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G_z Can Mediate the Acute Actions of μ- and κ-Opioids but Is Not Involved in Opioid-Induced Adenylyl Cyclase Supersensitization[1]

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ABSTRACT

The three subtypes of opioid receptors (δ, μ, and κ) are known to regulate multiple effectors through either pertussis toxin-sensitive or -insensitive G proteins. In opioid-induced inhibition of adenylyl cyclase, both G_i and G_z proteins can serve as the signal transducer. Our previous study showed that opioid-induced adenylyl cyclase supersensitization in human embryonic kidney (HEK) 293 cells expressing the δ-opioid receptor requires G_i but not G_z proteins. Herein, we studied the ability of μ- and κ-opioid receptors to regulate the activities of adenylyl cyclase through G_z. In HEK 293 cells coexpressing G_z with the μ- or κ-opioid receptors, opioid agonists induced inhibition of adenylyl cyclase in a pertussis toxin-insensitive manner. However, adenylyl cyclase supersensitization induced by chronic opioid treatments remained sensitive to pertussis toxin. We also showed that the responsiveness of cAMP-dependent response element-binding proteins to forskolin was not altered after prolonged opioid treatment but was higher in cells coexpressing G_z. Although the μ- and κ-opioid receptors mediated acute activation of extracellular signal-regulated protein kinase 1/2 via both G_i and G_z, these responses were abolished by chronic opioid treatment. These studies showed that G_z could mediate acute actions of μ- and κ-opioids but G_z alone was insufficient to mediate adenylyl cyclase supersensitization induced by the chronic activation of opioid receptors.

Opioids induce their biological effects by binding to three subtypes of opioid receptors (δ, μ, and κ). The opioid receptors are distributed throughout the central and peripheral nervous systems (Reisine and Bell, 1993). Although the three subtypes of opioid receptors share 60% amino acid sequence identity, their physiological functions are not identical. This is especially true for the μ- and κ-opioid receptors where recent studies suggest that they may even produce antagonistic effects (for review, see Pan, 1998). For example, κ-agonists can reverse the μ-opioid-induced inhibition of C-fiber-evoked responses in spinal horn neurons (Dickenson and Knox, 1987). At the cellular level, the opioid receptors interact with the G_i proteins to regulate a wide range of effectors, including ion channels and enzymes (Connor and Christie, 1999). However, some of these effectors are differentially regulated by the opioid receptors.

Adenylyl cyclase (AC) is the typical effector of opioid receptors. Acute stimulation of opioid receptors leads to inhibition of AC activity. A compensatory increase in AC activity is often induced after chronic opioid treatment. This phenomenon is observed in a variety of cell types, including the simian kidney fibroblast COS-7, Chinese hamster ovary, and neuroblastoma × glioma hybrid NG 108-15 cells (Law et al., 1982; Avidor-Reiss et al., 1995, 1997). Downstream of AC, cAMP-dependent response element binding proteins (CREBs) also play an important role in the physiology of opioid abstinence. The development of physical dependence to opiates is significantly reduced by intralocus ceruleus infusion of CREB antisense oligonucleotide (Lane-Ladd et al., 1997) or mutation in the gene encoding CREB (Maldonado et al., 1996). Apart from the activities of AC and CREB, extracellular signal-regulated protein kinases (ERK1/2) are involved in opiate addiction (Lane-Ladd et al., 1997). Activation of all three types of opioid receptors leads to ERK1/2 phosphorylation through the G_i-released $\beta\gamma$-complex (Burt et al., 1996), and the ERK1/2 activity has been shown to be involved in manifesting opioid addiction. For instance, chronic opioid administration can lead to a sustained increase in ERK1/2 phosphorylation in the rat ventral tegmental area (Berhow et al., 1996). Besides being regulated by the $\beta\gamma$-complex, the

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ABBREVIATIONS: AC, adenylyl cyclase; CREB, cAMP-dependent response element-binding protein; ERK, extracellular signal-regulated protein kinase; PTX, pertussis toxin; HEK, human embryonic kidney; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; DTT, dithiothreitol.

ERK1/2 activity can be modulated by cAMP (Faure et al., 1994). The compensatory increase in cAMP after opioid removal provides a possible link to the activation of ERK1/2. Both opioid receptor-regulated AC and ERK1/2 activities are completely abolished by pertussis toxin (PTX), indicating the involvement of G_i proteins.

Numerous studies have revealed that opioid receptors can use G_z to regulate AC (Chan et al., 1995; Lai et al., 1995; Tsu et al., 1995) and ERK1/2 activities (Tso et al., 2000). The δ-opioid receptors expressed in human embryonic kidney (HEK) 293 cells are capable of using G_i and G_z proteins to inhibit AC and to activate ERK1/2 phosphorylation. Chronic stimulation of the δ-opioid receptors leads to AC supersensitization in HEK 293 cells coexpressing G_z, but PTX pretreatment completely abolishes AC supersensitization. Given that the μ- and κ-opioids may produce opposing effects (for review, see Pan, 1998), we explored possible differences in the regulatory actions of the μ- and κ-opioid receptors through G_i or G_z on AC, ERK1/2, and CREB. Our results demonstrated that acute activation of the μ- or κ-opioid receptors in HEK 293 cells coexpressing G_z resulted in inhibition of AC and induction of ERK1/2 phosphorylation in a PTX-insensitive manner. Chronic activation of the μ- or κ-opioid receptors in the cells expressing G_z resulted in AC supersensitization, but it was completely blocked by PTX treatment, indicating that G_z was not required for the chronic action of the μ- and κ-opioid receptors on AC. Collectively, these results revealed that there was no difference in the regulatory actions of the δ-, μ-, and κ-opioid receptors on AC and ERK1/2, and that $G_{i/o}$ proteins were required to mediate AC supersensitization.

