Continuous Opioid Agonist Treatment Dose-Dependently Regulates μ-Opioid Receptors and Dynamin-2 in Mouse Spinal Cord

QIUYU ZHANG, VISHAL PUROHIT, AND BYRON C. YOBURN*
Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, New York 11439

KEY WORDS μ opioid receptors (μOR); dynamin-2; mouse spinal cord; down-regulation; mRNA; etorphine

ABSTRACT Continuous opioid agonist treatment produces tolerance and in some cases μ opioid receptor (μOR) down-regulation. Previous studies indicate that down-regulation of μOR is more likely with high-efficacy opioid agonists (e.g., etorphine), whereas lower efficacy agonists (e.g., morphine) do not regulate μOR density. It has been suggested that μOR down-regulation may depend upon increases in Dynamin-2 (DYN-2) proteins. Therefore, the present study examined the effect of various infusion doses of etorphine on μOR density, DYN-2 protein, and DYN-2 mRNA abundance in mouse spinal cord. Mice were implanted sc with an osmotic pump that infused etorphine (50–250 μg/kg/day). Controls were implanted with inert placebo pellets. At the end of 7 days, mice were sacrificed, spinal cord removed and processed for radioligand binding, quantitative Western blotting, or RT-PCR assay. Results indicate that etorphine induced dose-dependent regulation of μOR density, DYN-2 proteins, and mRNA abundance in mouse spinal cord. Higher infusion doses significantly down-regulated μOR density, increased DYN-2 protein abundance, and decreased DYN-2 mRNA. Analysis of these results indicated a significant correlation between μOR down-regulation and DYN-2 abundance in mouse spinal cord. Taken together, μOR regulation may depend on changes in DYN-2 abundance induced by high-efficacy opioid agonists in mouse spinal cord. **Synapse 56:123–128, 2005.** © 2005 Wiley-Liss, Inc.

INTRODUCTION

High-efficacy opioid agonists such as etorphine have been shown to induce μOR internalization in vitro (Whistler et al., 1999; Zaki et al., 2000) and down-regulation in vivo (Patel et al., 2002b; Yoburn et al., 2004). Studies indicate that trafficking proteins such as dynamin (DYN) and G-protein receptor kinase (GRK) are involved in μOR internalization and down-regulation (Whistler et al., 1998; Zhang et al., 1998; Patel et al., 2002b). Cell culture studies indicate that dominant negative mutants of DYN inhibit etorphine-induced μOR internalization (Whistler et al., 1998; Zhang et al., 1998). Previous studies in our lab indicate that μOR down-regulation is accompanied by an increase in DYN-2 abundance and a decrease of DYN-2 mRNA levels in mouse spinal cord, whereas morphine-induced tolerance does not produce μOR down-regulation or changes in DYN-2 (Patel et al., 2002b; Yoburn et al., 2004). Similarly, Noble et al. (2000) have shown that chronic morphine treatment produces no change in total immunoreactive DYN in mouse or rat whole brain, although increases in the caudate putamen were observed. Furthermore, Rajeshekara et al. (2003) have shown that chronic opioid antagonist (e.g., naloxone) treatment dose-dependently up-regulated μOR density and decreased DYN-2 abundance in mouse spinal cord. These findings suggest that μOR regulation by opioid agonists and antagonists may depend upon changes of DYN-2 abundance in intact animals.

Contract grant sponsor: National Institutes of Health; Contract grant number: 12868.

*Correspondence to: Byron C. Yoburn, Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, NY 11439. E-mail: yoburnb@stjohns.edu

Received 12 November 2004; Accepted 10 January 2005

DOI 10.1002/syn.20137

Published online in Wiley InterScience (www.interscience.wiley.com).

© 2005 WILEY-LISS, INC.

To date, only one agonist infusion dose protocol has been examined for changes in μOR density and DYN-2 abundance in mouse spinal cord (Patel et al., 2002a,b; Yoburn et al., 2004). A dose-dependent protocol is one approach to evaluate the link between μOR down-regulation and DYN-2 abundance. Therefore, we have explored the effects of different doses of etorphine treatment on μOR density and DYN-2 abundance in mouse spinal cord. The current data suggest that there is a significant correlation between μOR down-regulation and DYN-2 abundance in mouse spinal cord. These data are in agreement with our prior suggestion that μOR regulation in the intact animal may depend upon DYN-2 trafficking (e.g., Rajeshekara et al., 2003; Patel et al., 2002b).

