA}.** 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_{oA} α-subunit with leucine (Leu) resulted in full efficacy of the mutant to transduce receptor signal (Bahia et al., 1998). Hence, the Cys891 of G_{oA} and Cys942 of G_{oB} of G_{oA} was mutated to Leu. Point mutation primers were designed as follows: for G_{oA}, 5′-GAAACACTGTAAGGGACCTGCTGGCTTCCTGG-3′; for G_{oB}, 5′-CAACTTAAGGAGCTCGGGCTTTACTGAGAG-3′; and for G_{i1} and G_{i3}, 5′-CAAATCTCCGGGGCCTAGGCTTGTACTGACC-3′.

**Adenovirus Construction.** G_{i2}, G_{i3}, or G_{oA} mutant was cloned into pShuttle-CMV vector (Stratagene) following the manufacturer's protocol. In brief, the recombinant plasmids were cotransfected with pAdEasy-1 into BJ 5183 cells by electroporation. The recombinant plasmids were then sequenced, and 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-time 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 cm² 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 cm² flask of N2A 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 x 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 added to a final 50-µl scale. The PCR conditions were as follows: 2 µl of RT reaction solution and 2 units of Taq polymerase (QIAGEN) 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_{i2} and G_{i3,5}), 30 s at 72°C for 25 cycles (35 cycles for G_{oA} and G_{oB}), and 10 min at 72°C. The PCR primers for each specific Gα subunit were designed as follows: G_{i2}: forward, ATGGAAAGGTATGCAGAGAAGA; reverse, GCTGTTCTTCCTGGGTGCAGA; G_{i3,5}: forward, GCAGAACCTGAGCAAGAGGCA; reverse, GCTGTTCTGGGTAGGCTGC; G_{oA}: forward, GCCCTGTTCCCGAGCTGATGGA; reverse, GTTGTAGCTTGGGTACAGCA; G_{oB}: forward, GCCCTGTTCCCGAGCTGATGGA; reverse, GCTGTTCTTGGGTACAGCA; G_{i2}: forward, GCTGTTCTTGAGCTGAGTC; reverse, GCTGTTCTTGAGCTGAGTC; G_{i3,5}: forward, GCTGTTCTTGAGCTGAGTC; reverse, GCTGTTCTTGAGCTGAGTC; G_{oA}: forward, AATGGAATGAGATGTGCACTGG; reverse, GCTGTTCTTGAGCTGAGTC; G_{oB}: forward, AATGGAATGAGATGTGCACTGG; reverse, GCTGTTCTTGAGCTGAGTC; G_{i2}: forward, CCCCTGTTCCCGAGCTGATGGA; reverse, CATGACAGAATCCCTGAGA.
Expression Pattern of Different \( \alpha \)-Subunits in N2A Cells. Before our investigation on the identities of \( \alpha \) proteins involved in the short- and long-term action of DOR, the expression patterns of the \( \alpha \)-subunits in N2A 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 \( \alpha_{1} \), \( \alpha_{2} \), and \( \alpha_{3} \) were detected in N2A cells. Even under much less stringent conditions (53°C annealing temperature and 35 cycles, compared with 60°C annealing temperature and 25 cycles for \( \alpha_{1} \), \( \alpha_{2} \), and \( \alpha_{3} \)) \( \alpha_{1} \) and \( \alpha_{3} \) were not detected. The inability to detect these \( \alpha \)-subunits in N2A was not due to the primer sequences, because both \( \alpha_{1} \) and \( \alpha_{3} \) were detected with mouse brain RNAs using the same PCR conditions and primers (data not shown). Quantification of the RT-PCR results suggested that \( \alpha_{2} \) and \( \alpha_{3} \) were the most abundant subunits and that \( \alpha_{0} \) was expressed in a much lower level. Similar expression patterns for \( \alpha_{2} \), \( \alpha_{3} \), and \( \alpha_{5} \) proteins could be detected by Western blotting analysis (Fig. 2B, control). Thus, in subsequent studies, only the PTX-insensitive mutants of \( \alpha_{2} \), \( \alpha_{3} \), and \( \alpha_{5} \) were used to investigate their abilities to restore DOR functions after PTX treatment.