Experimental Procedures

Materials. The rat μ- and κ-opioid receptor cDNAs were kindly provided by Dr. L. Yu (University of Cincinnati College of Medicine, Cincinnati, OH) and Dr. M. Satoh (Kyoto University, Kyoto, Japan), respectively. The rat G_z cDNA was a generous gift from Dr. Y. Kaziro (Tokyo Institute of Technology, Yokohama, Japan). HEK 293 cells were obtained from the American Type Culture Collection (CRL-1573; Manassas, VA). PTX and forskolin were purchased from List Biological Laboratories (Campbell, CA) and Research Biochemicals International (Natick, MA), respectively. [^{3}H]Adenine was obtained from Amersham (Buckinghamshire, UK). [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO) (63 Ci/mmol) and [^{3}H]U-69,593 (65 Ci/mmol) were from DuPont-NEN (Boston, MA). Plasmid purification columns were purchased from Qiagen Inc. (Hilden, Germany). $G\alpha_z$-specific antiserum sc-388 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$G\alpha_o$ (GC/2) anti-$G\alpha_{i1\text{-}2}$ (AS/7) antisera were purchased from Biotechnology Systems NEN Research Products (Boston, MA). p44/42 MAP kinase and phospho-p44/42 (Thr^{202}/Tyr^{204}) mitogen-activated protein kinase (MAPK) antibodies as well as CREB and phospho-CREB (Ser^{133}) antibodies were purchased from New England Biolabs (Beverly, MA). Cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Establishment of Stable Cell Lines. HEK 293 cells were grown in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (v/v), 50 U/ml penicillin, and 50 μg/ml streptomycin, in 5% CO_2. 293/MOR and 293/KOR cells expressing the μ- and κ-opioid receptor, respectively, were established by retroviral infection and selection under 500 μg/ml G418 as described previously (Chiu et al., 1996). The cDNA encoding rat $G\alpha_z$ in pcDNA1 was subcloned into pcDNA3.1 Zeo(+) as an *Eco*RI cassette. 293/MOR or 293/KOR cells were seeded at a density of 2.5×10^5 cells/10-cm plate 1 day before transfection. Qiagen-purified $G\alpha_z$/pcDNA3.1 Zeo(+) (35 μg) was transfected into 293/MOR or 293/KOR cells by standard calcium phosphate precipitation. Subsequently, stable 293/MOR-Z and 293/KOR-Z cell lines were selected and maintained with MEM containing 200 μg/ml zeocin.

cAMP Accumulation Assays. Cells were seeded onto 24-well plates at 5×10^4 cells/well in 0.5 ml of growth medium. One day later, cells were labeled with 0.5 ml of MEM containing 1 μCi of [^{3}H]adenine and 1% fetal calf serum (v/v) in the absence or presence of PTX (100 ng/ml) with or without opioid ligands (10 nM) as indicated. After 16 h of labeling, the cells were rinsed once with 0.5 ml of assay medium (MEM containing 20 mM HEPES, pH 7.4). The cells were then incubated at 37°C for 30 min with 1 ml of assay medium containing 1 mM 1-methyl-3-isobutylxanthine in the absence or presence of 50 μM forskolin with or without the respective opioid ligands (100 nM). Intracellular levels of [^{3}H]cAMP were subsequently measured as described previously (Wong et al., 1992). [^{3}H]cAMP was isolated by sequential chromatography and estimated by determining the ratios of [^{3}H]cAMP to total [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]cAMP pools. Absolute values for cAMP accumulation varied between experiments, but variability in any given experiment was normally less than 10%.

Membrane Preparation. Membranes were prepared from control or pretreated HEK 293, 293/MOR, 293/KOR, 293/MOR-Z, and 293/KOR-Z cells. Briefly, cells from five confluent 10-cm tissue culture dishes were harvested in PBS containing 1 mM EDTA. Cells were resuspended in lysis buffer [50 mM Tris-HCl containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 1 mM EGTA, and 1 mM dithiothreitol (DTT), pH 7.4] and lysed by one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle. After removal of nuclei by centrifugation, membranes were collected, washed, and resuspended in lysis buffer. Protein concentrations were determined by using the Bio-Rad Protein Assay kit. For each sample, 50 μg of membrane proteins was analyzed by Western blot analysis.

CREB and MAPK Assay. Each stable cell line was seeded onto 12-well plates at 10^5 cells/well in 1 ml of growth medium. One day later, for MAPK assay, the cells were treated with serum-free MEM with or without specific ligands (10 nM) in the presence or absence of PTX (100 ng/ml) for 24 h. For CREB assay, 1% fetal calf serum MEM was used instead of serum-free MEM. Subsequently, cells were washed with 1 ml of PBS and treated with 1 ml of serum-free MEM in the presence or absence of 50 μM forskolin or 100 nM specific ligands for 5 min. Reactions were terminated by adding 150 to 200 μl of ice-cold lysis buffer (50 mM Tris-HCL, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM NaP_2O_7, 1% Triton X-100, 1 mM DTT, 200 μM Na_3VO_4, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 4 μg/ml aprotinin). The supernatant of each lysate was obtained after spinning the lysate at 14,000 rpm for 8 min. Then 30 to 40 μl of 6× sample buffer was added to each lysate and 80 μl of each sample was analyzed by Western blot analysis. The intensities of immunoreactive signals were quantified by NIH Image 1.6.

Western Blot Analysis. Protein samples were separated on 12.5% polyacrylamide SDS gels and electrophoretically transferred to nitrocellulose membranes. Localization of protein markers on the nitrocellulose membrane was detected by Ponceau S staining. Immunodetection of $G\alpha_z$, $G\alpha_o$, $G\alpha_{i1,\ i2}$, CREB, and MAPK by the Gα-subunit specific-antisera sc-388, AS/7, GC/2, anti-CREB, and anti-p42/p44 MAPK, respectively, were visualized by chemiluminescence with the ECL kit from Amersham.

Receptor-Binding Assays. Membranes (30–50 μg) from transfected COS-7 cells were incubated for 1 h at 30°C with the appropriate labeled ligand at 0.01 to 15 nM concentration in a final volume of 500 ml of 50 mM Tris-HCl (pH 7.4), containing 1 mM benzamidine-HCl, 0.001% (w/v) soybean trypsin inhibitor, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM DTT, and 0.1% (w/v) BSA. Bound ligand was separated by filtration through Whatman GF/B glass filters with a PHD cell harvester (Cambridge Technology Inc., Watertown, MA), and

washed with 3 × 4 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM cold ligand. K_d and B_{max} values were calculated by Scatchard analysis.

Data Analysis. For the results of cAMP assay, data shown in each figure represent the mean ± S.D. from triplicate samples assayed in one experiment. Each experiment was repeated at least three times. The cAMP levels were interpreted as the ratios of counts per minute (cpm) of [^{3}H]cAMP fractions to those of the total labeled nucleotide fractions and expressed as [cAMP/total (×1000)]. All data were analyzed with paired Bonferroni t test for comparison of two independent sets of data. The level of significance was set at $P < .05$.