MATERIALS AND METHODS

Subjects

Male Swiss-Webster mice (20–30 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 Institutional Animal Care and Use Committee.

Procedure

Osmotic minipumps (Alzet model 2001; Durect Corp., Cupertino, CA) that infused etorphine (50, 100, 150, 200, and 250 μg/kg/day) were implanted subcutaneously in mice for 7 days. Controls were implanted with an inert placebo pellet (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, mice were sacrificed and spinal cords were collected for Western blotting assays or reverse transcriptase-polymerase chain reaction (RT-PCR) assays. For receptor binding assays, osmotic minipumps and pellets were removed on day 7 of etorphine and placebo treatment. Mice were sacrificed 16 h after termination of etorphine treatment (see Yoburn et al., 1993) and spinal cord collected for radioligand binding assay.

Western blotting assay

Mice (n = 5–8/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 (3% SDS, 1.5 mM sodium orthovanadate, 6.25 mM Tris, pH 7.4), centrifuged at 10,000 rpm (15°C) for 10 min and the supernatant was removed for analysis. Protein concentration was determined by the Bradford method (Bradford, 1976) with reagent purchased from Bio-Rad (Hercules, CA) using an Opsys MR™ Microplate Reader (DYNEX Technologies, Chantilly, VA). Samples were diluted using a mixture of equal volume of lysis and 2 × sample buffer (4% SDS, 2% β-mercaptoethanol, 10% Glycerol, 125 mM Tris base, loading dye). Samples were incubated in boiling water for 5 min. An aliquot of the sample (8 μl; 0.5 μg of protein for DYN-2; 16 μg of protein for actin) was loaded on the polyacrylamide gels (Pager Gels 10% Tris-Glycine, Biowhittaker Molecular Applications, Rockland, ME) and samples separated by electrophoresis (150 V for 60 min). A sample from one spinal cord from each mouse was loaded on each lane. Protein was 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 (overnight at 4°C) in blocking buffer (0.2% Aurora Blocking Reagent from ICN Biomedicals, Costa Mesa, CA; 1× Phosphate Buffered Saline: 0.058 M Na_2HPO_4, 0.017 M NaH_2PO_4, 0.068 M NaCl; 0.05% Tween-20) followed by incubation (1–1.5 h, 24°C) with primary antibody (Goat polyclonal IgG for DYN-2 [1:300] or actin [1:300]; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. Membranes were washed twice with blocking buffer and then incubated (1 h, 24°C) with secondary antibody (Donkey anti-goat IgG-AP [1:5,000], Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. 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 for DYN-2; 4.0–32.0 μg/lane for actin) was included on each gel. This allowed conversion of optical density into valid estimates of percent change in protein. All data were converted to protein equivalents and were expressed as percent of control. Each experiment for DYN-2 was replicated two to three times.

μOR binding

Binding was performed as described previously (Yoburn et al., 1995). Since previous studies (Stafford et al., 2001; Patel et al., 2002b) have indicated that etorphine significantly regulates B_{MAX} and not K_D, the present study used a single concentration of radioligand so that μOR binding could be determined in individual spinal cords from each mouse. Mice (n = 6/group) were sacrificed and spinal cord removed. Each spinal cord was placed in an individual tube and homogenized in 4 ml ice-cold 50 mM Tris buffer (50 mM Tris-HCl, pH 7.4) for 20 sec. Homogenates were centrifuged at 16,500 rpm (2–4°C) for 20 min, supernatants were discarded, and pellets were resuspended with the same volume of ice-cold 50 mM Tris buffer and incubated for

30 min at 25°C. Homogenates were centrifuged again at 16,500 rpm (2–4°C) for 20 min and the pellets were finally resuspended in 1.6 ml 42 mM phosphate buffer (75 mM Dibasic potassium phosphate, 100 mM Monobasic potassium phosphate, pH 7.2). An aliquot (200 μl) of homogenate from each cord was assayed in triplicate in tubes containing 1 nM [^{3}H] [D-Ala^2, *N*-MePhe^4, Gly^5-ol] enkephalin (DAMGO) (μ ligand, PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was determined in the presence of 1,000 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). Binding studies were repeated 1–2 times for each dose of etorphine and controls.

