

Role of $G_{i\alpha 2}$ -Protein in Opioid Tolerance and μ -Opioid Receptor Downregulation In Vivo

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ABSTRACT Although opioid receptors are G-protein coupled, the role that specific G-protein subunits play in the development of opioid tolerance and the regulation of opioid receptor number is not well understood. In the present study, we used a $G_{i\alpha 2}$ antisense oligodeoxynucleotide (ODN) to examine the contribution of $G_{i\alpha 2}$ proteins to μ -opioid tolerance and receptor downregulation in the mouse. Mice were injected intracerebroventricularly (ICV) and into the spinal intrathecal space (IT) for 4–5 consecutive days (30 μ g/site/day), with an antisense ODN or a mismatch ODN directed at mRNA for the $G_{i\alpha 2}$ subunit of G-proteins. Controls were treated with dH₂O. On the second day of ODN treatment continuous subcutaneous (SC) infusion of etorphine (200 μ g/kg/day) or morphine (40 mg/kg/day + 25 mg pellet) was begun. Control mice were implanted with inert placebo pellets. Three days later, pumps and pellets were removed and mice were tested for morphine analgesia or μ -opioid receptor density was determined in whole brain. Etorphine produced significant tolerance (ED₅₀ shift = ~11-fold) and downregulation of μ -opioid receptors (~25%). Morphine treatment produced significant tolerance (ED₅₀ shift \approx 9-fold), but no μ -opioid receptor downregulation. Antisense treatment reduced $G_{i\alpha 2}$ protein levels in striatum and spinal cord by ~25%. $G_{i\alpha 2}$ antisense reduced the acute potency of morphine. $G_{i\alpha 2}$ antisense blocked the development of tolerance to morphine treatment and reduced the development of tolerance to etorphine treatment. Antisense did not have any effect on etorphine-induced μ -opioid receptor downregulation. In another experiment, 7-day treatment with morphine or etorphine similarly increased $G_{i\alpha 2}$ mRNA and protein abundance in spinal cord. Overall, these results support an important role for $G_{i\alpha 2}$ -protein in the acute effects of opioids and opioid tolerance. However, $G_{i\alpha 2}$ is not required for agonist-induced μ -opioid receptor density regulation in vivo. **Synapse 47:109–116, 2003.** © 2002 Wiley-Liss, Inc.

INTRODUCTION

Opioid receptors are coupled to guanine-nucleotide binding proteins (G-proteins). These proteins are required for many intracellular signaling events (Gomes et al., 2002; Sanchez-Blazquez et al., 1995; Rossi et al., 1995; Raffa et al., 1994). Interference with the function of G-proteins has been shown to reduce the acute potency of opioid agonists and to inhibit tolerance and dependence (Gomes et al., 2002; Parolaro et al., 1990). Conversely, pertussis toxin (PTX) treatment, which reduces opioid tolerance and the acute potency of opioids, does not interfere with opioid agonist-induced μ -opioid receptor downregulation in vivo (Gomes et al., 2002). Similarly, cell culture studies have found that G-proteins are not a requirement for receptor downregulation (Zaki et al., 2000; Law et al., 1985; Kato et al., 1998; Yabaluri and Medzihradsky, 1997).

Opioid agonist-induced receptor downregulation depends upon the intrinsic efficacy of the agonist. Many studies have reported that high intrinsic efficacy opioid agonists (e.g., etorphine) will produce μ -receptor internalization and downregulation in cell culture and whole animal, while low intrinsic efficacy agonists (e.g., morphine) do not (Gomes et al., 2002; Shen et al., 2000; Zaki et al., 2000; Whistler et al., 1999). Although opioid

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tolerance does not depend upon receptor downregulation (receptor density-independent mechanisms), recent data (Stafford et al., 2001) supports a role for decreased μ -opioid receptor density in tolerance (receptor density-dependent mechanisms). At present, it is not clear at what point in intracellular signaling the message for downregulation and receptor density-independent mechanisms of tolerance diverge.

It is generally agreed that morphine and etorphine produce their effects through the same second-messenger systems. Since both agonists produce substantial tolerance and etorphine causes downregulation, while morphine does not (Shen et al., 2000; Gomes et al., 2002; Keith et al., 1996), it is likely that tolerance and downregulation depend upon separate and, perhaps, overlapping mechanisms. In the present study, we used an antisense approach to examine the role of a specific G-protein subunit ($G_{i\alpha 2}$) in opioid tolerance and μ -opioid receptor downregulation in the intact mouse. $G_{i\alpha 2}$ has been suggested to play a particularly important role in mediating μ -opioid receptor signaling of many opioid agonists (Standifer et al., 1996).

MATERIALS AND METHODS

Subjects

Male, Swiss-Webster mice (22–40 g) from Taconic Farms (Germantown, NY) were used in all experiments. The animals were housed 10 per cage with free access to food and water for at least 24 h prior to experimentation. Mice were used only once.

Procedure

To examine the effects of chronic opioid treatment on $G_{i\alpha 2}$ abundance and transcription, mice were treated with morphine or etorphine for 7 days. Mice were implanted subcutaneously (SC) with an osmotic minipump (1 μ l/h) that infused etorphine (200 μ g/kg/day). For morphine treatment, mice were implanted SC with an osmotic minipump (1 μ l/h) that infused morphine (40 mg/kg/day) plus one 25 mg morphine implant pellet. Controls were implanted with placebo pellets. The pumps and pellets were implanted at the nape of the neck while mice were lightly anesthetized with halothane:oxygen (4:96%). Following 7 days of treatment with etorphine, morphine, or placebo, mice were sacrificed and the spinal cord collected for quantitative immunoblot or reverse transcriptase – polymerase chain reaction (RT-PCR) assays (see below).