Results

Establishment of Stable 293/MOR and 293/KOR Cell Lines. Because the HEK 293 cells do not express the μ- or κ-opioid receptor, they are a suitable experimental platform for the study of the cloned μ- and κ-opioid receptors. Stable cell lines (293/MOR and 293/KOR) expressing the rat μ- and κ-opioid receptors were established by retroviral infection as described previously (Chiu et al., 1996). Binding of the μ-selective agonist [^{3}H]DAGO to membranes prepared from 293/MOR cells yielded a B_{max} of 673 ± 56 fmol/mg of protein (n = 3). Scatchard analysis with [^{3}H]U-69,593 revealed that the κ-opioid receptor was expressed at a similar level in 293/KOR cells (B_{max} = 758 ± 41 fmol/mg of protein). The K_d values of [^{3}H]DAGO (1.05 ± 0.17 nM) and [^{3}H]U-69,593 (5.18 ± 0.53 nM) for their respective receptors were comparable with those reported previously in other cell types (Raynor et al., 1994). Activation of the μ- and κ-opioid receptors with DAGO and U50,488 in 293/MOR and 293/KOR cells led to inhibition of AC (Fig. 1). [D-Pen^2,D-Pen^5]-enkephalin, a δ-selective opioid agonist was incapable of inhibiting the forskolin-stimulated cAMP accumulation in 293/MOR and 293/KOR cells (data not shown), but PTX treatment of the cells completely abolished DAGO- and U50,488-induced inhibition of AC (Fig. 1). These results indicate that the 293/MOR and 293/KOR cells expressed functional μ- and κ-opioid receptors that inhibited cAMP accumulation in a ligand-selective and PTX-sensitive manner.

Chronic Exposure of 293/MOR and 293/KOR Cells to Opioids Potentiate Forskolin Responses. To demonstrate the phenomenon of AC supersensitivity in 293/MOR and 293/KOR cells, forskolin-stimulated cAMP accumulation was measured after chronic exposure of the cells to specific opioid ligands. When 293/MOR and 293/KOR cells were treated with opioid agonists (10 nM) for 8 h or more, forskolin-induced cAMP accumulations were stimulated by about 4- to 5-fold (Fig. 2). Continuous exposure of these two cell lines to agonists for about 10 h maximized the compensatory stimulation of forskolin response. Parental HEK 293 cells did not respond to chronic opioid treatment (data not shown). To determine whether this compensatory increase in forskolin-stimulated cAMP accumulation was mediated by opioid receptors, the 293/MOR and 293/KOR cells were treated with various opioid agonists and antagonists for 24 h. None of the δ- or κ-selective opioid agonist was able to trigger an increase in responsiveness of 293/MOR cells to forskolin, whereas DAGO treatment significantly potentiated the forskolin response (Fig. 2). Likewise, only chronic U50,488 treatment induced the compensatory increase in forskolin response in

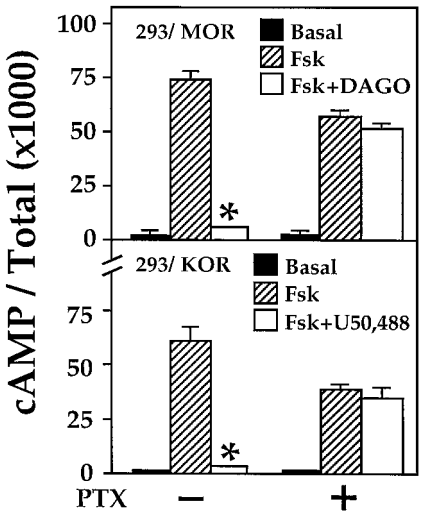

Fig. 1. Opioid-induced inhibition of forskolin-stimulated cAMP accumulation in 293/MOR and 293/KOR cells. 293/MOR and 293/KOR cells were labeled with [^{3}H]adenine (1 μCi/ml) with or without PTX (100 ng/ml) and then assayed for cAMP accumulation in presence of 50 μM forskolin (Fsk) with or without 100 nM opioid ligands as indicated. Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. *, forskolin response was significantly inhibited by opioid agonists; Bonferroni t test, $P < .05$.

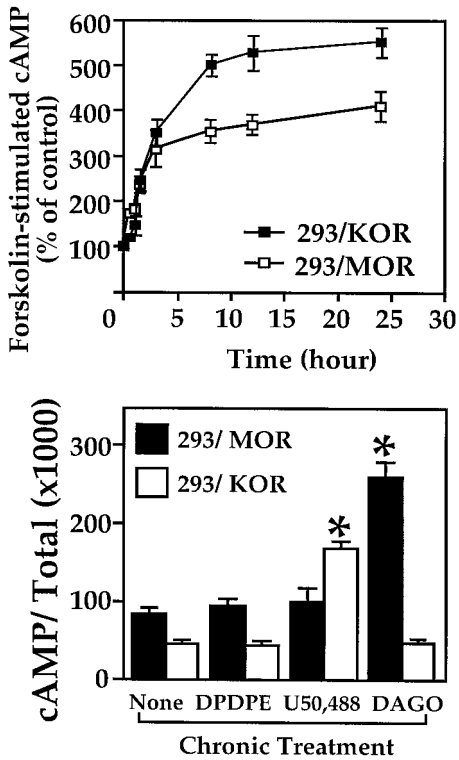

Fig. 2. Opioid-induced AC supersensitization in 293/MOR and 293/KOR cells. Top, 293/MOR and 293/KOR cells were labeled with 1 μCi of [^{3}H]adenine for 24 h and exposed to DAGO and U50,488 (10 nM), respectively, for different intervals as indicated. Treated cells were challenged with 50 μM forskolin and their responses were expressed as a percentage of that obtained with control cells (time zero). Bottom, 293/MOR and 293/KOR cells were labeled with 1 μCi of [^{3}H]adenine in the absence or presence of [D-Pen^2,D-Pen^5]-enkephalin, DAGO, or U50,488 (10 nM) for 24 h. The cells were subsequently stimulated with forskolin (50 μM). Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. *, forskolin-elevated cAMP accumulation was significantly increased compared with that obtained from cells without prolonged opioid treatment; Bonferroni t test, $P < .05$.

293/KOR cells, whereas δ- and μ-selective opioid agonists were ineffective (Fig. 2). In the 293/MOR and KOR/293 cells, pretreatment with PTX or a nonselective opiate antagonist, naloxone, completely abolished the enhancement on forskolin-induced cAMP accumulation (Fig. 3). These results suggest that the compensatory increase in AC sensitivity on chronic opioid treatment was mediated by μ- and κ-opioid receptors through the PTX-sensitive $G_{i/o}$ proteins. Interestingly, chronic opioid treatment of 293/MOR and 293/KOR cells did not affect the ability of the same agonist to inhibit the forskolin-stimulated cAMP accumulation (Fig. 3). Our results showed that the opioid-induced inhibition of forskolin-stimulated cAMP accumulation in pretreated 293/MOR and 293/KOR cells, respectively, exhibited maximal inhibitions of more than 80%.