RT-PCR Assay

Total RNA was extracted from individual mouse spinal cords (n = 5–8/treatment) using TRIzol reagent (Invitrogen, Carlsbad, CA) and precipitated in 100% isopropyl alcohol. Yeast tRNA (25 μg) was added as a carrier to facilitate the precipitation of RNA prepared from a single spinal cord. The pellet was washed in 75% ethanol, dried, and suspended in 75 μl of RNase-free water. The tubes were then treated with 1.5 U of RNase-free DNase (MAXI Script kit; Ambion, Austin, TX) at 37°C for 30 min. RNA was stored at −80°C until analysis. The yield of RNA was determined using UV-spectrometry (260 and 280 nm).

The RT-PCR assay was based on the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, CA). The primers for mouse DYN-2 were CAG TTT GGA GTG GAC TTT GAG AAGC (forward) and AGA CAG GGC TCT TTC AGC TTG ACC (backward) corresponding to bases 131 to 155 and 1,379 to 1,402 (272-bp product) (GenBank, L31398).

The reverse transcription reaction used 0.2 μg of spinal RNA. The two-step RT reaction mixture contained 1.25 μM random hexamers, 2.5 mM $MgCl_2$, 250 μM dNTP blend, 10 U/20 μl RNase inhibitor, 10 mM DTT, 15 U/20 μl MultiScribe reverse transcriptase and 1× RT-PCR buffer (Applied Biosystems, Foster City, CA). Total volume was brought to 20 μl with RNase-free water and tubes placed in a Techne Progene thermal cycler (Techne, Princeton, NJ) at 25°C for 10 min, followed by 42°C for 12 min. The product of the RT step was added to the PCR reaction mix (0.15 μM upstream primer and 0.15 μM downstream primer, 1.75 mM $MgCl_2$, 200 μM dNTP blend, 2.5 U/50μl AmpliTaq Gold DNA polymerase, and 1× RT-PCR buffer). Total volume is brought to 50 μl with diethyl pyrocarbonate treated water. A wax bead was placed in each tube to prevent evaporation. Tubes were then placed in the cycler under the following conditions: a 10-min incubation at 95°C, followed by amplification for 30 cycles at 94°C for 20 sec and 62°C for 1 min, followed by a final extension at 72°C for 7 min. Tubes were brought to 4°C, 1 μl 10× loading dye (Ambion) was added to each tube, and then a 10-μl aliquot containing amplified product was loaded on 1.8% agarose gel and electrophorised (100 V, 60 min). The agarose gel was stained in 0.5 μg/ml Ethidium Bromide for 1 h. The RT-PCR assay yielded the predicted size amplification products for DYN-2 (272 bp). A standard curve for DYN-2 using 0.05, 0.1, 0.2, and 0.4 μg of total RNA was included in each assay so as to validly estimate percentage changes in target mRNAs. The standard curves were used to convert optical densities to arbitrary RNA units for DYN-2 amplified products.

Drugs

Etorphine hydrochloride and corresponding placebo pellets were obtained from Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse. Etorphine were dissolved in 0.9% saline and doses are expressed as the base.

Data analysis

The gel images were digitized (Gel-Pro version 3.0) and bands analyzed for optical density. Optical densities from Western blot data or RT-PCR data were converted to protein or RNA equivalents using the standard curves and evaluated using t test or ANOVA ($P < 0.05$) with appropriate post-hoc comparisons (Fisher LSD test).

RESULTS

Continuous etorphine treatment produced a dose-dependent ($F_{5,121} = 4.9$, $P < 0.05$) increase in DYN-2 protein abundance in mouse spinal cord (Fig. 1A). Lower dose infusions of etorphine (50–150 μg/kg/day) had no effect on DYN-2 protein abundance. Continuous treatment with higher etorphine doses (200, 250 μg/kg/day) significantly (Fisher LSD test, $P < 0.05$) increased DYN-2 immunoreactive abundance in mouse spinal cord (+32%, +46%). Control studies indicated that the highest dose of etorphine (250 μg/kg/day) treatment had no effect ($t < 1.0$; $P > 0.05$) on actin protein abundance (Fig. 2). The standard curves for DYN-2 (Fig. 1C) and actin protein (Fig. 2) were linear and included the range of optical densities employed for unknowns.