For antisense studies, mice were injected intracerebroventricularly (ICV) and into the spinal intrathecal space (IT), as described previously (Yoburn et al., 1988). Mice were anesthetized briefly with halothane:oxygen (4:96%) prior to ICV and IT injections. ICV injections (4 μ l) were directed to the right lateral ventricle ~2 mm caudal and ~2 mm lateral to bregma at a depth of 3 mm. IT injections (2 μ l) were by lumbar

puncture. Mice treated with ODNs were injected ICV and IT (30 μ g/site/day, total dose 60 μ g/mouse) every 24 h for 4 (morphine) or 5 (etorphine) consecutive days with either an antisense ODN (5'-CTT GTC GAT CAT TTT AGA-3') (Shen et al., 1998; Standifer et al., 1996) complementary to murine $G_{i\alpha 2}$ mRNA (43rd–60th nucleotides following ATG start site) or a mismatch ODN (5'-TCT TGC GAT CTA TTT AAG-3'; underline represents bases with reversed order compared to antisense). Controls were treated on the same schedule with distilled water. On the second day of ODN treatment, chronic treatment with etorphine (200 μ g/kg/day) or morphine (40 mg/kg/day infusion + 25 mg morphine pellet) was begun. Controls were implanted with a single inert placebo pellet.

The pumps and pellets were removed 72 h following the start of morphine, etorphine, or placebo treatment in the ODN study. The shorter exposure time to morphine and etorphine in this antisense study was employed to limit the number of IT and ICV ODN injections. We have previously shown (Shen et al., 2000) that this protocol effectively produces tolerance and receptor regulation. Etorphine treated mice and controls were injected a fifth time with ODN or dH₂O when pellets and pumps were removed, while morphine-treated mice were not. Sixteen hours after termination of etorphine infusion and 4 h after the end of morphine treatment, mice were tested for morphine antinociception or mice were sacrificed and whole brain removed for μ -opioid receptor binding studies (see below). The interval between termination of treatment and binding studies was used to allow for elimination of residual agonist, as reported previously (Yoburn et al., 1993). Other mice were sacrificed, striatum and spinal cords removed, and samples prepared for Western blot.

RT-PCR

Total RNA was extracted from mouse spinal cord ($n = 3/\text{treatment}$) using TRIzol reagent according to the manufacturer's instructions (Invitrogen Corp, Carlsbad, CA). Yeast tRNA (12.5 μ g) was added as a carrier to facilitate the precipitation of RNA. The yield of RNA was determined using UV spectrometry (260 nm and 280 nm). RNA was stored at -80°C until analysis.

The RT-PCR assay was carried out using thermostable rTth RNA PCR kit (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (see Shen et al., 1998). The primers (Midland Certified, Midland, TX) for mouse $G_{i\alpha 2}$ were TGA GGA TGA GGA GAT GAA CCGC (Forward) and AAC ACA AAC TGC ACG TTC TTGG (Backward) corresponding to bases 805–826 and 1086–1107 (302 bp product). Following amplification, tubes were brought to 4°C , 1 μ l loading dye (Ambion, Austin, TX) was added to each tube, and a 5 μ l aliquot was loaded on 1.8% agarose gels and electrophoresed (85 V, 1 h). DNA

products were then visualized with UV light and captured using FluorChem v. 2.0 Imaging System (Alpha Innotech, San Leandro, CA). The gel image was digitized (Gel-Pro v. 3.0) and bands analyzed for optical density. A standard curve for G_{1A2} mRNA using increasing amounts of total RNA (0.06–0.5 µg) was included in each gel assay. This allowed conversion of optical density into valid estimates of percent changes in mRNA. The unknowns were included in the linear range of optical densities for standards.

Western blot assay

At the end of treatment (16 h following 5 days of ODN treatment; or following 7-day morphine or etorphine treatment), spinal cord and striatum were rapidly removed and homogenized (Brinkman Polytron Homogenizer, 20,000 rpm 30 sec) in ice-cold 50 mM Tris buffer and centrifuged at 20,000 rpm (2°C) for 15 min. The pellet was resuspended in ice-cold Tris buffer and stored at –80°C until analysis. The homogenate was thawed on ice and an aliquot was assayed for protein using the Bradford (1976) assay with reagents purchased from Bio-Rad (Richmond, CA). Samples for Western analysis were initially diluted in sample buffer (4–10% SDS, 1% β-mercaptoethanol, 20% glycerol, loading dye; in 125 mM Tris). Final sample concentrations for loading were diluted using lysis buffer (1 mM sodium orthovanadate, 2% SDS in 12.5 mM Tris), and the samples were boiled for 5 min.

An aliquot (8 µl, 14–25 µg protein) of the boiled sample (one spinal cord / lane; or four pooled pairs of striata/lane) was loaded on polyacrylamide gels (Pager Gels 10% Tris-Glycine; BioWhittaker Molecular Applications, Rockland, ME) and electrophoresed at 0.02 Amp for 85 min. Proteins were transferred onto PVDF membranes (Bio-Rad). Nonspecific binding sites on the membrane were blocked by overnight incubation in blocking buffer at room temperature (0.2% Aurora™ blocking reagent: 1× phosphate-buffered saline, 0.1% Tween-20; ICN Biomedicals, Costa Mesa, CA) followed by incubation (1 h, 24°C) with primary (1:750) mouse G_{1A2} antibody (Chemicon, Temecula, CA) in blocking buffer. Membranes were washed in blocking buffer and incubated for 1 h with goat antimouse alkaline phosphate-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted in blocking buffer (1:5,000). The blot was washed and visualized with chemiluminescence (Aurora™ Western Blotting kit, ICN Biomedicals). The image was captured (FluorChem v. 2.0 Imaging System; Alpha Innotech) and quantitated (GelPro 3.0) for optical density. A standard curve (minimum three points) using increasing concentrations (8–18 ng) of recombinant G_{1A2} protein (Santa Cruz Biotech) was included in each gel. This allows the conversion of optical density into valid estimates of percent changes in protein.

