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Chronic opioid antagonist treatment dose-dependently regulates μ-opioid receptors and trafficking proteins in vivo

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Abstract

Chronic opioid antagonist treatment increases the density of μ-opioid receptors (μOR) in many model systems. In previous studies, naltrexone treatment produced an increase in μOR density accompanied by decreases in GRK-2 and DYN-2 protein abundance. To examine the relationship between changes in receptor density and proteins involved in receptor trafficking, the dose-dependent effect of chronic naloxone infusion was determined. Dose-dependent antagonism of morphine analgesia was also examined. Mice were infused with naloxone (0.1, 1.0, 5.0 mg/kg/day sc) for 7 days via osmotic pump. Controls were treated with placebo pellets. On the 7th day, morphine dose–response studies were determined using the tail flick. Other mice were sacrificed at the end of the treatment and spinal cords were collected for determination of μOR density and GRK-2 and DYN-2 protein abundance. Naloxone infusion dose-dependently increased spinal μOR density with no change in affinity. The increases in μ-receptor density were proportional to dose-dependent decreases in GRK-2 and DYN-2 protein levels. Furthermore, naloxone dose-dependently antagonized morphine. These data suggest that opioid antagonist-induced μOR up-regulation in mouse spinal cord is associated with regulation of proteins involved in receptor trafficking and support suggestions that opioid antagonist-induced receptor up-regulation is due to reduced constitutive internalization of opioid receptors.

Keywords: μ-Opioid receptor; Up-regulation; Trafficking proteins; Analgesia; Antagonist; Spinal cord

1. Introduction

Chronic treatment with opioid receptor antagonists (e.g., naloxone or naltrexone) increases the density of opioid receptors in cell culture and in vivo models (Zukin and Tempel, 1986; Zadina et al., 1994; Bhargava et al., 1993; Castelli et al., 1997; Yoburn et al., 1986; Duttaroy et al., 1999; Unterwald et al., 1995). Studies have demonstrated increased μ, δ and κ receptor density following chronic opioid antagonist treatment in both cell culture and in vivo systems, although up-regulation of μ-opioid receptors appears to be of the largest magnitude (e.g., Yoburn et al., 1995; Zadina et al., 1994). Although opioid antagonist-induced up-regulation is a robust and highly reliable phenomenon, the mechanisms that account for the increase in opioid receptor density remain to be determined. Previous studies have implicated proteins involved in receptor trafficking such as GRK, DYN and β-arr in regulation of opioid receptors (Patel et al., 2002b; Zhang et al., 1998; Tsao and von Zastrow, 2001). GRKs and DYNs have been shown to play an important role in opioid agonist-induced receptor internalization in cell culture (Zhang et al., 1998; Whistler and von Zastrow, 1998). Recently, it has been proposed that these proteins also are important in opioid antagonist-induced receptor up-regulation in mouse spinal cord (Patel et al., 2002a).

To date, opioid antagonist-induced regulation of trafficking proteins in the mouse spinal cord has been studied using one antagonist (naltrexone) and a single chronic dosing protocol (Patel et al., 2002a). Therefore, the current study examined if regulation of trafficking proteins (GRK-2, DYN-2) occurs with a different opioid antagonist (nalox-

Abbreviations: GRK, G-protein receptor kinase; DYN, dynamin; β-arr, β arrestin; DAMGO, [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin; GPCR, G-protein coupled receptor; PVDF, polyvinylidene difluoride; μOR, μ-opioid receptor.

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one). We also examined the dose-dependent relationship between antagonism of morphine analgesia and regulation of μOR density and trafficking protein abundance. This in vivo study suggests that opioid antagonist-induced up-regulation of μORs depends upon regulation of intracellular proteins implicated in receptor trafficking.

2. Materials and methods

2.1. Subjects

Male, Swiss–Webster mice (22–24 g) (Taconic Farms, Germantown, NY) were used in all experiments. Animals were housed 10 per cage for at least 24 h after arrival with free access to food and water prior to experimentation. Each mouse was used only once. All procedures were approved by the St. John's University Animal Care and Use Committee.

2.2. General procedure

Osmotic minipumps (Alzet model 2001; Alza, Palo Alto, CA) that infused naloxone (0.1, 1.0, 5.0 mg/kg/day, 1 μl/h) were implanted subcutaneously in mice for 7 days. To reduce costs, controls were implanted with an inert placebo pellet, which has no significant effect on any outcome measure (Duttaroy and Yoburn, 1995). The pellets and pumps were implanted at the nape of the neck while mice were lightly anesthetized with halothane/oxygen (4:96). At the end of the 7 days naloxone or placebo treatment, mice were tested in morphine-induced analgesia assays or sacrificed and spinal cords were collected for receptor binding and western blotting assays.