In radioligand binding studies, continuous treatment with etorphine (50–250 μg/kg/day) dose-dependently ($F_{5,59} = 19.8$, $P < 0.05$) decreased μOR density in mouse spinal cord (Fig. 1A). The density of μOR was significantly (Fisher LSD test, $P < 0.05$) reduced for

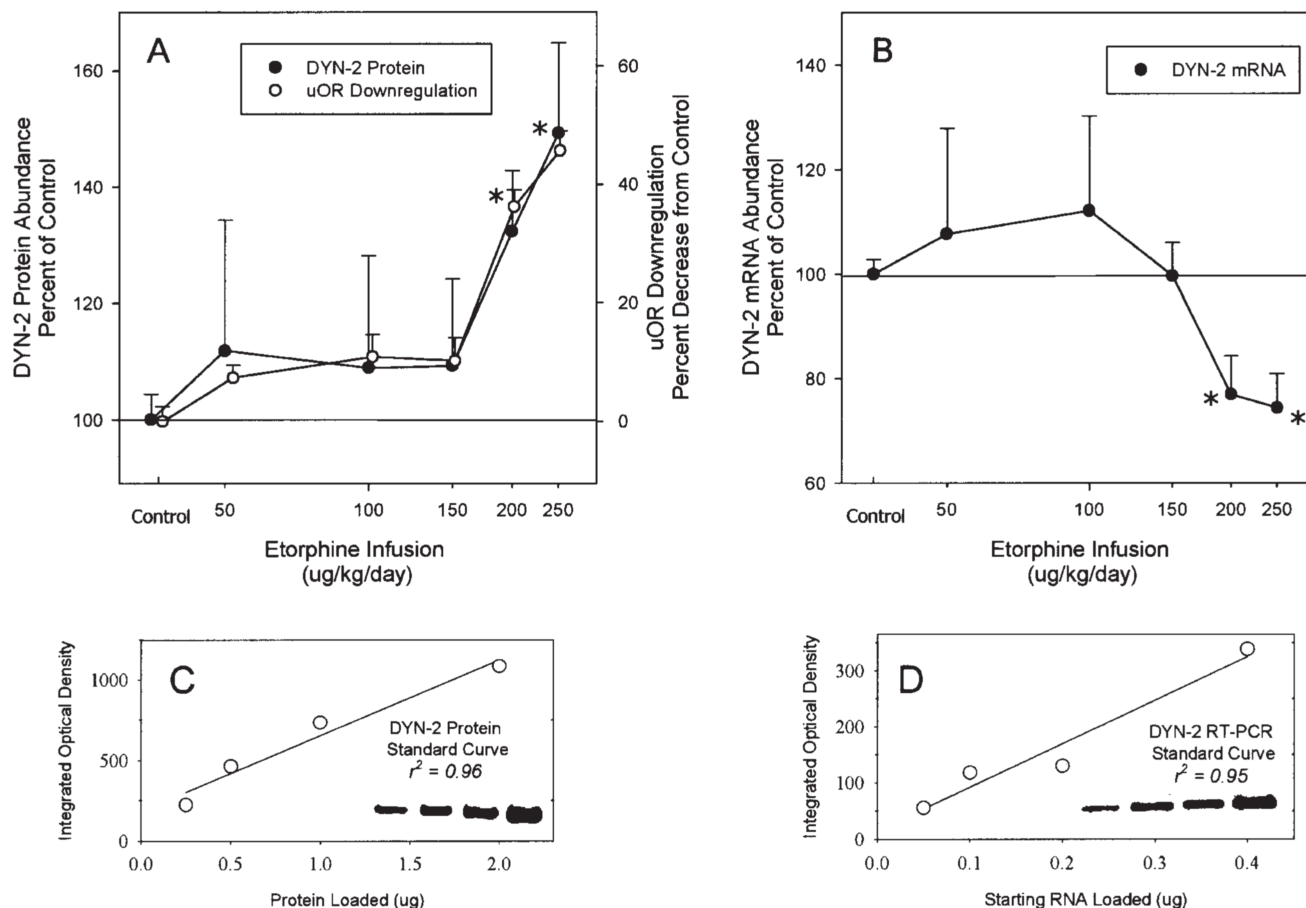

Fig. 1. **A:** Effect of etorphine treatment on DYN-2 protein abundance (left axis) and μOR down-regulation (right axis) in mouse spinal cord. **B:** Effect of etorphine treatment on DYN-2 mRNA abundance. **C,D:** Representative standard curves for DYN-2 Western blots and DYN-2 RT-PCR assays, respectively. Mice (n = 5–8/treatment, 2–3 independent experiments) were continuously infused (sc) for 7 days with etorphine (50, 100,150, 200, and 250 μg/kg/day). Controls were implanted with placebo pellets. At the end of treatment, mice were sacrificed and individual spinal cords were collected for Western blot or RT-PCR studies. For radioligand binding assays (n = 6/treatment, 1–2 independent experiments), pumps and pellets were removed, and 16 h later individual spinal cords collected for [^{3}H]DAMGO binding (see Methods). Data are expressed as the mean ± SEM of all experiments. *Significantly different from control ($P < 0.05$).

the 200–250 μg/kg/day dosing groups compared to the control. Finally, continuous treatment with etorphine dose-dependently ($F_{5,69} = 4.1$, $P < 0.05$) decreased DYN-2 mRNA abundance (Fig. 1B). High infusion doses of etorphine (200 and 250 μg/kg/day) significantly (Fisher LSD test, $P < 0.05$) decreased DYN-2 mRNA abundance (−23%, −26%, respectively) in mouse spinal cord, but lower doses of etorphine (50–150 μg/kg/day) had no effect on DYN-2 mRNA. The standard curves for DYN-2 mRNA (Fig. 1D) were linear and included the range of optical densities for unknowns.

DISCUSSION

High-efficacy opioid agonists have been shown to induce internalization and down-regulation of μOR in cell culture and in the intact animal (Whistler and von Zastrow, 1998; Yoburn et al., 1993; Tao et al., 1989). Since regulation of μOR is associated with changes in trafficking protein abundance, it has been suggested that they may play a role in agonist-mediated receptor internalization and down-regulation of opioid receptors (Whistler and von Zastrow, 1998; Zhang et al., 1998; Patel et al., 2002a). Consistent with this possibility, studies have shown that expression of dominant negative mutants of DYN in HEK 293 cells inhibited opioid agonist-induced μOR internalization (Zhang et al., 1998; Whistler and von Zastrow, 1998).

In the intact mouse spinal cord, agonist-induced μOR down-regulation is accompanied by an increase in DYN-2 protein abundance (Patel et al., 2002a,b; Yoburn et al., 2004), while antagonist-induced μOR up-regulation is associated with a decrease in DYN-2 abundance (Rajashekara et al., 2003). These changes in DYN-2 abundance may alter the rate of μOR internalization and thus affect μOR density. While there is currently no direct way to regulate DYN-2 levels in vivo, evaluating the dose-dependent changes in DYN-2 and μOR density is one strategy to evaluate the role of DYN-2 in μOR