Analgesia assay

Analgesia (antinociception) was determined using the tail flick assay in which a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail (Shen et al., 1998; D'Amour et al., 1941). The intensity of the light was adjusted so that baseline flick latencies were 2–4 sec. If a mouse failed to flick its tail by 10 sec during morphine dose–response testing, the test was terminated and the mouse was defined as analgesic. Mice were tested for analgesia 30 min following morphine administration. All testing was conducted in a blind manner.

A cumulative dose–response protocol was used for all studies. All mice in a treatment group (n = 7 per group) were injected SC with a starting dose (0.5 mg/kg) of morphine and tested for antinociception 30 min later. All mice that were not analgesic were given a second dose of morphine within 5 min of testing and tested for antinociception again 30 min later. This cumulative dose–response procedure was continued until all mice were analgesic (0.5 mg/kg increment, range of doses = 0.5–45.5 mg/kg). The morphine doses used were determined in a previous study (Duttaroy et al., 1997).

µ-Opioid receptor binding

Whole brain was rapidly removed, weighed, and homogenized in 80 volumes of ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged at 15,000 rpm for 15 min, the supernatant discarded, and the pellet resuspended in buffer and centrifuged again. The pellet was resuspended and incubated (30 min at 25°C), centrifuged a third time, and finally resuspended in 20–80 volumes of phosphate buffer (50 mM, pH 7.2). An aliquot (200 µl) of the homogenate was assayed in triplicate in tubes containing 0.04–5.0 nM [³H] [D-Ala², N-MePhe⁴, Gly-ol⁵] enkephalin (DAMGO). Nonspecific binding was determined in the presence of 1,000 nM levorphanol. Tubes were incubated for 90 min at 25°C. Incubation was terminated by ice-cold phosphate buffer followed by filtration over GF/B glass fiber filters. Filters were transferred to vials and scintillation cocktail was added and vials were counted. Counts per minute (cpm) were converted to disintegrations per minute (dpm) using the external standard method. Protein was determined using the Bradford (1976) method. Each treatment was examined in 2–4 binding experiments.

Drugs and reagents

Etorphine HCl, morphine pellets, and inert placebo pellets were obtained from the Research Triangle Institute (Research Triangle Park, NC). Morphine sulfate was obtained from Penick Laboratories (Newark, NJ). [³H] DAMGO was obtained from NEN Lifesciences (Boston, MA). ODNs were synthesized by Midland Certified Reagent Co. (Midland, TX). All compounds were

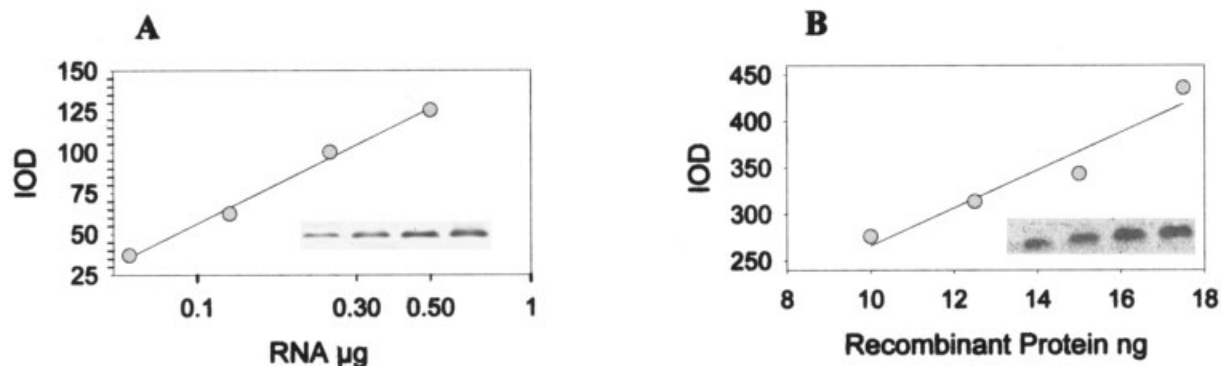


Fig. 1. Typical standard curves for $G_{i\alpha 2}$ mRNA (A) and protein (B). IOD = integrated optical density. See Methods.