G_z Alone Is Insufficient to Mediate Opioid-Induced AC Supersensitization. Because we have previously shown that G_z does not support the δ-opioid receptor-induced AC supersensitization (Tso et al., 2000), we asked whether the μ- and κ-opioid receptors can use G_z to mediate their chronic actions. $G\alpha_z$ was introduced into 293/MOR and 293/KOR cells by calcium phosphate transfection and selection in zeocin. Expression of $G\alpha_z$ in 293/MOR-Z and 293/KOR-Z cells was confirmed by Western blotting with the $G\alpha_z$-specific antiserum sc-388 (Fig. 4). Functional coupling of the μ- and κ-opioid receptors to G_z was demonstrated by the ability of DAGO and U50,488, respectively, to inhibit the forskolin-stimulated cAMP accumulation in the presence of PTX. The

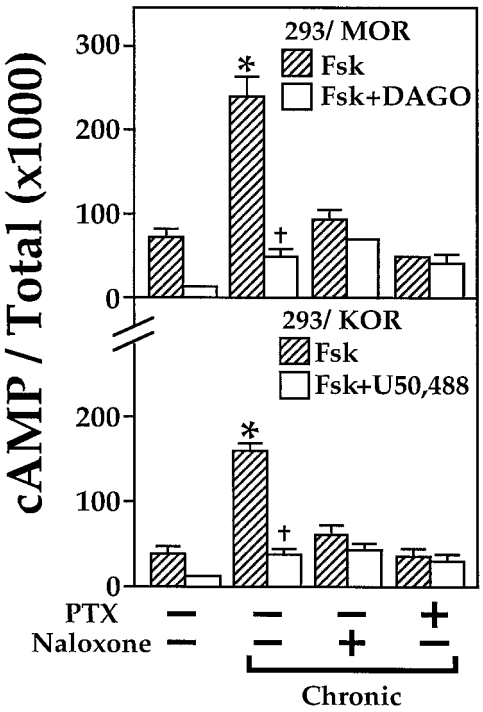

Fig. 3. Prevention of AC supersensitization by PTX and naloxone. 293/MOR and 293/KOR cells were labeled with 1 μCi of [^{3}H]adenine in the presence of DAGO or U50,488 with or without naloxone and PTX for 24 h. cAMP accumulation was determined in the presence of forskolin (Fsk) with or without opioid ligands (100 nM) as indicated. Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. *, forskolin-elevated cAMP accumulation was significantly increased compared with that obtained from cells without chronic treatment. †, acute opioid treatment significantly inhibited the forskolin-stimulated cAMP accumulation; Bonferroni t test, $P < .05$.

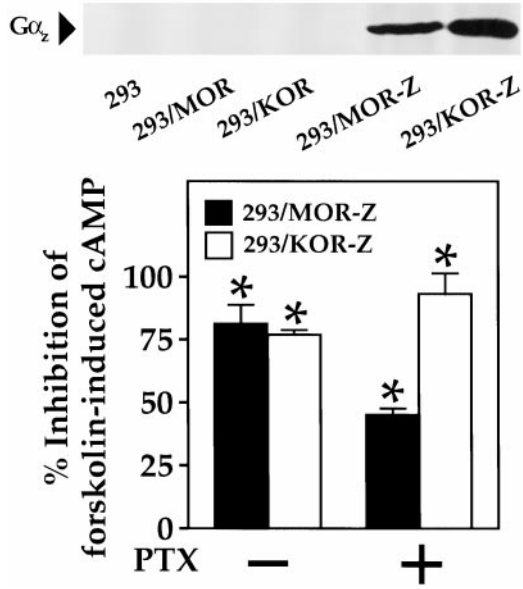

Fig. 4. AC regulation in 293/MOR-Z and 293/KOR-Z cells. 293/MOR-Z and 293/KOR-Z cells were labeled with 1 μCi of [^{3}H]adenine in the absence or presence of PTX for 24 h. The cells were then assayed for cAMP accumulation in the presence of 50 μM forskolin with or without 100 nM DAGO or U50,488. Results were expressed as percentage of inhibition of the forskolin-stimulated response. Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. *, agonists significantly inhibited the forskolin-stimulated cAMP accumulation; Bonferroni t test, $P < .05$. Expression of $G\alpha_z$ in 293/MOR-Z and 293/KOR-Z cells was determined by immunodetection with the $G\alpha_z$-specific antiserum sc-388. Membrane proteins (50 μg) prepared from HEK 293, 293/MOR, 293/KOR, 293/MOR-Z, and 293/KOR-Z cells were resolved on a 12.5% SDS gel, transferred to nitrocellulose membrane, and probed with sc-388. Two independent experiments with different batches of membrane proteins gave similar results.

response of MOR-Z cells to PTX differed from that of KOR-Z cells (Fig. 4). MOR-Z cells exhibited partial sensitivity to PTX, implying participation of G_i proteins, whereas KOR-Z cells exhibited no sensitivity to PTX. There appeared to be a shift toward G_z proteins when the κ-opioid receptors were expressed with G_z. These results indicate that both μ- and κ-opioid receptors were able to interact with G_z because they mediated inhibition of AC in the presence of PTX.

We then asked whether the μ- and κ-opioid receptors can use G_z to mediate AC supersensitization. The 293/MOR-Z and 293/KOR-Z cells were treated with 10 nM DAGO and U50,488, respectively, for 24 h in the absence or presence of PTX (100 ng/ml). Chronic exposure of 293/MOR-Z and 293/KOR-Z cells to specific opioids resulted in AC supersensitization (Fig. 5). Chronic opioid treatment enhanced the forskolin-mediated cAMP accumulation by about 4- and 1-fold in 293/MOR-Z and 293/KOR-Z cells, respectively (Fig. 5). The AC supersensitization was completely abolished by PTX pretreatment in both cell lines (Fig. 5). In 293/MOR-Z and 293/KOR-Z cells, the μ- and κ-opioid receptors were capable of interacting with G_z to inhibit AC activity after chronic opioid treatment because the inhibition of AC remained resistant to PTX (Fig. 5). These results clearly show that prolonged activation of G_z-regulated effectors was insufficient to trigger AC supersensitization.