2.3. μOR binding

Binding was performed as described previously (Yoburn et al., 1995). Briefly, mice (n = 12/group) were sacrificed and spinal cord removed, pooled and homogenized in 80 volumes of ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged at 15,000 rpm (2–8 °C) for 15 min, supernatants were discarded and pellets were resuspended and incubated for 30 min at 25 °C in 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged again and the pellets were finally resuspended in 80 volumes of 50 mM phosphate buffer (pH 7.2). An aliquot (200 μl) of homogenate was assayed in triplicate in tubes containing 0.03–5 nM [^{3}H] [D-Ala^2, N-MePhe^4, Gly^5-ol] enkephalin (DAMGO) (μ ligand, New England Nuclear, Boston, MA). Nonspecific binding was determined in the presence of 1000 nM levorphanol. Tubes were incubated for 90 min at 25 °C and the incubation terminated by filtration of samples over GF/B glass fiber filters (Brandel, Gaithersburg, MD). Filters were washed three times with cold phosphate buffer and transferred to vials containing scintillation cocktail and counted. Counts per minute (CPMs) were converted to disintegration per minute (DPMs) using the external standard method. Protein was assayed by the Bradford method (Bradford, 1976) using reagent purchased from Bio-Rad (Richmond, CA). Independent saturation binding studies were repeated 2–4 times for each dose of naloxone and controls.

2.4. Analgesia assay

Analgesia testing was conducted with placebo pellets and pumps still implanted on the 7th day following the start of treatment. Analgesia 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. The intensity of the light was adjusted so that baseline tail flick latencies were 2–4 s. Mice (n = 7–9/treatment) were injected with 0.5 mg/kg sc morphine and tested for antinociception 30 min later. If a mouse failed to flick by 10 s, it was defined as analgesic. Mice that were not analgesic, were injected within 5 min of testing with a second dose of morphine (0.5 mg/kg) and tested again 30 min later. Testing was continued in this manner until all mice were analgesic (cumulative dose range = 0.5–5.5 mg/kg). All testing was conducted in a blind manner.

2.5. Western-blotting assay

Mice (n = 4/treatment) were sacrificed, individual spinal cords were rapidly removed on ice and homogenized (Brinkman Polytron Homogenizer, 20,000 rpm 30 s) in 500 μl lysis buffer [2% SDS, 1 mM sodium orthovanadate, 12.5 mM Tris (pH 7.4)], boiled for 5 min and centrifuged at 10,000 rpm (15 °C) for 10 min. The supernatant was removed for analysis and protein concentration was determined (Bradford, 1976). Samples were diluted using a mixture of equal volume of lysis and sample buffer (4% SDS, 1% β-mercaptoethanol, 20% Glycerol, 125 mM Tris base, loading dye). An aliquot of the diluted sample (8 μl, 0.25–2 μg of protein) was loaded on polyacrylamide gels (Pager Gels 10% Tris-Glycine, Biowhittaker Molecular Applications, Rockland, ME) and samples were separated by electrophoresis (150 V for 60 min). A sample from an individual spinal cord was loaded on each lane. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using the miniprotean II (Bio-Rad) at 100 V for 85 min. Nonspecific binding sites on the membrane were blocked by incubation (2 h at 24 °C or overnight at 4 °C) in blocking buffer (0.2% Aurora Blocking Reagent; 1X Phosphate Buffered Saline: 0.058 M Na_2HPO_4, 0.017 M NaH_2PO_4, 0.068 M NaCl; 0.05% Tween-20 from ICN Biomedicals, Costa Mesa, CA) followed by incubation (1 h, 24 °C) with primary antibody in blocking buffer (Rabbit polyclonal IgG for GRK-2 (1:200); Goat polyclonal IgG for DYN-2 (1:300), Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed twice with blocking buffer and then incubated (1 h, 24