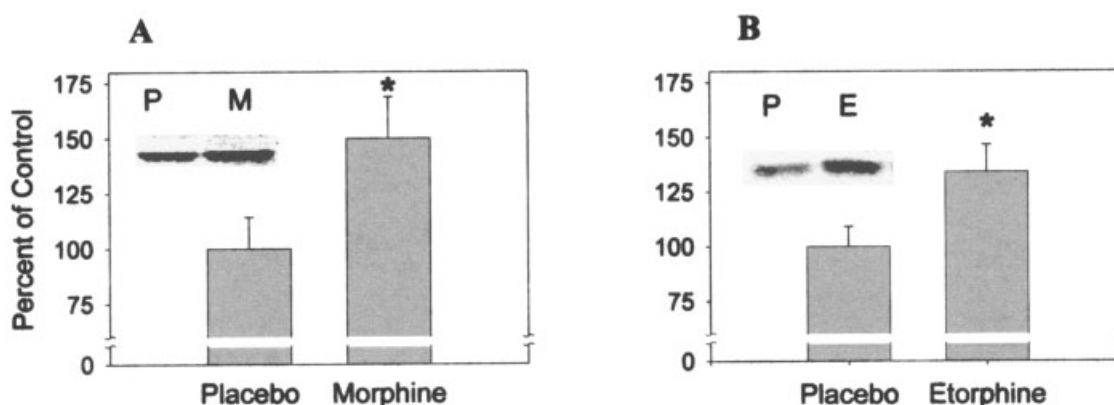


Fig. 2. The effect of chronic morphine (A) and etorphine (B) on $G_{i\alpha 2}$ protein abundance in spinal cord. Etorphine-treated (200 $\mu\text{g}/\text{kg}/\text{day}$), morphine-treated (40 $\text{mg}/\text{kg}/\text{day}$ + one 25 mg morphine pellet), and control (placebo) were infused sc for 7 days ($n = 6/\text{group}$). At the end of treatment spinal cords were removed and levels of $G_{i\alpha 2}$ were

determined using Western blotting. Insets are representative blots (P = placebo; E = etorphine; M = morphine). Data are mean \pm SEM from six independent experiments. *Significantly different from placebo ($P < 0.05$).

dissolved in normal saline (0.9%), except the ODNs, which were dissolved in distilled water. Doses were calculated as the free base.

Data analysis

Dose–response data were analyzed by probit analysis (Finney, 1973) using a computerized program that estimated the ED_{50} 's, standard errors, and 95% confidence limits. Statistical differences among dose–response parameters were analyzed based on the results of probit analysis using the Z-test. B_{max} and K_d were obtained from saturation studies using nonlinear regression analysis (Prism 3.0, Graphpad software, San Diego, CA). All saturation data were best fit by a one-site model. Significant differences ($P < 0.05$) among the groups were analyzed using ANOVA and appropriate post-hoc tests.

RESULTS

Opioid agonist-induced changes in $G_{i\alpha 2}$ protein and mRNA abundance

A typical standard curve for RT-PCR assays is shown in Figure 1A (from three assays mean \pm SD

$r^2 = 0.95 \pm 0.01$) and a representative standard curve for Western blot assays is shown in Figure 1B (from six assays mean \pm SD $r^2 = 0.98 \pm 0.01$). In both cases, linear or log-linear (RT-PCR, Fig. 1A) functions fit the data well.

Chronic treatment (7 days) with both morphine and etorphine significantly increased the abundance of $G_{i\alpha 2}$ ($\approx 50\%$, $\approx 34\%$) in spinal cord (Fig. 2). Similarly, mRNA abundance of $G_{i\alpha 2}$ was significantly increased for both morphine- and etorphine-treated groups ($\approx 128\%$, $\approx 121\%$) in spinal cord (Fig. 3). Thus, both morphine and etorphine similarly upregulated $G_{i\alpha 2}$.

Effect of antisense on $G_{i\alpha 2}$ protein abundance in spinal cord and the striatum

Western blot analysis indicated that $G_{i\alpha 2}$ antisense treatment significantly reduced target protein abundance in striatum by $\approx 25\%$, whereas mismatch had no effect (Fig. 4). Similar results were obtained for the spinal cord (data not shown). Thus, antisense treatment appeared effective at both injection sites in brain (striatum) and spinal cord.

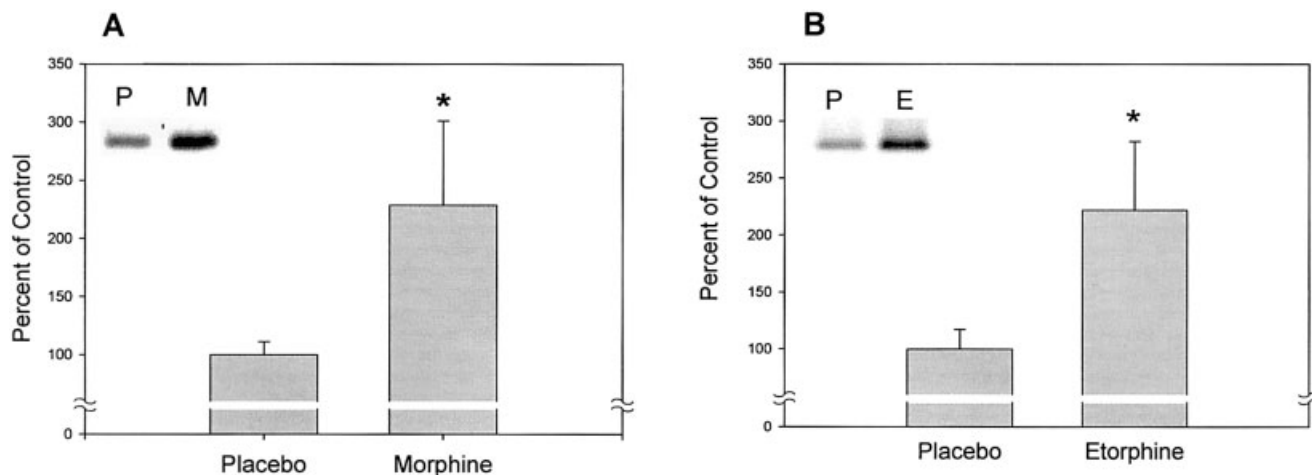
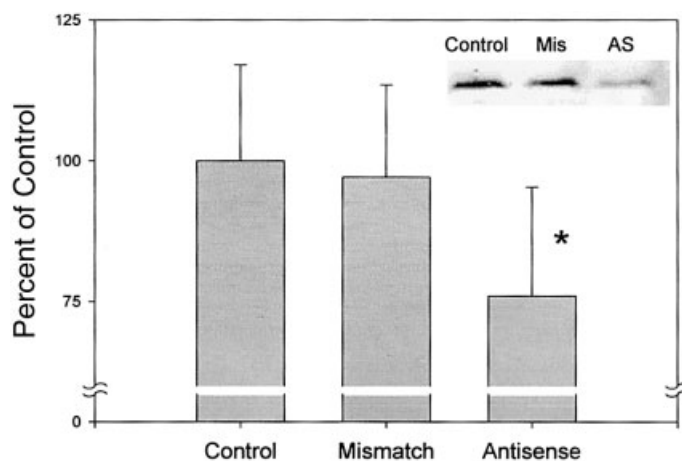


