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μ-Opioid receptor down-regulation and tolerance are not equally dependent upon G-protein signaling

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Abstract

In the present study, the contribution of pertussis toxin (PTX)-sensitive $G_{i/o}$-proteins to opioid tolerance and μ-opioid receptor down-regulation in the mouse were examined. Mice were injected once intracerebroventricularly and intrathecally with PTX (0.1 μg/site). Controls were treated with saline. On the 10th day following PTX treatment, continuous subcutaneous infusion of etorphine (150 or 200 μg/kg/day) or morphine (40 mg/kg/day + 25 mg slow-release pellet) was begun. Control mice were implanted with inert placebo pellets. Pumps and pellets were removed 3 days later, and mice were tested for morphine analgesia or μ-opioid receptor density was determined in the whole brain, spinal cord, and midbrain. Both infusion doses of etorphine produced significant tolerance (ED_{50} shift = ≈ 4–6-fold) and down-regulation of μ-opioid receptors (≈ 20–35%). Morphine treatment also produced significant tolerance (ED_{50} shift = ≈ 5–8-fold), but no μ-opioid receptor down-regulation. PTX dramatically reduced the acute potency of morphine and blocked the further development of tolerance by both etorphine and morphine treatments. However, PTX had no effect on etorphine-induced μ-opioid receptor down-regulation in brain, cord, or midbrain. These results suggest that PTX-sensitive G-proteins have a minimal role in agonist-induced μ-opioid receptor density regulation in vivo, but are critical in mediating acute and chronic functional effects of opioids such as analgesia and tolerance.

Keywords: Pertussis toxin, PTX; μ-Opioid down-regulation; G-proteins; Opioid tolerance

1. Introduction

Activation of guanine–nucleotide binding proteins (G-proteins) is the first step in the cascade of receptor-mediated effects of G-protein-coupled receptors (GPCR), including the opioid receptors (e.g., Sanchez-Blazquez et al., 1995; Rossi et al., 1995; Raffa et al., 1994). Numerous studies indicate that opioid receptors are coupled to pertussis toxin (PTX)-sensitive $G_{i/o}$-proteins (Shah et al., 1997; Goode and Raffa, 1997; Hoehn et al., 1988). PTX catalyzes the ADP ribosylation of a cysteine side chain on the α-subunit of $G_{i/o}$-proteins (Resine, 1990) and inactivates it. Antisense targeting studies confirm the importance of G-proteins in opioid receptor signaling and have shown that $G_{i\alpha}$-subunits play a dominant role in opioid coupling to intracellular events (Standifer et al., 1996). However, the role that G-proteins play in chronic opioid effects such as the regulation of opioid receptor density and tolerance is less understood.

Down-regulation of opioid receptors is readily observed following chronic exposure to high-intrinsic-efficacy opioid agonists (e.g., etorphine), but not following low-intrinsic-efficacy agonists (e.g., morphine) (Shen et al., 2000; Zaki et al., 2000; Whistler et al., 1999; Yabaluri and Medzihradsky, 1997; Duttaroy and Yoburn, 1995). While, it is clear that down-regulation is not required for opioid tolerance (Whistler et al., 1999; Duttaroy and Yoburn, 1995), evidence suggests that it contributes to the magnitude of opioid tolerance (Shen et al., 2000; Stafford et al., 2001). As such, it is important to understand the events that mediate opioid receptor down-regulation and its contribution to chronic opioid effects such as tolerance.

Several investigators have examined the role that PTX-sensitive G-proteins play in opioid receptor internalization and down-regulation in cell culture. In some cases, agonist-induced receptor regulation is independent of G-protein function (Law et al., 1985; Kato et al., 1998; Remmers

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et al., 1998); while other reports indicate that receptor regulation may be partially blocked by PTX (Zaki et al., 2000; Yabaluri and Medzihradsky, 1997; Chakrabarti et al., 1997) and that sensitivity differs for μ- and δ-opioid receptors (e.g., Chakrabarti et al., 1997). These cell culture results make it difficult to predict if PTX-sensitive G-proteins are critical in opioid receptor regulation in the intact animal. On the other hand, the functional effects of opioid agonists in cell culture and in vivo (e.g., inhibition of adenylyl cyclase, analgesia) are uniformly inhibited by interference with G-protein function (e.g., Remmers et al., 1998; Shen et al., 1998).