Expressions of Gα-Subunits Were Not Altered by Prolonged Agonist Treatment. Because chronic opioid treatment has been shown to alter the expression level of $G_{i/o}$

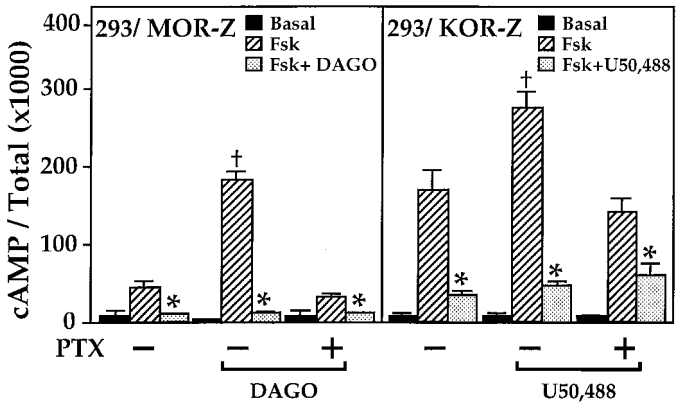

Fig. 5. PTX-sensitivity of AC supersensitization. 293/MOR-Z and 293/KOR-Z cells were labeled with 1 μCi of [^{3}H]adenine in the absence or presence of 10 nM DAGO and U50,488, respectively, with or without PTX for 24 h. Pretreated 293/MOR-Z and 293/KOR-Z cells were then assayed for cAMP accumulation in the absence (basal) or presence of forskolin (Fsk) with or without opioid agonists. DAGO (100 nM) and U50,488 (100 nM) were used for 293/MOR-Z and 293/KOR-Z cells, respectively. Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. *, agonists significantly inhibited the forskolin-stimulated cAMP accumulation. †, forskolin-stimulated cAMP accumulation was significantly enhanced by chronic opioid treatment compared with that obtained from untreated cells; Bonferroni t test, $P < .05$.

proteins in various cell lines, we examined the relative abundance of Gα-subunits in the different stable cell lines before and after chronic opioid treatment. 293/MOR and 293/MOR-Z cells were treated in the absence or presence of 10 nM DAGO with or without PTX for 24 h. Membrane proteins obtained from the different cell lines were resolved in denaturing gel electrophoresis and probed with anti-Gα-antisera. Endogenous G$\alpha_{i1\text{-}2}$ and Gα_o were detected by antisera AS/7 and GC/2, respectively (Fig. 6). Neither PTX treatment nor chronic opioid treatment altered the expression of G$\alpha_{i1\text{-}2}$ and Gα_o in the two cell lines. No endogenous Gα_z was detected by the Gα_z-specific antiserum sc-388 in 293/MOR cells (Fig. 6). Like the $G_{i/o}$ proteins, the expression of Gα_z in 293/MOR-Z cells was not affected by PTX or chronic opioid treatment. Therefore, the lack of involvement of G_z in opioid-induced AC supersensitization was not due to reduced expression of G_z. Similar results were obtained from 293/KOR and 293/KOR-Z cells (data not shown).

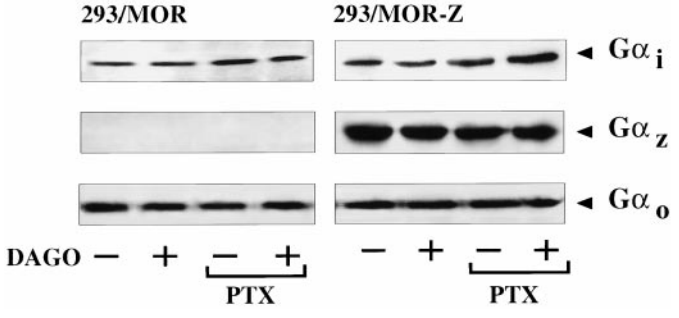

Fig. 6. Immunodetection of G$\alpha_{i1\text{-}2}$, Gα_o, and Gα_z in 293/MOR and 293/MOR-Z cells. 293/MOR and 293/MOR-Z cells were treated in the absence or presence of 10 nM DAGO with or without PTX (100 ng/ml) for 24 h. Membrane proteins (50 μg) prepared from the different cell lines were resolved on a 12.5% SDS gel and then transferred to nitrocellulose membranes. G$\alpha_{i1\text{-}2}$, Gα_o, and Gα_z were probed with antisera AS/7, GC/2, and sc-388, respectively. Two independent experiments with different batches of membrane proteins gave similar results.

AC Supersensitization Does Not Enhance the Response of CREB to Forskolin. There is increasing evidence to suggest that CREB may play a role in the development of opiate addiction (Maldonado et al., 1996; Lane-Ladd et al., 1997). The enhancement of AC responsiveness to forskolin on chronic opioid treatment in HEK 293 cells coexpressing opioid receptors and G_z may lead to changes in the state of phosphorylation of CREB, which is activated by cAMP-dependent protein kinase A. We used a phospho-specific CREB^{133} antiserum to detect the phosphorylated form of CREB. In 293/MOR, 293/MOR-Z, 293/KOR, and 293/KOR-Z cells, only forskolin, but not acute opioid stimulation, induced CREB phosphorylation by about 2- to 3-fold (Fig. 7; Table 1). The forskolin-mediated CREB phosphorylation was not enhanced by chronic opioid treatment. The response of CREB to forskolin stimulation was higher in 293/MOR-Z and 293/KOR-Z cells than that in 293/MOR and 293/KOR cells (Fig. 7; Table 1), suggesting that the coexpression of G_z somehow enhanced the response of CREB to forskolin. The increased response of CREB to forskolin in 293/KOR-Z was attenuated by PTX treatment, whereas PTX had no effect on the forskolin-induced CREB phosphorylation in the other cell lines (Fig. 7; Table 1). Phosphorylation of ATF-1 also was detected after forskolin stimulation in these four cell lines. In parental HEK 293 cells, only forskolin stimulated CREB and ATF-1 phosphorylation (Table 1). These results show that although prolonged opioid treatment enhanced the responsiveness of AC to forskolin, it did not potentiate phosphorylation of CREB. Also, the coexpressed Gα_z might interact with regulators of CREB to increase its response to forskolin.