Fig. 3. The effect of chronic morphine (A) and etorphine (B) on $G_{i\alpha 2}$ mRNA abundance in spinal cord. Etorphine-treated (200 $\mu\text{g}/\text{kg}/\text{day}$), morphine-treated (40 $\text{mg}/\text{kg}/\text{day}$ + one 25 mg morphine pellet), and control (placebo) were infused sc for 7 days ($n = 3/\text{group}$). At the end of treatment spinal cords were removed and mRNA levels of $G_{i\alpha 2}$

were determined using RT-PCR. Insets are representative blots of $G_{i\alpha 2}$ amplification product after morphine and etorphine. (P = placebo; E = etorphine; M = morphine). Data are mean \pm SEM from three independent experiments. *Significantly different from placebo ($P < 0.05$).

Fig. 4. The effect of oligodeoxynucleotide treatment on $G_{i\alpha 2}$ protein abundance in mouse striatum. Mice were injected with $G_{i\alpha 2}$ antisense for 5 successive days with dH₂O, mismatch, or antisense ($n = 3/\text{group}$) as described. 16 h following the final injection, mice were sacrificed, brain removed, and striatum dissected and $G_{i\alpha 2}$ protein abundance determined using Western analysis. Results are mean \pm SEM from two combined, independent experiments. *Significantly different from control and mismatch ($P < 0.05$). The inset is a representative blot (control, Mis = mismatch, AS = antisense).



Effect of $G_{i\alpha 2}$ antisense on morphine analgesic potency and tolerance

Baseline tail flick latencies did not differ significantly between saline and $G_{i\alpha 2}$ antisense-treated mice. Daily $G_{i\alpha 2}$ antisense treatment significantly reduced morphine's analgesic potency by ~ 3 -fold, whereas mismatch had no effect (Figs. 5, 6; second and third bars). Both etorphine infusion and morphine treatment produced significant tolerance to morphine analgesia (Figs. 5, 6; fourth bar). When antisense and opioid agonist treatment was combined, there was significantly reduced development of tolerance. $G_{i\alpha 2}$ antisense treatment appeared to completely block morphine-induced tolerance relative to the $G_{i\alpha 2}$ antisense-placebo group (Fig. 5), whereas etorphine induced tolerance was partially blocked (Fig. 6). The mismatch ODN did not alter tolerance to morphine or etorphine.

Effect of $G_{i\alpha 2}$ antisense on μ -opioid receptor density in brain

$G_{i\alpha 2}$ antisense and mismatch did not alter μ -opioid receptor density in placebo-treated mice (Fig. 7). Continuous etorphine infusion (200 $\mu\text{g}/\text{kg}/\text{day}$) produced a significant decrease (28%) in μ -receptor B_{max} with respect to control. K_d 's were not significantly or systematically changed from control by etorphine, morphine, or ODN treatment (range of K_d 's = 0.8–1.4 nM). When antisense and etorphine treatments were combined, $G_{i\alpha 2}$ antisense treatment did not alter downregulation produced by etorphine. As previously reported (Yoburn et al., 1993), morphine treatment did not cause any change in receptor density. When morphine treatment was combined with $G_{i\alpha 2}$ antisense there was no significant change in the B_{max} or K_d . Thus, knockdown of $G_{i\alpha 2}$ protein did not block downregulation of μ -opioid receptors induced by etorphine.

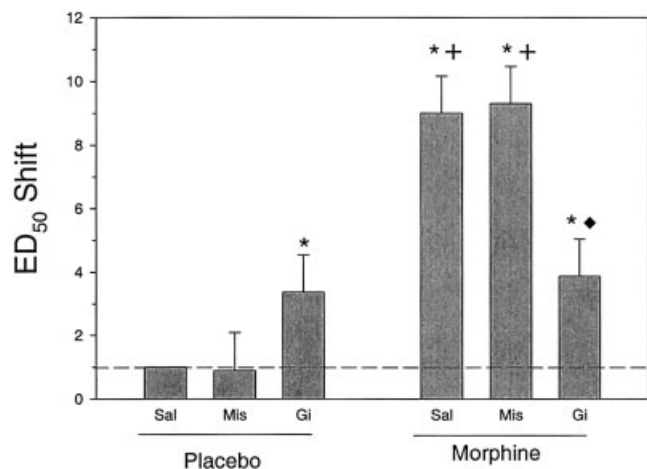


Fig. 5. The effect of $G_{i\alpha 2}$ antisense on morphine-induced tolerance. Mice were injected with $G_{i\alpha 2}$ antisense for 4 successive days. On day 2, morphine treatment was started. Morphine treatment was terminated after 72 h and morphine ED_{50} 's determined (see Methods). Data are presented as the ED_{50} shift, which is the ratio of the ED_{50} of the treated groups to that of the control (saline-placebo). Each bar represents the ED_{50} shift (\pm SEM) from a single study ($n = 7/\text{group}$). The mean ED_{50} for the control group was 1.92 ± 1.14 mg/kg. *Significantly different from the control (saline-placebo). †Significantly different from $G_{i\alpha 2}$ -Antisense-Placebo. ‡Significantly different from Saline-Morphine and Mismatch-Morphine groups ($P < 0.05$). Sal = saline, Mis = mismatch, Gi = $G_{i\alpha 2}$ -antisense. Similar results were found in a separate independent experiment.