Expression of PTX-Resistant Adeno-Ga\( \alpha_{2} \)-Leu, Adeno-Ga\( \alpha_{3} \)-Leu, and Adeno-Ga\( \alpha_{5} \)-Leu Mutants in N2A Cells. To determine the efficiency of adenosivirus infection of N2A cells, various cell–adeno-GFP virus ratios were used to infect the N2A 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 adenosivirus

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**Fig. 1.** RT-PCR analysis showing the expression of \( \alpha_{1} \), \( \alpha_{2} \), \( \alpha_{3} \), \( \alpha_{5} \), and \( \alpha_{0} \) in N2A cells. The relative transcript levels of various \( \alpha \)-subunits were determined by RT-PCR as described under Materials and Methods. Ten microliters of each PCR reaction (50 \( \mu \)l) was resolved on 1.5% NuSieve CTG agarose gel (Intermontain Scientific/BioExpress, Kaysville, UT) run in Tris-borate/EDTA buffer. The positions of molecular markers are indicated on the left side.

**Fig. 2.** Expression of PTX-resistant adeno-Ga\( \alpha_{2} \)-leu, adeno-Ga\( \alpha_{3} \)-leu, and adeno-Ga\( \alpha_{5} \)-leu mutants in N2A cells. A, adenosivirus infection efficiency of N2A cells. N2A cells were cultured on six-well plates to 70% confluence and were infected with adeno-GFP; on the right, cells were infected with individual mutants expressing PTX-resistant adenosivirus proteins. B, Western analysis of adeno-Ga\( \alpha_{2} \)-leu, adeno-Ga\( \alpha_{3} \)-leu, and adeno-Ga\( \alpha_{5} \)-leu mutants expressed in N2A cells. Similar to the studies summarized in A, N2A 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 \( \mu \)g of cell membrane extracts was loaded. The Ga\( \alpha \) bands were probed by specific anti-Ga\( \alpha_{1} \), Ga\( \alpha_{2} \), or Ga\( \alpha_{3} \) antibodies (1:1000). C, the relative density of each Ga\( \alpha \) mutant subunits expressed in N2A cells. The relative density of the \( \alpha \) in N2A 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-Ga\( \alpha_{2} \)-leu, adeno-Ga\( \alpha_{3} \)-leu, and adeno-Ga\( \alpha_{5} \)-leu mutants, respectively, compared with the control (\( \alpha \) = 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 Gα22, Gα23, and Gαo were overexpressed in N2A cells. The Cys^{351} of Gα23 and Gαo and the Cys^{352} of Gα22 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 Gα22 mutants were confirmed by Western blotting using specific antibodies for Gα22, Gα23, and Gαo (Fig. 2B).

Cells infected with the adeno-Gα22-leu mutant showed a 353.0 ± 82.9% increase in Gα22 protein compared with control cells that were infected with adeno-GFP (Fig. 2C). Likewise, cells infected with adeno-Gα23-leu or adeno-Gαo-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 Adenyl Cyclase Activity.** To determine the identity of the G protein involved in DOR regulation of AC activity in N2A cells, the ability of the PTX-insensitive Gα mutants to restore DPDPE inhibition was investigated. As summarized in Fig. 3 and Table 1, overexpression of the PTX-insensitive Gα mutants affected neither the potency nor the maximal inhibition level of DPDPE. From the DPDPE concentration-dependent inhibition of forskolin-stimulated cAMP production in N2A cell studies, the maximum inhibition levels were calculated to be 63 ± 2.7% (n = 4) for cells infected with GFP-adenovirus, and in Gα23 mutant-expressing cells, 61 ± 2.4% (n = 4) for cells infected with adeno-Gα22-leu, 62 ± 2.6% (n = 4) for cells infected with adeno-Gα23-leu, and 64 ± 2.5% (n = 4) for cells infected with adeno-Gαo-leu.