In the present study, we investigated the role of PTX-sensitive G-proteins in opioid effects in the intact animal. Agonist-induced changes in μ-opioid receptor density, opioid agonist potency, and tolerance following PTX treatment were examined. We show that G-protein function does not impact on μ-opioid receptor down-regulation, but plays a significant role in opioid tolerance.

2. Material and methods

2.1. Subjects

Male Swiss–Webster mice (35–40 g; Taconic farms, Germantown, NY) were used throughout. Mice were housed 10 per cage for at least 24 h prior to experimentation with free access to food and water. Mice were used once.

2.2. Procedure

Mice were lightly anesthetized with halothane:oxygen (4:96%) and injected intracerebroventricularly (4 μl) in the right lateral ventricle (~2 mm caudal and ~2 mm lateral to bregma at a depth of 3 mm) and intrathecally (2 μl) as described previously (Yoburn et al., 1988). Mice treated with PTX were injected once intracerebroventricularly and intrathecally with PTX (0.1 μg/site). All controls were treated with saline. On the 10th day following PTX treatment, mice were implanted subcutaneously with osmotic minipumps that infused etorphine (150 or 200 μg/kg/day). Other mice were implanted subcutaneously with osmotic minipumps infusing morphine (40 mg/kg/day) and a slow-release morphine pellet (25 mg morphine, 122 mg cellulose, 2.5 mg silicon dioxide, and 1.5 mg magnesium stearate). 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. 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 brain and spinal cord removed for μ-opioid receptor binding studies (see below). The interval between the termination of dosing and collection of tissue is included to allow agonist to be eliminated and to eliminate possible contamination of binding results by residual drug (see Yoburn et al., 1993).

2.3. Analgesia assay and dose–response testing

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 and Smith, 1941). The intensity of the light was adjusted so that baseline flick latencies were 2–4 s. If a mouse failed to flick its tail by 10 s following morphine, 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 the mice in a treatment group (seven per group) were injected subcutaneously 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 (range of doses = 0.5–45.5 mg/kg) 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. The morphine doses used were determined in a previous study (Duttaroy et al., 1997).

2.4. μ-Opioid receptor binding studies

Whole brain and spinal cord were rapidly removed, weighed, and homogenized in 80 vol. of ice-cold 50 mM Tris buffer (pH 7.4). In some cases, midbrain was isolated before homogenization. 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 vol. of phosphate buffer (50 mM, pH 7.2). An aliquot (200 μl) of the whole brain or spinal cord homogenate was assayed in triplicate in tubes containing 0.04–5.0 nM [^{3}H]-DAMGO. For midbrain samples, an aliquot of homogenate was assayed in triplicate using a single saturating concentration (5 nM) of [^{3}H]-DAMGO. Nonspecific binding was determined in the presence of 1000 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, the 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 method (1976) with reagents purchased from Bio-Rad (Richmond, CA).

2.5. 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). PTX

was obtained from List Biological Laboratories (Campbell, CA). [^{3}H]-DAMGO was obtained from NEN Life Sciences (Boston, MA). All compounds were dissolved in normal saline (0.9%). Doses were calculated as the free base.

2.6. Data analysis

Dose response data were analyzed by probit analysis (Finney, 1973) using a computerized program that estimates the ED_{50} and 95% confidence limits. B_{max} and K_d were calculated from saturation studies using nonlinear regression analysis (Prism ver. 3.0, GraphPad software, San Diego, CA). Significant differences among the data were analyzed using ANOVA with appropriate post hoc comparisons. All saturation data were best fit by a one-site model.