Chronic Opioid Treatment Abolishes Acute Opioid Stimulation-Induced ERK1/2 Phosphorylation. A series of studies shows that ERK1/2 may be involved in opiate addiction (Berhow et al., 1996; Schulz and Hollt, 1998). The activity of ERK1/2 is regulated by opioids through the $\beta\gamma$-

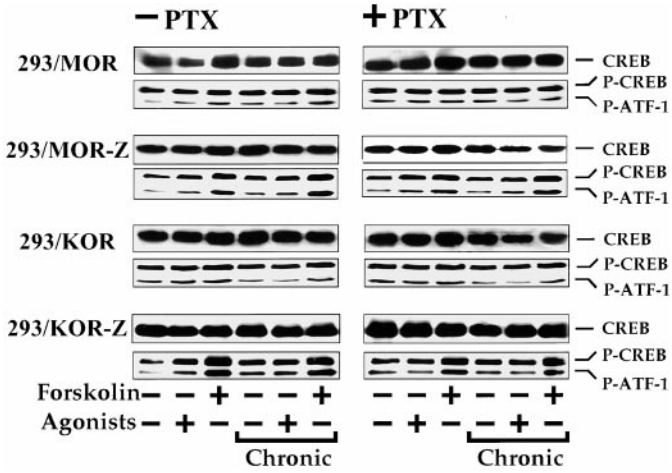

Fig. 7. Effects of various treatments on CREB phosphorylation. 293/MOR, 293/MOR-Z, 293/KOR, and 293/KOR-Z cells were pretreated in the absence or presence of 10 nM DAGO or U50,488 with or without PTX for 24 h. Subsequently, the cells were stimulated by 100 nM DAGO, U50,488, or 50 μM forskolin for 5 min. Cell lysates (80 μl) were resolved on a 12.5% SDS gel and transferred to nitrocellulose membranes. Total CREB and phospho-CREB were immunodetected by specific antisera. Immunoreactive bands were visualized by enhanced chemiluminescence system. Each panel shows representative immunoblots for CREB, phospho-CREB^{133}, and phospho-ATF-1 (activating transcription factor-1). The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

TABLE 1

Effects of chronic opioid treatment on CREB phosphorylation

Pretreatments for transfected HEK 239 cells, acute drug treatments, and the determination of CREB phosphorylation were as described in the legend to Fig. 7. The intensities of immunoreactive signals were quantified by NIH Image 1.6. Results are expressed as percentage of stimulation of CREB phosphorylation compared with the corresponding basal activity. Data shown represent the mean ± S.D. of three sets of immunoblots performed independently.

Cells	PTX Treatment	CREB Activation (% of Basal)			
		No Pretreatment		Chronic Opioid	
		Agonists	Forskolin	Agonists	Forskolin
293/MOR	−PTX	111 ± 27	167 ± 4[a]	106 ± 27	148 ± 9[a]
	+PTX	120 ± 18	162 ± 37[a]	134 ± 46	176 ± 26[a]
293/MOR-Z	−PTX	111 ± 23	250 ± 60[a,b]	102 ± 14	276 ± 20[a,b]
	+PTX	111 ± 22	278 ± 18[a,b]	107 ± 22	208 ± 17[a]
293/KOR	−PTX	104 ± 12	185 ± 9[a]	102 ± 22	167 ± 18[a]
	+PTX	93 ± 32	227 ± 40[a]	89 ± 46	213 ± 16[a]
293/KOR-Z	−PTX	112 ± 37	315 ± 74[a,b]	102 ± 74	365 ± 52[a,b]
	+PTX	102 ± 16	187 ± 27[a,c]	106 ± 11	190 ± 13[a,c]

[a] Forskolin significantly induced CREB phosphorylation; Bonferroni t test, $P < .05$.
[b] Significantly different from the corresponding value obtained in cells lacking $G\alpha_z$ but expressing the same opioid receptor; Bonferroni t test, $P < .05$.
[c] PTX significantly inhibited the forskolin-stimulated CREB phosphorylation; Bonferroni t test, $P < .05$.

complex of PTX-sensitive $G_{i/o}$ proteins (Burt et al., 1996; Fukuda et al., 1996; Belcheva et al., 1998). Moreover, G_z is capable of activating ERK1/2 in a PTX-resistant manner (Morales et al., 1998; Tso et al., 2000). Herein, we assessed the ability of μ- and κ-opioid receptors to regulate ERK1/2 through G_z. Application of 100 nM DAGO or U50,488 stimulated ERK1/2 phosphorylation by 2.5- to 5-fold in the four cell lines (Fig. 8; Table 2). In cells coexpressing G_z, PTX was unable to block stimulation of ERK1/2 phosphorylation by opioid agonists, suggesting that G_z can substitute for G_i in regulating the ERK1/2 activity. Chronic opioid treatment of the four cell lines did not affect the basal ERK1/2 activity but completely abolished the agonist-induced stimulation of ERK1/2 phosphorylation (Fig. 8).

Because elevation of intracellular cAMP level can stimulate ERK1/2 activity (Faure et al., 1994), the induction of AC supersensitization may indirectly potentiate the phosphorylation of ERK1/2. Hence, we examined the effects of chronic opioid treatment on the ability of forskolin to stimulate ERK1/2 in the four cell lines. Forskolin-induced cAMP accumulation could trigger ERK1/2 phosphorylation in 293/MOR-Z and 293/KOR cells but not in 293/MOR and 293/KOR-Z cells. Surprisingly, PTX treatment of 293/MOR and 293/KOR-Z cells allowed them to become responsive to forskolin but the bacterial toxin reduced (~25%) the forskolin response in 293/MOR-Z and 293/KOR cells (Table 2). Chronic opioid treatment had little effect on the forskolin responses except in 293/MOR-Z cells, where it potentiated the forskolin-induced ERK1/2 phosphorylation (Fig. 8; Table 2). In all cell lines, the various acute and chronic opioid treatments did not alter the total ERK1/2 immunoreactivity. Collectively, our results suggest that chronic opioid treatment diminished the ERK1/2 responsiveness to opioid stimulation and the μ- and κ-opioid-expressing cells differed in terms of their forskolin-induced ERK1/2 phosphorylation (Table 2).