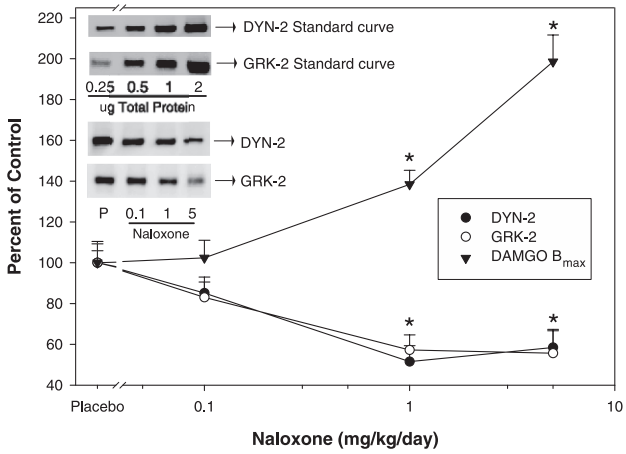

Fig. 1. Effects of chronic naloxone on μOR density, GRK-2 and DYN-2 protein abundance in mouse spinal cord. Mice ($n=4$/treatment in three independent experiments) were chronically treated with naloxone (0.1, 1.0, 5.0 mg/kg/day) or placebo (P) for 7 days. At the end of treatment, mice were sacrificed and spinal cords were collected. Binding data (Control $B_{max} \pm$ S.E.M. $= 189 \pm 17$ fmol/mg protein) represents the mean percent of control from two to four experiments. Protein data are mean (± S.E.M. of all experiments) percent of placebo control from three independent experiments comprising four individual spinal cords per treatment (total $n=12$/treatment; mean ± S.E.M. protein equivalents (μg/lane) for each placebo control group $= 0.61 \pm 0.06$ and 0.85 ± 0.07 for GRK-2 and DYN-2, respectively). * Significantly different from placebo and 0.1 mg/kg/day naloxone ($P<.05$). The inset presents representative blots from standard curves (0.25, 0.5, 1.0, 2.0 ug total protein) and blots demonstrating the dose-dependent decrease in GRK-2 and DYN-2 protein abundance.

°C) with secondary antibody in blocking buffer (Anti-rabbit IgG-AP for GRK-2 (1:5000), ICN Biomedicals; Anti-goat IgG-AP for DYN-2 (1:5000), Santa Cruz Biotechnology). Membranes were then washed thrice with blocking buffer, followed by two quick rinses with Assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM $MgCl_2$). Bands were visualized using a Chemiluminescence assay (CDP Star Substrate, Novagen, Madison, WI). A standard curve using increasing amounts of spinal cord protein from controls (0.25-2.0 μg/lane) was included on each gel. This allowed conversion of optical density into valid estimates of percent change in protein. All data are expressed as percent of control. Each experiment was replicated three times with new groups of mice.

2.6. Drugs

Naloxone HCl and corresponding placebo pellets were from Dupont Pharmaceuticals (Wilmington, DE) and Research Triangle Institute (Research Triangle Park, NC). [^{3}H] DAMGO was obtained from NEN Life Sciences (Boston, MA). Naloxone was dissolved in 0.9% saline and doses are expressed as the free base.

2.7. Data analysis

Gel images were captured using a FluorChem ver 2.0 Imaging System (Alpha Innotech, San Leandro, CA). The images were digitized and analyzed for optical density using GelPro image analysis software (ver 3.1, Media Cybernetics, Silver Spring, MD). Optical densities from Western blot data were converted to protein equivalents using the standard curves and evaluated using ANOVA ($P<.05$). B_{max} and K_D values were estimated from saturation studies using nonlinear regression (Prism ver 3.02, Graphpad Software, San Diego, CA). Binding data were best fit by a one-site model. Significant differences ($P<.05$) among the groups were analyzed using ANOVA with appropriate post hoc comparisons. Dose–response data were analyzed by Probit analysis (Finney, 1971) using a computerized program (BLISS 21, Department of Statistics, University of Edinburgh, Edinburgh, Scotland) that estimates ED_{50}s ($P<.05$) and relative potencies. Probit analysis was used to determine significant differences among relative potencies of morphine.

3. Results

In saturation binding studies, chronic naloxone treatment dose-dependently increased [$F(3,11)=14.7$, $P<.0$] μOR density in mouse spinal cord (Fig. 1) without altering affinity [$F(3,11)=1.7$, $P>.05$; mean $K_D \pm$ S.E.M. $= 0.71 \pm 0.0$, 0.74 ± 0.04, 0.71 ± 0.02 and 0.71 ± 0.01 nM; Placebo, Naloxone 0.1, 1.0 and 5.0 mg/kg/day, respectively]. Chronic naloxone treatment dose-dependently decreased GRK-2 and DYN-2 protein abundance in mouse spinal cord [$F(3,17)>10.5$, $P<.01$]. The standard curves for GRK-2 and DYN-2 proteins were linear and included the range of optical densities employed for unknowns. A representative