3. Results

3.1. The effect of PTX on morphine analgesic potency and tolerance

The mice tolerated PTX treatment well. Although PTX-treated mice weighed less than saline-treated mice ($\approx$3 g) prior to dose–response testing, this difference was not significant ($P > .05$). Furthermore, PTX treatment resulted in less than 3% mortality versus $\approx$1% for saline-treated mice. Baseline tail-flick latencies did not differ significantly ($P > .05$) between saline- and PTX-treated mice.

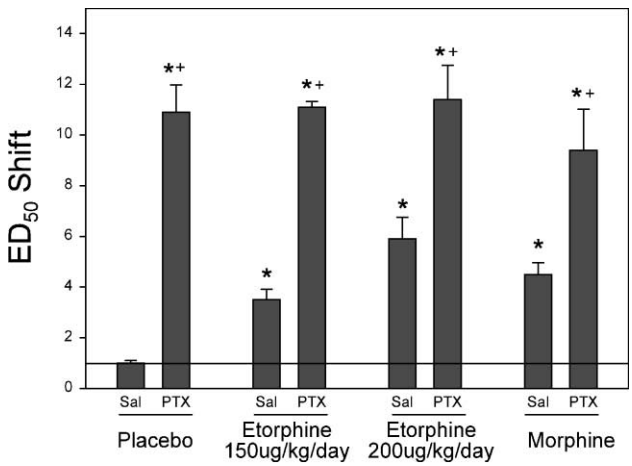

Fig. 1. The effect of PTX on opioid tolerance. Mice were injected with saline (Sal) or PTX (0.1 μg/site). After 10 days, mice were treated with etorphine (150 or 200 μg/kg/day) or morphine for 3 days (see Materials and Methods). At the end of treatment, the morphine ED_{50} was determined. Data are presented 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 is the mean (±S.E.M) of 3–10 experiments. The mean morphine analgesic ED_{50} for the control (saline–placebo) was 2.40±0.22 mg/kg. * Significantly different from the control. $^+$PTX–placebo, PTX–etorphine (150 and 200 μg/kg/day) and PTX–morphine are significantly different from saline–etorphine (150 and 200 μg/kg/day) and saline–morphine, but not significantly different from each other ($P < .05$).

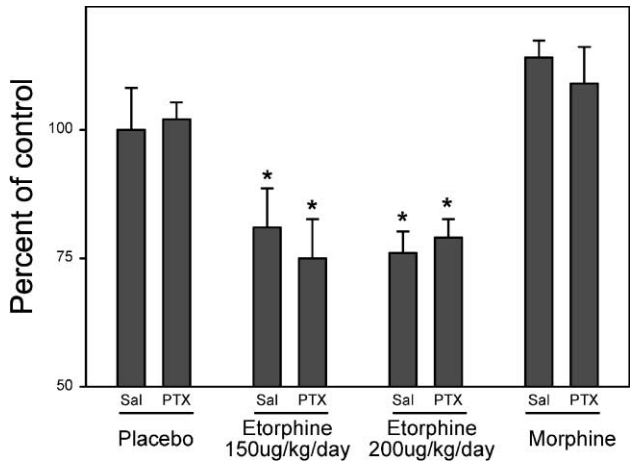

Fig. 2. The effect of PTX treatment on opioid agonist-induced μ-opioid receptor regulation in brain. Mice were injected with saline (Sal) or PTX (0.1 μg/site). After 10 days, mice were treated with etorphine (150 or 200 μg/kg/day) or morphine for 3 days. At the end of treatment, μ-opioid receptor binding ([^{3}H]-DAMGO) was determined in the whole brain (see Materials and Methods). Each bar is the mean (±S.E.M) percent of control (saline–placebo) B_{max} from 3 to 12 experiments determined in saturation binding studies. The mean B_{max} of the control group was 240±20 fm/mg protein. * Significantly different from the control ($P < .05$).