PTX-pre-treatment totally abolished the DPDPE-induced inhibition effect on cAMP accumulation in control cells, adeno-Gα22-leu–, and adeno-Gα23-leu– expressing cells. It is noteworthy that the inhibition effect of CAMP accumulation can still be observed after PTX-pre-treatment only in adeno-Gα22-leu mutant expressing cells (41 ± 3.1%, n = 4; Fig. 3B). The potency of DPDPE in N2A cells overexpressing the adeno-Gα22-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 Gα mutants to restore the DPDPE inhibition of AC activity in N2A cells could not be caused by low expression of these Gα mutants, because a similar level of increase in the adeno-Gα22-leu, compared with that of adeno-Gα23-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 Gα23 mutant-expressing cells, 53 ± 2.8% and 58 ± 3.0% (n = 2), respectively, for cells infected with adeno-Gα22-leu, 55 ± 1.4% and 55 ± 6.0% (n = 2), respectively, for cells infected with adeno-Gα23-leu, and 59 ± 1.6% and 55 ± 3.7% (n = 2), respectively, for cells infected with adeno-Gαo-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-Gα23-leu–, and adeno-Gαo-leu–expressing cells. But the DPDPE inhibition effect of cAMP accumulation can still be observed after PTX pretreatment only in adeno-Gα22-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 Gα22 mutants affected neither the potency nor 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 Gα23 mutant-expressing cells, 53 ± 2.8% and 58 ± 3.0% (n = 2), respectively, for cells infected with adeno-Gα22-leu, 55 ± 1.4% and 55 ± 6.0% (n = 2), respectively, for cells infected with adeno-Gα23-leu, and 59 ± 1.6% and 55 ± 3.7% (n = 2), respectively, for cells infected with adeno-Gαo-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-Gα23-leu–, and adeno-Gαo-leu–expressing cells. But the DPDPE inhibition effect of cAMP accumulation can still be observed after PTX pretreatment only in adeno-Gα22-leu mutant expressing cells (55 ± 3.0% and 61 ± 4.8%, n = 2, in NG108-15 and NIE115 cells, respectively).
adeno-Gαo-leu mutant after PTX pretreatment (1.7 \pm 0.6 and 1.5 \pm 0.3 \text{nM} for NG108-15 and NIE115 cells, respectively) was not significantly different from those of control cells without PTX treatment (0.7 \pm 0.2 and 0.6 \pm 0.1 \text{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 Gαo subunit in N2A 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 N2A 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 Gβγ (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 Gβγ proteins after long-term agonist treatment. Hence, the PTX-insensitive Gβγ 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, N2A cells were infected with the Gα mutants first, and were subjected to long-term treatment with 1 \text{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 Gβγ proteins from the receptor. Treating the N2A cells according to this paradigm has resulted in a complete uncoupling of Gβγ proteins from DOR as reflected in the absence of agonist high-affinity binding states (Law et al., 1991; Chakraborti et al., 1997).

When N2A cells were treated with 1 \text{μM DPDPE} for 18 h, complete receptor desensitization was observed. After long-term treatment, 1 \text{μM DPDPE} elicited 4.8 \pm 2.2\% inhibition of forskolin-stimulated cAMP production compared with 64.3 \pm 2.3\% in cells not subjected to long-term treatment with the agonist. Similar levels of receptor desensitization were observed in N2A cells infected either with adeno-Gαo, adeno-Gαo-leu, or adeno-Gαo. 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 N2A cells infected with various adenoviruses and treated with DPDPE. There was no statistically significant difference in the maximal level of AC superactivation in N2A cells infected either with the adeno-GFP

![Fig. 4. Gαo 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-Gαo-leu, adeno-Gαo-leu, and adeno-Gαo-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 \text{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.](molpharm.aspetjournals.org)
virus (215 ± 12%, n = 4), adeno-Gαi2-leu virus (204 ± 14% n = 4), adeno-Gαi3-leu virus (200 ± 7%, n = 4), or adeno-Gαo-leu virus (230 ± 11%, n = 4). There was also no dramatic difference in the concentration of naloxone to induce 50% of maximal level (EC50) of AC superactivation in the N2A cells infected with these viruses (Table 2). When the N2A cells infected either with adeno-GFP, adeno-Gαi2-leu, or adeno-Gαo-leu were treated with PTX after long-term DPDPE treatment, AC superactivation observed in the presence of antagonist was completely eliminated (Fig. 5A, C, and D). Coincident with the short-term treatment experiment results, only the cells expressing with adeno-Gαi2-leu showed superactivation after PTX treatment. In these N2A 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-Gαi2-leu virus (316 ± 28% and 296 ± 2%, respectively, n = 2), adeno-Gαi3-leu virus (293 ± 16% and 252 ± 41%, respectively, n = 2), and adeno-Gαo-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 (EC50) 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-Gαi2-leu, adeno-Gαi3-leu, or adeno-Gαo-leu virus, respectively). Coincident with the result from N2A cells, only the cells expressing with adeno-Gαi2-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 Gαi2 after long-term DPDPE treatment, the same Gαi2 protein was responsible for the expression of AC superactivation.