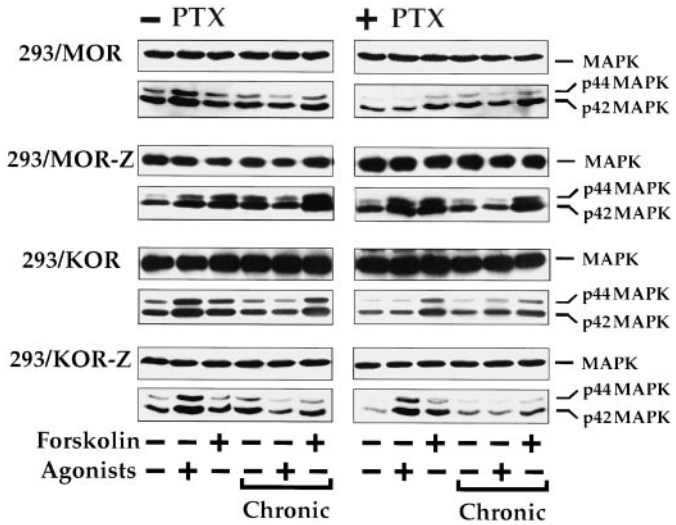

Fig. 8. Effects of acute and chronic opioid treatments on ERK1/2 phosphorylation. 293/MOR, 293/MOR-Z, 293/KOR, and 293/KOR-Z cells were pretreated in the absence or presence of 10 nM DAGO or U50,488 with or without PTX for 24 h. Subsequently, the cells were stimulated by 100 nM DAGO, U50,488, or 50 μM forskolin for 5 min. Cell lysates (80 μl) were resolved on a 12.5% SDS gel and transferred to nitrocellulose membranes. Total ERK1/2 (MAPK) and phospho-ERK1/2 (p42 and p44 MAPK) were immunodetected by specific antisera. Immunoreactive bands were visualized by enhanced chemiluminescence system. Each panel shows representative immunoblots for MAPK and phospho-ERK1/2. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

Discussion

G_z has recently been implicated in opioid-induced supraspinal analgesia (Sanchez-Blazquez et al., 1995) and can functionally associate to all three types of opioid receptors (Chan et al., 1995; Lai et al., 1995; Tsu et al., 1995). Despite the ability of G_z to mediate opioid-induced inhibition of AC and stimulation of ERK1/2, it could not support AC supersensitization elicited by opioid pretreatment in HEK 293 cells expressing the μ-, κ- (this study), or δ-opioid receptor (Tso et al., 2000). The inability of G_z to mediate opioid-induced AC supersensitization suggests that PTX-sensitive $G_{i/o}$ proteins may possess additional functions not performed by G_z, or that multiple signals are required for the induction of AC supersensitivity. Although G_z can regulate AC (Wong et al., 1992), ERK1/2 (Morales et al., 1998), and ion channels (Jeong and Ikeda, 1998) in much the same way as $G_{i/o}$ proteins, it remains to be shown whether G_z can indeed perform all other functions of $G_{i/o}$ proteins. For example, it would be interesting to determine whether G_z can activate the signal transducer and activator of transcription 3 (Stat3) as demonstrated for G_o (Ram et al., 2000).

Although all three types of opioid receptors are capable of interacting with multiple members of the G_i subfamily, in-

TABLE 2

Effects of chronic opioid treatment on ERK1/2 phosphorylation

Pretreatments for transfected HEK 293 cells, acute drug treatments, and the determination of ERK1/2 phosphorylation were as described in the legend to Fig. 8. The intensities of immunoreactive signals were quantified by NIH Image 1.6. Results are expressed as percentage of stimulation of ERK1/2 phosphorylation compared with the corresponding basal activity. Data shown represent the mean ± S.D. of three sets of immunoblots performed independently.

Cells	PTX Treatment	ERK1/2 Activation (% of Basal)			
		No Pretreatment		Chronic Opioid	
		Agonist	Forskolin	Agonist	Forskolin
293/MOR	−PTX	259 ± 38[a]	125 ± 57	93 ± 45	164 ± 57
	+PTX	102 ± 22[b]	214 ± 38[a]	137 ± 34	257 ± 27[a]
293/MOR-Z	−PTX	252 ± 20[a]	284 ± 26[a,c]	95 ± 34	449 ± 9[a,c]
	+PTX	216 ± 32[a,c]	209 ± 40[a]	78 ± 45	223 ± 22[a,b]
293/KOR	−PTX	453 ± 22[a]	375 ± 28[a]	117 ± 31	363 ± 31[a]
	+PTX	113 ± 53[b]	262 ± 40[a]	144 ± 29	248 ± 39[a]
293/KOR-Z	−PTX	434 ± 55[a]	148 ± 47[c]	87 ± 53	168 ± 45[c]
	+PTX	507 ± 51[a,c]	340 ± 35[a]	150 ± 32	274 ± 39[a]

[a] ERK1/2 phosphorylation was significantly increased compared with the corresponding basal activity; Bonferroni t test, $P < .05$.
[b] PTX significantly attenuated agonist/forskolin-induced ERK1/2 phosphorylation; Bonferroni t test, $P < .05$.
[c] Significantly different from the corresponding value obtained in cells lacking $G\alpha_z$ but expressing the same opioid receptor; Bonferroni t test, $P < .05$.

cluding $G_{i1\text{-}3}$, G_o, and G_z (Murthy and Makhlouf, 1996; Garzon et al., 1998), the opioid receptor-G protein selectivity appears to differ between various cell types. Nevertheless, it is conceivable that simultaneous activation of multiple G proteins is required for opioid-induced AC supersensitization, and thus G_z alone is inadequate to accomplish the task. The exact permutation of linkage between opioid receptors and multiple $G_{i/o}$ proteins might influence the ultimate physiological responses, including AC supersensitization. The fact that 293/KOR-Z and 293/MOR-Z exhibited differential PTX sensitivity supports such a notion. In terms of the magnitude of agonist-induced AC supersensitization, the δ-opioid receptor appears to be far more effective than the μ- or κ-opioid receptors in HEK 293 cells. In HEK 293 cells, chronic δ-opioid treatment induces a 20-fold enhancement of the forskolin stimulation (Tso et al., 2000), whereas the μ- and κ-opioid receptors can only potentiate the forskolin response by 3- to 4-fold (Fig. 2). It remains to be determined whether this property of the δ-opioid receptor has any correlation with its ability to induce tolerance and dependence. It is noteworthy that the δ-opioid receptor can spontaneously adopt a ligand-independent active conformation in HEK 293 cells (Chiu et al., 1996). Coexpression of $G\alpha_z$ with the δ- and κ-opioid receptors, but not with the μ-opioid receptor, reduced the agonist-induced enhancement of the forskolin response. Because the three opioid receptors were expressed at comparable levels, these results suggest that the magnitude of AC supersensitization may be determined in part by the receptor's preference for different G proteins. Among the three opioid receptors, the μ-subtype may have a higher preference for G_z (Garzon et al., 1998).