DISCUSSION

It is well established that chronic morphine and etorphine treatment induce significant tolerance to the analgesic (and other) effects of opioids (e.g., Law et al., 2000; Williams et al., 2001). However, only high intrinsic efficacy opioid agonists produce μ -opioid receptor downregulation (e.g., Shen et al., 2000; Yoburn et al., 1993). In the present study, we have shown that chronic treatment with morphine or etorphine produced tolerance and selective μ -opioid receptor downregulation. Antisense-induced reduction of $G_{i\alpha 2}$ protein abundance resulted in decreased acute effects of morphine and inhibition of opioid tolerance. However, $G_{i\alpha 2}$ antisense had no effect on etorphine-induced μ -opioid receptor downregulation. On the other hand, both agonists upregulated $G_{i\alpha 2}$ -protein abundance and mRNA in spinal cord. These results suggest that $G_{i\alpha 2}$ plays an important role in a select group of receptor mediated effects, but not in receptor downregulation.

Chronic morphine and etorphine treatment increased $G_{i\alpha 2}$ -protein abundance and mRNA in mouse spinal cord. Although regulation of G-protein subunits by opioids has been reported previously (Kaewsuk et al., 2001; Vogel et al., 1990; Terwilliger et al., 1991; Nestler et al., 1989), the fact that both morphine and etorphine produce tolerance and that both agonists similarly regulate $G_{i\alpha 2}$ suggests that selective receptor downregulation by etorphine is mediated independently of $G_{i\alpha 2}$. The failure of $G_{i\alpha 2}$ antisense to interfere

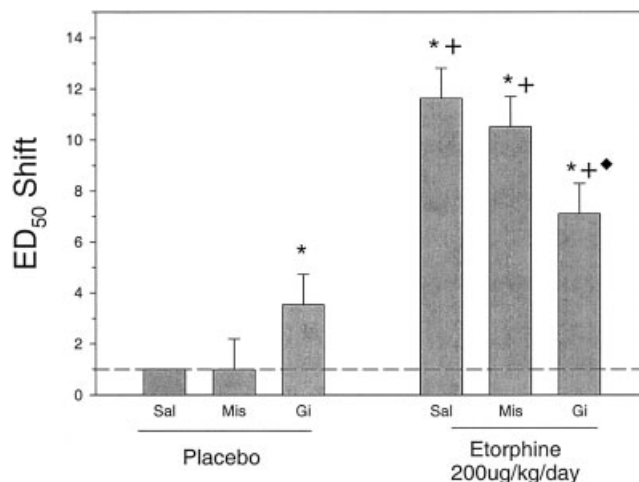


Fig. 6. The effect of $G_{i\alpha 2}$ antisense on etorphine-induced tolerance. Mice were injected with $G_{i\alpha 2}$ antisense for 5 successive days. On day 2, etorphine treatment was started. Etorphine treatment was terminated after 72 h and morphine ED_{50} 's determined (see Methods). Data are presented as the ED_{50} shift, which is the ratio of the ED_{50} of the treated groups to that of the control (saline-placebo). Each bar represents the ED_{50} shift (\pm SEM) from a single study ($n = 7/\text{group}$). The mean ED_{50} for the control group was 1.94 ± 1.15 mg/kg. *Significantly different from the control (saline-placebo). †Significantly different from $G_{i\alpha 2}$ -Antisense-Placebo. ‡Significantly different from Saline-Etorphine and Mismatch-Etorphine ($P < 0.05$). Sal = saline, Mis = mismatch, Gi = $G_{i\alpha 2}$ -antisense. Similar results were found in a separate independent experiment.

with etorphine-induced downregulation supports this suggestion.

The lack of effect of $G_{i\alpha 2}$ antisense on downregulation cannot be attributed to ineffectiveness of the ODN. Antisense to $G_{i\alpha 2}$ reduced the abundance of this protein in brain (striatum) and spinal cord and reduced the acute analgesic potency of systemic morphine. In addition, antisense to $G_{i\alpha 2}$ reduced tolerance following chronic etorphine treatment and eliminated tolerance following chronic morphine treatment. Our results confirm and extend previous reports implicating G-proteins in acute and chronic opioid effects such as tolerance and dependence in vivo (Gomes et al., 2002; Funada et al., 1993; Parolaro et al., 1990; Raffa et al., 1994; Sanchez-Blazquez et al., 1994, 1997; Shen et al., 1998; Standifer et al., 1996).

Overall, the present results are consistent with an in vivo study that employed PTX to inactivate $G_{i/o}$ proteins (Gomes et al., 2002). In that report, PTX reduced the acute effects of opioids and inhibited tolerance, but did not affect etorphine-induced downregulation. Similarly, cell culture studies suggest only partial involvement of G-proteins in μ -opioid receptor downregulation (Zaki et al., 2000; Kato et al., 1998; Yabaluri and Medzihradsky, 1997; Law et al., 1985).