PTX significantly reduced the analgesic potency of morphine by approximately 11-fold in placebo-treated mice (Fig. 1). Both morphine and etorphine treatment produced tolerance to the analgesic effects of morphine. Etorphine infusion (150 and 200 μg/kg/day) produced a significant 3.5- and 5.9-fold shift in morphine analgesic potency with respect to control (Fig. 1). Similarly, morphine treatment produced a significant 4.9-fold shift in morphine analgesic potency with respect to control. When PTX was combined with opioid agonist treatment, there was no further development of tolerance relative to the PTX-treated controls. Neither PTX–etorphine nor PTX–morphine groups were significantly different from PTX–placebo. However, both groups were significantly different from mice not treated with PTX. Taken together, PTX pretreatment blocked the development of further tolerance to both etorphine and morphine.

3.2. The effect of PTX on μ-opioid receptor density

PTX treatment had no effect on μ-receptor density in the whole brain (Fig. 2), spinal cord, or midbrain (Table 1). Continuous etorphine infusion (150 and 200 μg/kg/day) produced a significant decrease (19% and 25%, respectively) in the B_{max} for [^{3}H]-DAMGO in the whole brain (Fig. 2). Similar changes were observed for spinal cord and midbrain from etorphine-treated (200 μg/kg/day) mice (Table 1). Morphine had no effect on μ-opioid receptor density either in the absence or presence of PTX. When PTX and etorphine treatments were combined, etorphine-induced down-regulation was not altered (Fig. 2, Table 1). Morphine treatment did not produce any change in receptor

Table 1
The effect of PTX treatment on opioid receptor regulation in the spinal cord and midbrain

Treatment	Specific binding (fm/mg protein)	Percent of control binding
Spinal cord	B_{max}	
Saline–placebo	211.5 ± 9.1	100
PTX–placebo	198.6 ± 7.7	93.9
Saline–morphine	192.0 ± 4.3	90.8
PTX–morphine	196.0 ± 5.5	92.7
Saline–etorphine	149.7 ± 11.1	70.8*
PTX–etorphine	127.6 ± 3.0	60.3*
Midbrain		
Saline–placebo	185.2 ± 2.9	100.0
PTX–placebo	164.0 ± 6.0	88.6
Saline–morphine	203.9 ± 6.3	110.1
PTX–morphine	165.2 ± 5.2	89.2
Saline–etorphine	122.1 ± 5.3	66.0*
PTX–etorphine	98.9 ± 3.3	53.4*

Mice were treated with etorphine (200 μg/kg/day) or morphine as described in Fig. 2. Saturation binding parameters (± S.E.M.) for spinal cord ($n = 12$ per group) were determined using nonlinear regression analysis of [^{3}H]-DAMGO binding. Data for midbrain ($n = 5$ per group) are from a single concentration (5 nM) studies using [^{3}H]-DAMGO.

* Significantly different ($P < .05$) from control (saline–placebo).

density or affinity. K_d's were not significantly different from control for any group ($K_d = 0.9–1.5$).

4. Discussion

Opioid receptors, like other GPCR, can undergo adaptations following agonist treatment (Law et al., 2000). These include receptor desensitization, internalization, and down-regulation. Although the initial step in opioid receptor signal transduction involves G-protein-mediated activation of intracellular systems (e.g., adenylyl cyclase, ion channels), the role that G-proteins play in receptor internalization, down-regulation, and tolerance in the intact animal was unclear. In the present study, the contribution of PTX-sensitive G-proteins to these effects was examined using an intact animal model of μ-opioid receptor regulation and tolerance. Our results indicate a role for PTX-sensitive G-proteins in tolerance but not in the down-regulation of μ-receptors.