The mechanism by which chronic activation of opioid receptors leads to AC supersensitization has not been fully delineated. Different isoforms of AC appear to be sensitized to different extents (Avidor-Reiss et al., 1997; Nevo et al., 1998), and it may be correlated to their responsiveness to $G\beta\gamma$-subunits (Bayerwitch et al., 1998). As explained in our previous report (Tso et al., 2000), isozyme specificity could not account for the inability of G_z to mediate PTX-insensitive AC supersensitization in MOR-Z/293 and KOR-Z/293 cells. The involvement of $G\beta\gamma$-subunits in chronic opioid actions has been demonstrated (Thomas and Hoffman, 1996). Because the exact permutation of a G protein heterotrimer can determine the fidelity of signaling (Kleuss et al., 1992), activation of G_z may release the inappropriate $G\beta\gamma$-subunits for AC supersensitization. As yet, it remains unclear whether $G\alpha_z$ and $G\alpha_i$ have the same preference for different $G\beta\gamma$-subunits. It is noteworthy that the two subtypes of $G\alpha_o$ exhibit different preferences for $G\beta\gamma$-complexes (Kleuss et al., 1992). Moreover, PTX-catalyzed ADP-ribosylation of $G_{i/o}$ proteins may reduce the pool of opioid-releasable $G\beta\gamma$-subunits to such an extent that activation of G_z alone cannot mobilize sufficient amounts of $G\beta\gamma$.

Similar to our previous observations on the δ-opioid receptor (Tso et al., 2000), opioid pretreatments did not alter forskolin-stimulated CREB phosphorylation in HEK 293 cells expressing the μ- or κ-opioid receptors with or without G_z. Other regulatory factors for CREB phosphorylation may be involved. Cross talk between ERK and protein kinase A may be required for CREB phosphorylation. The loss of opioid-induced phosphorylation of ERK1/2 after opioid pretreatment (Fig. 8) suggests that the control of the ERK pathway has been compromised. Indeed, the MAPK/ERK kinase (MEK) inhibitor PD98059 can block glutamate-induced phosphorylation of CREB (Vanhoutte et al., 1999). Because opioid pretreatment of the four cell lines resulted in the abrogation of opioid-induced ERK activity, it may offset the stimulatory effects of forskolin on CREB phosphorylation despite the sensitization of AC.

The present study also demonstrated an interesting observation: increased responses of CREB to forskolin in cells coexpressing $G\alpha_z$ with the μ- or κ-opioid receptors. In contrast, coexpression of $G\alpha_z$ attenuated the forskolin-mediated CREB phosphorylation in HEK 293 cells stably expressing δ-opioid receptors (Tso et al., 2000). The molecular basis of this discrepancy is not clear. One possibility is that the δ-opioid receptor is more prone to adopt a spontaneously active conformation (Chiu et al., 1996) and the provision of $G\alpha_z$ might further accentuate this constitutive activity. It also should be noted that the Raf-MEK pathway is involved in forskolin-induced CREB activation independent of CREB phosphorylation at Ser^{133} (Seternes et al., 1999). Given that G_z supports opioid-induced activation of ERK1/2, forskolin-induced stimulation of CREB might be affected. Additional studies are needed to examine this possibility.

The blockade of opioid-induced ERK1/2 phosphorylation after opioid pretreatment suggests the presence of a negative feedback mechanism. Receptor desensitization was not involved because acute inhibition of cAMP accumulation by

agonists was still observed in the different cell lines. As indicated in the immunoblots, the expressions of ERK1/2 were not down-regulated. Also, the ability of forskolin to activate ERK1/2 via cAMP was unaffected by opioid pretreatment. A possible negative feedback mechanism involves the disassociation of the Ras guanyl-nucleotide exchange protein (SOS)/small adapter protein (Grb2) complex upstream of ERK via phosphorylation of SOS by ERK (Dong et al., 1996). Prolonged activation of ERK by opioids may similarly affect ERK signaling. Alternatively, AC supersensitization and elevated cAMP may suppress other signaling components such as Raf, which lies upstream of ERK (Cook and McCormick, 1993). G$\beta\gamma$-subunits are known to activate ERK in a Ras/Raf-dependent manner (Crespo et al., 1994). Because neither Gα_i nor Gα_z is capable of stimulating ERK1/2 activity (Y. H. Wong, unpublished data), opioid-induced ERK1/2 phosphorylation is likely to be mediated through the G$\beta\gamma$-subunits and thus might involve Ras and Raf. Involvements of G$\beta\gamma$, Ras, protein kinase C, and tyrosine kinases have been implicated in opioid-induced activation of ERK (Fukuda et al., 1996; Belcheva et al., 1998). Hence, chronic opioid treatment may invoke a number of complex mechanisms that ultimately inhibit subsequent opioid-induced, but not forskolin-induced, stimulation of ERK.

The ability of forskolin to stimulate ERK1/2 phosphorylation differed between the various cell lines. The phenotype of 293/MOR resembled that of 293/KOR-Z, whereas 293/MOR-Z was more akin to 293/KOR. For the δ-opioid receptor, coexpression of Gα_z did not affect the forskolin-induced ERK activation (Tso et al., 2000). Enhancement of the forskolin stimulation on ERK was observed for the μ-opioid receptor, whereas a reduction of response was seen with the κ-opioid receptor. The differential effects of forskolin on ERK activation in these cell lines did not correlate with forskolin-induced cAMP accumulation. We cannot fully explain the observed differences in forskolin-induced ERK1/2 phosphorylation. It might be attributed to clonal variations in signaling components of the cell lines. Alternatively, the three opioid receptors may use different mechanisms to activate the ERK pathway. For example, receptor internalization is required for δ-opioid, but not κ-opioid, induced stimulation of ERK (Ignatova et al., 1999; Li et al., 1999). Moreover, G_i proteins may use a novel $\beta\gamma$- and Ras-independent pathway to activate the ERK pathway (Hedin et al., 1999). Other regulatory factors such as protein kinase C, tyrosine kinases, B-raf, and the regulator of G protein signaling proteins may be involved in the activation of ERK and thus influence the ability of forskolin to stimulate ERK1/2 activity under different cellular environments.

In summary, G_z has been demonstrated to interact with the μ- and κ-opioid receptors to inhibit AC and stimulate ERK1/2. However, prolonged regulation of these effectors by G_z alone is insufficient to trigger AC supersensitization. Our results indicate the indispensable involvement of PTX-sensitive $G_{i/o}$ proteins in mediating AC supersensitization. Further studies are needed to establish whether individual PTX-sensitive $G_{i/o}$ proteins can support opioid-induced AC supersensitization or whether collaborative actions between several $G_{i/o}$ proteins are essential.

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