While opioid receptors are characterized as G-protein-coupled receptors, agonist-induced receptor downregulation does not depend on $G_{i\alpha 2}$ or on PTX-sensitive G-proteins (Gomes et al., 2002). At present, it is not clear how agonist-induced μ -opioid receptor downregu-

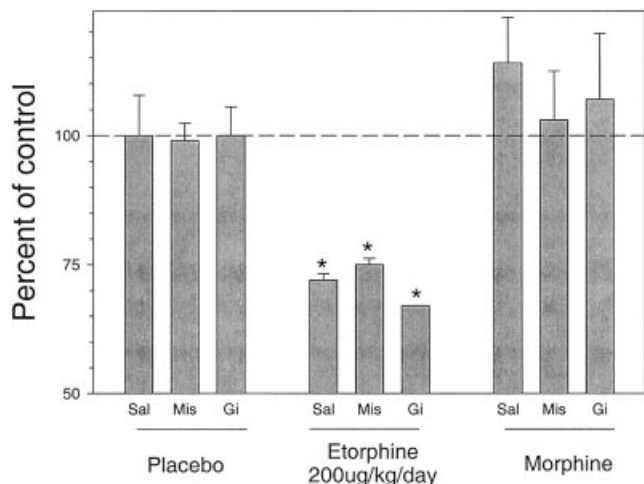


Fig. 7. The effect of $G_{1\alpha 2}$ -Antisense on μ -opioid receptor density in brain following etorphine or morphine treatment. Mice were treated as described in Figures 5 (morphine) and 6 (etorphine). Each bar is the mean (\pm SEM) percent of control B_{max} (saline-placebo) from 2–4 experiments ($n = 3$ /group). The mean B_{max} of the control groups was 254 ± 20 fm/mg protein. *Significantly different from control ($P < 0.05$). Sal = saline, Mis = mismatch, Gi = $G_{1\alpha 2}$ -Antisense.

lation is mediated. However, a previous study reported that opioid antagonist-induced upregulation is also independent of PTX-sensitive G-proteins (Chang et al., 1991). Thus, we propose that opioid receptor regulation in vivo is generally independent of G-protein signaling. It remains to be determined if PTX-insensitive G-proteins mediate receptor regulation.

Since G-proteins appear to play a minimal role in μ -opioid receptor regulation, other mechanisms must be important in opioid agonist-induced receptor regulation. It is possible that some opioid agonists (etorphine) are able to place the μ -opioid receptor into a conformation that is targeted for downregulation and simultaneously activate G-protein coupled second-messenger signaling. Other agonists (morphine) apparently are effective only in activating G-protein-coupled signaling. These conformational variations may result in differences in suitability as a substrate for G-protein receptor kinases (GRKs) and β -arrestin. Previous studies in which morphine causes internalization of the μ -opioid receptor when GRK or β -arrestin are overexpressed in cells (Whistler and Von Zastrow, 1998; Zhang et al., 1998) are consistent with the suggestion that morphine-activated μ -receptors are a less than optimal target for β -arrestin and GRKs. Similarly, recent studies indicate that the β_2 adrenergic receptor has distinct conformations for agonists, partial agonists, and antagonists (Ghanouni et al., 2001). Lastly, it is also possible that assorted intrinsic efficacy agonists may differentially regulate trafficking proteins (GRKs, β -arrestin, Dyanmin) such that internalization and downregulation is favored.

Treatment with antisense reduced etorphine-induced tolerance and eliminated morphine-induced tol-

erance. These results strongly suggest that G-protein function, and in particular $G_{1\alpha 2}$ function, is critical to the development of tolerance. It is likely that the complete block of morphine tolerance is related to the reduction of intracellular signaling and consequent reduced desensitization of the μ -receptor. The incomplete block of etorphine-induced tolerance by antisense treatment may be due to μ -opioid receptor downregulation that was unaffected by antisense. Thus, etorphine produces tolerance via receptor downregulation and via receptor desensitization. These data support the role of both receptor density-dependent and receptor density-independent mechanisms of tolerance and agree with previous results using antisense to PKA (Shen et al., 2000).

Taken together, this study supports a role for G-proteins, such as $G_{1\alpha 2}$, in the development of receptor density-independent mechanisms of tolerance and the acute effects of opioids. However, PTX-sensitive G-proteins, and $G_{1\alpha 2}$ in particular, have minimal impact on agonist-induced μ -opioid receptor downregulation in vivo.

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REFERENCES

- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Chang SC, Luffy K, Sierra V, Yoburn BC. 1991. Dissociation of opioid receptor upregulation and functional supersensitivity. *Pharmacol Biochem Behav* 38:853–859.
- D'Amour FE, Smith DL. 1941. A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72:74–79.
- Duttaroy A, Kirtman R, Farrell F, Phillips M, Phillippe J, Monderson T, Yoburn BC. 1997. The effect of cumulative dosing on the analgesic potency of morphine in mice. *Pharmacol Biochem Behav* 58:67–71.
- Finney DJ. 1973. *Probit analysis*, 3rd ed. London: Cambridge University Press.
- Funada M, Narita M, Suzuki T, Misawa M. 1993. Effect of pretreatment with pertussis toxin on the development of physical dependence on morphine. *Naunyn Schmied Arch Pharmacol* 348:88–95.
- Ghanouni P, Steenhuis JJ, Farrens DL, Kobilka BK. 2001. Agonist-induced conformational changes in the G-protein-coupling domain of the beta 2 adrenergic receptor. *Proc Natl Acad Sci USA* 98:5997–6002.
- Gomes BA, Shen J, Stafford K, Patel M, Yoburn BC. 2002. μ -Opioid receptor downregulation and tolerance are not equally dependent upon G-protein signaling. *Pharmacol Biochem Behav* 72:273–278.
- Kaewsuk S, Hutamekalin P, Ketterman AJ, Khotchabhakdi N, Govitrapong P, Casalotti SO. 2001. Morphine induces short-lived changes in G-protein gene expression in rat prefrontal cortex. *Eur J Pharmacol* 411:11–16.
- Kato S, Fukuda K, Morikawa H, Shoda H, Mima H, Mori K. 1998. Adaptations to chronic agonist exposure of μ -opioid receptor ex-