**Coimmunoprecipitation of DOR and Gαi2 Subunits.** After long-term agonist treatment, the activation of the Gαo proteins by DOR as reflected by [35S]GTPγS binding was completely abolished (Eisinger et al., 2002). Because the α-subunits of Gαo proteins are not known to activate AC activity, the observed AC superactivation in N2A cells infected with adeno-Gαi2-leu after PTX treatment could be attributed to the release of the Gβγ 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 Gβγ subunits in this cellular response (Nevo et al., 2000). The Gαi2 could probably serve as a scaffold to recruit cellular proteins involved in AC superactivation. If this was the case, then Gαi2 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, Gαi2 was coimmunoprecipitated with DOR. Western blotting analyses of similar immunoprecipitates with Gαi2 and Gαo 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 Gαi2. Thus, only Gαi2 formed a tight complex with DOR that could be detected with the immunoprecipitation studies. PTX treatment lowered the amount of

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**Fig. 5.** Gαi2 mutant restored the adenylyl cyclase superactivation after long-term treatment with DPDPE followed by PTX treatment. N2A cells were infected with adeno-GFP (A), adeno-Gαi2-leu (B), adeno-Gαi3-leu (C), and adeno-Gαo-leu (D) mutants in a cell-to-virus ratio of 1:100. These cells were treated with 1 μM DPDPE for 18 h (C) or with 1 μM DPDPE for 12 h followed by the addition of PTX (100 ng/ml) for 6 h (D). 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 $G_{i2}$ proteins to 22\% ($n = 2$) compared with the control (designated as 100\%), whereas the amount of precipitated $G_{i2}$ proteins after long-term 1 $\mu$M DPDPE treatment was 69\% ($n = 2$). Treating the cells with 1 $\mu$M DPDPE and PTX also lowered the amount of $G_{i2}$ proteins coimmunoprecipitated with DOR to 24\% ($n = 2$). Again, as in the case with control cells, $G_{i3}$ and $G_{z}$ could not be coimmunoprecipitated with DOR in the above drug and/or PTX treatments (Fig. 7B).

The overexpression of $G_{i2}$-leu in N2A cells infected with aden-o-$G_{i2}$-leu significantly increased the amount of $G_{i2}$ proteins coimmunoprecipitated with DOR (Fig. 7C). PTX pretreatment did not significantly reduce the amount of $G_{i2}$ coimmunoprecipitated in these cells. Although no attempt to distinguish between the wild-type and mutant $G_{i2}$ in the coimmunoprecipitated was made, because PTX treatment reduced the amount of wild-type $G_{i2}$ that was coimmunoprecipitated (Fig. 7A), it is reasonable to assume that most of the $G_{i2}$ coimmunoprecipitated with DOR reflects the PTX-insensitive mutant. Overexpression of $G_{i2}$-leu or $G_{z}$-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 $G_{i2}$.

**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 $G_{i3}$ 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 $G_{x}$, 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 (Abdo 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 Ga-specific antibodies suggest that $G_{i2}$ mediates DOR inhibition of AC in NG108-15 cells (McKenzie and Milligan, 1990), although the cross activity between $G_{i1}$ and $G_{i2}$ of the antibodies used did not eliminate the role of $G_{z}$ in this process. Using antisense oligodeoxynucleotides, it was shown that opioid-induced intracellular Ca$^{2+}$ mobilization in ND8-47 neuroblastoma × DRG hybrid cells is mediated by $G_{i2}$ (Tang et al., 1995). Another study using antisense oligodeoxynucleotides showed that $G_{i2}$ and $G_{x}$ antisense probes blocked spinal $\mu$-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 $G_{x}$ transcripts and partial