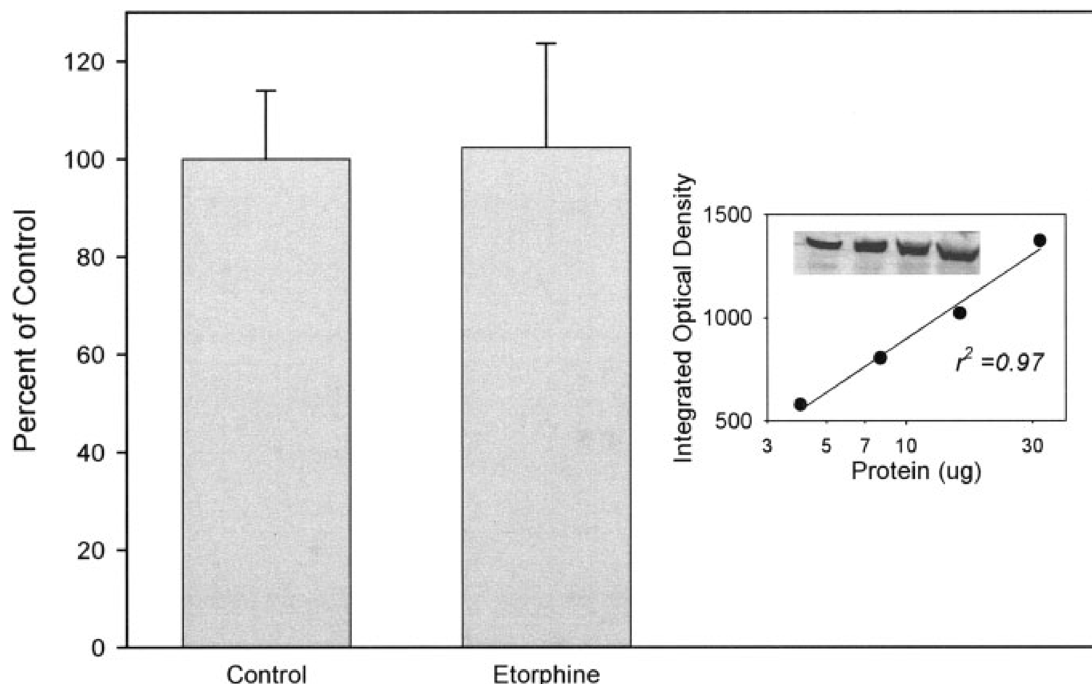

Fig. 2. Effect of etorphine treatment on actin abundance in mouse spinal cord. Mice (5/group) were infused with etorphine (250 μg/kg/day) for 7 days. Controls were implanted with a placebo pellet. At the end of treatment, mice were sacrificed and spinal cords were collected for Western blot studies. The data are the mean (±SEM) percent of control from two independent studies. **Inset:** A typical actin standard curve (4, 8, 16, and 32 μg of total protein).

regulation. Using this approach, Rajashekara et al. (2003) have shown that naloxone dose-dependently increases μOR density while decreasing DYN-2 abundance in the mouse spinal cord. However, to date, the regulation of DYN-2 and μOR density by opioid agonists in the intact mouse has been studied using one infusion dose of etorphine (Patel et al., 2002b; Yoburn et al., 2004). Therefore, the current study examined the dose-dependent effects of etorphine on μOR density, DYN-2 protein, and mRNA abundance in mouse spinal cord.

Etorphine produced a dose-dependent increase in DYN-2 abundance and a dose-dependent decrease in μOR density. In order to probe the relationship between DYN-2 abundance and etorphine-induced regulation of μOR, the correlation between these two outcomes was examined (Fig. 3). There was a significant correlation between DYN-2 abundance and μOR down-regulation in mouse spinal cord. This result supports a role for DYN-2 in etorphine-induced μOR down-regulation in mouse spinal cord. It is likely that the reduction in radioligand binding represents receptor degradation since recent studies indicate that a decrease in [^{3}H] DAMGO binding in mouse spinal cord is associated with a reduction in immunoreactive μOR abundance (Yoburn et al., 2004). Despite the fact that etorphine produces μOR down-regulation, it is important to note that down-regulation is not required for opioid tolerance (Duttaroy and Yoburn, 1995; Stafford et al., 2001). However, μOR down-regulation enhances the magnitude of tolerance in vivo (Stafford et al., 2001).