- pressing Chinese hamster ovary cells. *Eur J Pharmacol* 345:221–228.
- Keith DE, Murrariy SE, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, Von Zastrow M. 1996. Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* 271:19021–19024.
- Law P, Louie AK, Loh HH. 1985. Effect of pertussis toxin treatment on the downregulation of opiate receptors in neuroblastoma x glioma NG108-15 hybrid cells. *J Biol Chem* 260:14818–14823.
- Law P, Yung HW, Loh HH. 2000. Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* 40:389–430.
- Nestler EJ, Erdos JJ, Terwilliger R, Duman RS, Tallman JF. 1989. Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain Res* 476:230–239.
- Parolaro D, Patrini G, Giagnoni G, Massi P, Groppetti A, Parenti M. 1990. Pertussis toxin inhibits morphine analgesia and prevents opiate dependence. *Pharmacol Biochem Behav* 35:137–141.
- Raffa RB, Martinez RP, Connelly CD. 1994. G-protein antisense oligodeoxynucleotides and μ -opioid supraspinal antinociception. *Eur J Pharmacol* 258:R5–R8.
- Rossi GC, Standifer KM, Pasternak GW. 1995. Differential blockade of morphine and morphine-6-beta-glucuronide analgesia by antisense oligodeoxynucleotides directed against MOR-1 and G-protein alpha subunits in rats. *Neurosci Lett* 198:99–102.
- Sanchez-Blazquez P, Gracia-Espana A, Garzon J. 1994. Antibodies directed against α subunits of G_{12} , G_{13} , G_{16} , and G_{17} transducer proteins reduced the morphine withdrawal syndrome in mice. *Life Sci* 55:PL445–PL450.
- Sanchez-Blazquez P, Gracia-Espana A, Garzon J. 1995. In vivo injection of antisense oligodeoxynucleotides to G_{α} subunits and supraspinal analgesia evoked by mu and delta opioid agonists. *J Pharmacol Exp Ther* 275:1590–1596.
- Sanchez-Blazquez P, Garcia-Espana A, Garzon J. 1997. Antisense oligodeoxynucleotides to opioid mu and delta receptors reduced morphine dependence in mice: role of delta-2 opioid receptors. *J Pharmacol Exp Ther* 280:1423–1431.
- Shen J, Shah S, Hsu H, Yoburn BC. 1998. The effects of antisense to $G_{1\alpha 2}$ on opioid potency and $G_{1\alpha 2}$ protein and mRNA abundance in the mouse. *Mol Brain Res* 59:247–255.
- Shen J, Gomes BA, Stafford K, Yoburn BC. 2000. Role of cAMP-dependent protein kinase PKA in opioid agonist-induced μ -opioid receptor downregulation and tolerance in mice. *Synapse* 383:322–327.
- Stafford K, Gomes AB, Shen J, Yoburn BC. 2001. μ -Opioid receptor downregulation contributes to opioid tolerance in vivo. *Pharmacol Biochem Behav* 69:233–237.
- Standifer KM, Rossi GC, Pasternak GW. 1996. Differential blockade of opioid analgesia by antisense ODN's directed against various G-protein α subunits. *Mol Pharmacol* 50:293–298.
- Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ. 1991. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 548:100–110.
- Vogel Z, Barg J, Attali B, Simantov R. 1990. Differential effect of mu, delta, and kappa ligands on G protein alpha subunits in cultured brain cells. *J Neurosci Res* 27:106–111.
- Whistler JL, Von Zastrow M. 1998. Morphine-activated opioid receptors elude desensitization by β -arrestin. *Proc Natl Acad Sci USA* 95:9914–9919.
- Whistler JL, Chuang H, Chu P, Jan LY, Von Zastrow M. 1999. Functional dissociation of opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23:737–746.
- Williams JT, Christie MJ, Manzoni O. 2001. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* 81:299–343.
- Yabaluri N, Medzihradsky F. 1997. Down-regulation of μ -opioid receptor by full but not partial agonists are independent of G protein coupling. *Mol Pharmacol* 52:896–902.
- Yoburn BC, Luke MC, Pasternak GW, Inturrisi CE. 1988. Upregulation of opioid receptor subtypes correlates with potency changes of morphine and DADLE. *Life Sci* 43:1319–1324.
- Yoburn BC, Billings B, Duttaroy A. 1993. Opioid receptor regulation in mice. *J Pharmacol Exp Ther* 265:314–320.
- Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. 1997. Opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* 272:28869–28874.
- Zaki PA, Keith Jr DA, Brine GA, Carroll FI. 2000. Ligand-induced changes in surface μ -opioid receptor number: relationship to G protein activation? *J Pharmacol Exp Ther* 292:1127–1134.
- Zhang J, Ferguson SSG, Barak LS, Bodduluri SR, Laporte SA, Law PL, Caron MG. 1998. Role for G protein-coupled receptor kinase in agonist-specific regulation of μ -opioid receptor responsiveness. *Proc Natl Acad Sci USA* 95:7157–7162.