**TABLE 2**

The ability of various $G_{x}$, $\alpha$-subunit mutants to restore the superactivation of adenylyl cyclase activity after chronic DPDPE and PTX treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$t_{50}$ (nM)</th>
<th>+ PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno-GFP (Control)</td>
<td>2.0 ± 0.1 (215 ± 12)</td>
<td>N.E.</td>
</tr>
<tr>
<td>+ Adeno-$G_{i2}$-Leu</td>
<td>1.2 ± 0.2 (204 ± 14)</td>
<td>2.5 ± 0.2 (155 ± 6)</td>
</tr>
<tr>
<td>+ Adeno-$G_{i3}$-Leu</td>
<td>0.4 ± 0.1 (200 ± 7)</td>
<td>N.E.</td>
</tr>
<tr>
<td>+ Adeno-$G_{z}$-Leu</td>
<td>0.9 ± 0.1 (230 ± 11)</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

N.E., no excitation when naltrexone = $10^{-4}$ M.

**Fig. 6.** In NG108-15 (A) and NIE115 (B) cells, $G_{i2}$ mutant restored the adenylyl cyclase superactivation after long-term treatment with DPDPE followed by PTX treatment. Cells were infected with aden-o-GFP, aden-o-$G_{i2}$-leu, aden-o-$G_{i3}$-leu, and aden-o-$G_{z}$-leu mutants in a cell-to-virus ratio of 1:100. These cells were treated with 1 $\mu$M DPDPE for 18 h (□) or with 1 $\mu$M 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 naltrexone as described under Materials and Methods. The amount of cAMP produced in the presence of naltrexone was compared with that observed in the presence of 1 $\mu$M 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 Gα 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 Gα12 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 Gαo (Tso and Wong, 2000b), Gα12 (Tso and Wong, 2000a), Gα11, or Gαi1 (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, N2A cells were infected with individual genetically engineered PTX-resistant G proteins (Gα12, Gα13, and Gαi2) and were treated by DPDPE in the short or long term (18 h), then PTX was used to down-regulate all the endogenous Gα subunits. We could demonstrate that under this paradigm, before the PTX treatment, the DOR receptors in N2A 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 Gα12 by DOR receptors mediated both inhibition and superactivation of adenylyl cyclase activity. Expression of the Gα13 or Gαi2 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 Gα12 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 Gα12 and neither Gαi1 nor Gαo directly interacts with DOR tightly. Recent studies on rhodopsin suggested that there is a direct and tight interaction between rhodopsin dimers and Gαi (Fotiadis et al., 2003; Liang et al., 2003). Hence, the model that Gα12 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, Gα12, and downstream functional molecules.

Although the current studies suggest the important role of Gα12 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, Gβγ subunits are released. An indirect action of Gβγ with possible feedback regulatory functions, such as ERK/MAPK is possible. In support of this, Raf-1, a protein kinase in the MAPK feedback regulatory functions, is a possible target connecting the opioid receptors and downstream adenylyl cyclase activity. This could offer a plausible explanation for our current studies. Further investigation will certainly be needed to elucidate the relationship of DOR, Gα12, and downstream functional molecules.
Instead of serving as a signaling molecule, G_{\alpha}Q 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}Q complex, protein kinases mediate the observed AC superactivation. However, G_{\alpha}Q 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 Go-GTP and free G_{\beta} 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 opioid action in vivo, and opioid-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}Q recruiting specific RGS [for example, RGS4 (Cavalli et al., 2000)] to the vicinity of DOR, or the DOR receptor and G_{\alpha}Q together decide the selectivity of specific RGS (Hepler, 2003; Roy et al., 2003).

In conclusion, the present studies suggest that in N_{2}A cells, only G_{\alpha}Q is required for both DPDPE-induced short-term inhibition and superactivation of adenylyl cyclase after long-agonist treatment. Because agonist could not activate G proteins after long-agonist treatment, as indicated by GTP binding studies, G_{\alpha}Q has a dual functional role in two different opioid receptor signaling events resulting in opposite changes in adenylyl cyclase activities.

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In conclusion, the present studies suggest that in N_{2}A cells, only G_{\alpha}Q is required for both DPDPE-induced short-term inhibition and superactivation of adenylyl cyclase after long-agonist treatment. Because agonist could not activate G proteins after long-agonist treatment, as indicated by GTP binding studies, G_{\alpha}Q has a dual functional role in two different opioid receptor signaling events resulting in opposite changes in adenylyl cyclase activities.
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Address correspondence to: Lei Zhang, Department of Pharmacology, Medical School, University of Minnesota, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455. E-mail: zhang247@umn.edu