Virtually all opioid agonists have been shown to produce some degree of tolerance (Kato et al., 1998; Yu et al., 1997; Goode and Raffa, 1997; Duttaroy and Yoburn, 1995). Typically, only high-intrinsic-efficacy agonists promote receptor internalization and down-regulation in cell culture and intact animal systems (Zaki et al., 2000; Whistler et al., 1999; Kato et al., 1998; Yabaluri and Medzihradsky, 1997; Law et al., 1985). This distinction in the ability of opioid agonists to regulate receptor trafficking implies that there is a difference between the intracellular mechanisms activated by high and low intrinsic efficacy opioid agonists. Since G-protein activation is believed to be the first step in opioid receptor signaling (Shen et al., 1998; Standifer et al., 1996; Raffa et al., 1994; Cox, 1993), it represents an important intracellular target to evaluate for possible differential activation by opioid agonists.

The inactivation of $G_{i/o}$-proteins dramatically reduces acute opioid agonist potency (e.g., Shen et al., 1998; Standifer et al., 1996; Sanchez-Blazquez et al., 1995; Raffa et al., 1994). The present results confirm that PTX-sensitive G-proteins are involved in the acute potency of opioids. Based on the reduction in acute opioid potency, it was anticipated that interference with $G_{i/o}$-protein function would also affect tolerance. When animals treated with PTX were infused with morphine or etorphine, there was no further decrease in agonist potency. Thus, treatment with PTX, which shifted the ED_{50} by approximately 11-fold, blocked any further reductions in opioid potency by chronic morphine or etorphine exposure. As such, there was no opioid tolerance in PTX-treated mice as compared to PTX-alone groups. These results are in agreement with data that indicate that PTX treatment partially reduces opioid dependence produced by morphine (Parolaro et al., 1990). Thus, chronic opioid effects such as tolerance and dependence appear to be mediated by PTX-sensitive G-proteins.

As noted in earlier reports (Keith et al., 1996; Yabaluri and Medzihradsky, 1997; Stafford et al., 2001), morphine treatment had no significant effect on μ-receptor density, regardless of whether mice were treated with PTX. Similarly, PTX treatment alone did not affect μ-opioid receptor number or affinity, as reported previously (see Chang et al., 1991). In contrast, the high-intrinsic-efficacy agonist etorphine down-regulated μ-opioid receptors in brain, cord, and midbrain. Furthermore, unlike opioid-induced tolerance, which was absent in PTX-treated mice, down-regulation induced by etorphine was intact following PTX treatment (Fig. 2, Table 1). Taken together, these data indicate that high-intrinsic-efficacy agonists down-regulate μ-opioid receptors via a PTX-insensitive mechanism. Interestingly, opioid receptor up-regulation induced by chronic opioid antagonist treatment is also unaffected by PTX treatment (Chang et al., 1991), a finding that suggests that opioid receptor regulation in vivo may be generally independent of G-protein signaling.

The present results extend observations that PTX-sensitive G-proteins are required for the acute and chronic functional effects of opioids (Burford et al., 1998; Parolaro et al., 1990; Shah et al., 1997; Yabaluri and Medzihradsky, 1997; Zaki et al., 2000). In addition, these results are consistent with cell culture studies that show G-proteins are, at best, only partially involved in down-regulation induced by high-intrinsic-efficacy agonists (e.g., Zaki et al., 2000; Kato et al., 1998; Yabaluri and Medzihradsky., 1997; Law et al., 1985). However, the exact mechanisms that mediate down-regulation are unclear. It is possible that high-intrinsic-efficacy opioid agonists may shift the μ-opioid receptor into a conformation that is a more optimal target for phosphorylation. Agonists such as DAMGO and etorphine that cause down-regulation have been shown to

produce more phosphorylation of receptors than morphine (Yu et al., 1997; Zhang et al., 1998). Receptor phosphorylation by G-protein receptor kinases (GRK) may allow adapter proteins like β-arrestin to bind and direct receptor trafficking (Ferguson et al., 1998). That morphine-activated μ-receptors are a less than optimal target for β-arrestin and GRKs is supported by cell culture 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).

Taken together, this study provides insight into the relationship between G-protein activation and regulation of receptor number and tolerance in the intact animal. Our findings indicate that G-proteins have a minimal role in the regulation of the μ-opioid receptor but appear to play an important role in the development of tolerance and the acute effects of opioids.

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