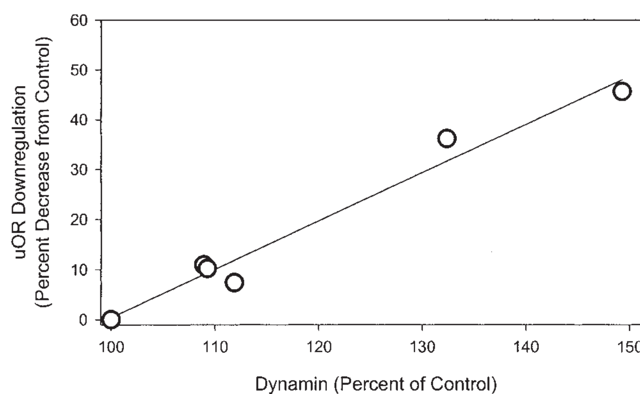

Fig. 3. Correlation between μOR down-regulation and DYN-2 protein abundance in mouse spinal cord. μOR down-regulation data represent the mean percent decrease from control. DYN-2 data are the mean percent increase relative to control. The correlation is statistically significant ($r^2 = 0.97$, $P < 0.05$).

In contrast with the increase in DYN-2 protein abundance, etorphine-induced a significant dose-dependent decrease DYN-2 mRNA levels. This result agrees with our prior findings indicating an inverse relationship between DYN-2 protein and mRNA abundance (Patel et al., 2002b). It is possible that this reduction in mRNA is a consequence of feedback inhibition of transcription (Patel et al., 2002b).

In summary, continuous etorphine treatment dose-dependently regulated μOR density and DYN-2 abundance in mouse spinal cord. The current results suggest

that DYN-2 mediated internalization may be necessary for agonist-induced down-regulation of μOR. Taken together, with previous results using opioid antagonists (e.g., Rajashekara et al., 2003), these data suggest that changes in the trafficking protein DYN-2 induced by opioid agonists and antagonists may contribute to μOR regulation in the intact mouse.

ACKNOWLEDGMENTS

We thank Dr. M.T. Turnock and Kaushal Patel for assistance. These data represent portions of a thesis presented by Qiuyu Zhang to the faculty of the College of Pharmacy and Allied Health Professions, St. John's University, in partial fulfillment of the requirements for the M.S. degree in Pharmaceutical Sciences.

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.

Duttaroy A, Yoburn BC. 1995. The effect of intrinsic efficacy on opioid tolerance. Anesthesiology 82:1226–1235.

Noble F, Szucs M, Kieffer B, Roques BP. 2000. Overexpression of dynamin is induced by chronic stimulation of mu- but not delta-opioid receptors: relationships with mu-related morphine dependence. Mol Pharmacol 58:159–166.

Patel M, Gomes B, Patel C, Yoburn BC. 2002a. Antagonist-induced μ-opioid receptor upregulation decreases G-protein receptor kinase-2 and dynamin-2 abundance in mouse spinal cord. Eur J Pharmacol 446:37–42.

Patel MB, Patel CN, Rajashekara V, Yoburn BC. 2002b. Opioid agonists differentially regulate mu-opioid receptors and trafficking proteins in vivo. Mol Pharmacol 62:1464–1407.

Rajashekara V, Patel CN, Patel K, Purohit V, Yoburn BC. 2003. Chronic opioid antagonist treatment dose-dependently regulates mu-opioid receptors and trafficking proteins in vivo. Pharmacol Biochem Behav 75:909–913.

Stafford K, Gomes GA, Shen J, Yoburn BC. 2001. μ-Opioid receptor down-regulation contributes to opioid tolerance in vivo. Pharm Biochem Behav 69:233–237.

Tao PC, Chang LR, Law PY, Loh HH. 1989. Decrease in delta and mu opioid receptor binding capacity in rat brain after chronic etorphine treatment. J Pharmacol Exp Ther 240:809–819.

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 HH, Chu P, Jan LY, von Zastrow M. 1999. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23:737–746.

Yoburn BC, Billings B, Duttaroy A. 1993. Opioid receptor regulation in mice. J Pharmacol Exp Ther 265:314–320.

Yoburn BC, Shah S, Chan K, Duttaroy A, Davis T. 1995. Supersensitivity to opioid analgesics following chronic opioid antagonist treatment: relationship to receptor selectivity. Pharmacol Biochem Behav 51:535–639.

Yoburn BC, Purohit V, Patel K, Zhang Q. 2004. Opioid agonist and antagonist treatment differentially regulates immunoreactive μ-opioid receptors and dynamin-2 in vivo. Eur J Pharmacol 498:87–96.

Zaki PA, Keith DE Jr, 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 SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, 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.