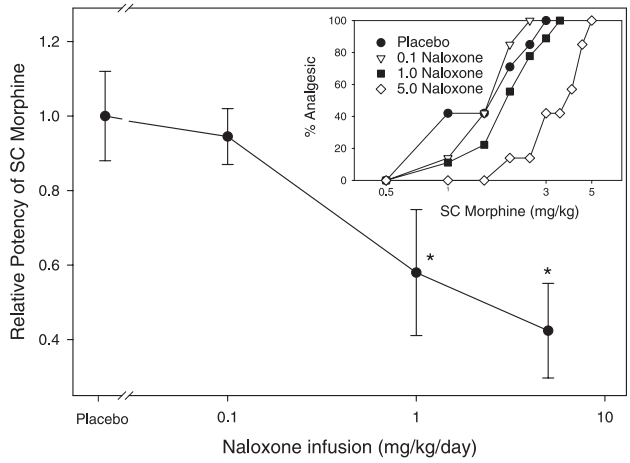

Fig. 2. Effect of chronic naloxone on morphine analgesia. Mice were implanted with placebo pellets or infused with naloxone (0.1, 1.0, 5.0 mg/kg/day sc) for 7 days. On the 7th day, cumulative dose–response studies were conducted for morphine analgesia using the tail flick. Mice were subcutaneously administered morphine and tested for analgesia 30 min later. Testing was continued until all mice were analgesic. The relative potency data are summarized from four experiments. The inset depicts representative cumulative dose–response functions for each condition. * Significantly different from placebo by Probit analysis ($P<.05$).

blot for standard curves for GRK-2 and DYN-2 is shown in the insets in Fig. 1 (mean ± S.E.M., r^2=.96 ± .01 and .96 ± .02, three assays GRK-2 and DYN-2, respectively). Chronic naloxone treatment dose-dependently shifted the morphine analgesia dose response curve to the right (Inset, Fig. 2) and decreased morphine relative potency (Fig. 2).

4. Discussion

Opioid antagonists reliably produce μOR up-regulation in whole animal and in cell culture (Unterwald et al., 1995, 1998; Zadina et al., 1994; Patel et al., 2002a; Narita et al., 2001). However, the mechanisms that mediate opioid antagonist-induced up-regulation of μOR have not been determined. Recent studies have shown that increases in μOR density and potency of μ-agonists are associated with regulation of several proteins involved in GPCR trafficking. Specifically, GRK-2 and DYN-2 protein have shown to be decreased following chronic naltrexone treatment (Patel et al., 2002a). To date, only one dosing protocol and one opioid antagonist have been utilized to regulate these proteins and μOR density in the mouse spinal cord. Therefore, in this study, the effect of various infusion doses of naloxone on GRK-2, DYN-2 and μOR density were determined.

The μ-opioid antagonist naloxone produced dose-dependent up-regulation of μOR in mouse spinal cord and dose-dependent decreases in GRK-2 and DYN-2 protein abundance. At the lowest naloxone dose (0.1 mg/kg/day), there were no significant changes in μOR, GRK-2 or DYN-2. In addition, the lowest dose of chronic naloxone was ineffective in antagonizing subcutaneous morphine analgesia. With increasing naloxone dose, μOR density was up-regulated in parallel with decreases in GRK-2 and DYN-2 and significant antagonism of morphine potency.

Previous reports suggest that opioid antagonist-induced up-regulation in vivo may be due to inhibition of constitutive internalization (Patel et al., 2002a). Similarly, studies indicate that antagonist-induced up-regulation of some μOR splice variants and mutants depend on proteins involved in GPCR trafficking and interference with constitutive internalization (Koch et al., 2001; Li et al., 2001). The current results are consistent with these data. The reduction in GRK-2 and DYN-2 protein abundance observed in the present study may be required for μOR up-regulation; much like opioid agonist induced μOR down-regulation depends upon these trafficking proteins (Whistler and von Zastrow, 1998; Zhang et al., 1998; Patel et al., 2002b). Finally, there was no significant regulation of GRK-2 or DYN-2 or up-regulation of μOR density at the lowest naloxone infusion dose. Since the low naloxone dose did not antagonize opioid agonist effects, it may be the case that regulation of proteins implicated in receptor trafficking and μOR up-regulation requires a minimal level of receptor occupancy by opioid antagonists. It should be noted that Diaz et al. (2002) reported increases in GRKs following antagonist treatment in rat brain. The apparent difference between those results and the present data may be due to the 24-h interval between treatment and protein analysis. Studies have shown that opioid antagonist-induced up-regulation declines rapidly following treatment (Tempel et al., 1982; Yoburn and Inturrisi, 1988). It is possible that GRK levels rebound above control soon after the end of treatment.

In summary, the current data indicate that there is a close association between opioid antagonist-induced up-regulation of μOR and decreases GRK-2 and DYN-2 protein abundance. These results support suggestions that chronic opioid antagonist induced up-regulation may depend upon inhibition of constitutive cycling of μOR in mouse spinal cord.

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