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μ-Opioid receptor downregulation contributes to opioid tolerance in vivo

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Abstract

The present study examined the contribution of downregulation of μ-opioid receptors to opioid tolerance in an intact animal model. Mice were implanted subcutaneously with osmotic minipumps that infused etorphine (50–250 μg/kg/day) for 7 days. Other mice were implanted subcutaneously with a morphine pellet (25 mg) or a morphine pellet plus an osmotic minipump that infused morphine (5–40 mg/kg/day) for 7 days. Controls were implanted with an inert placebo pellet. At the end of treatment, pumps and pellets were removed, and saturation binding studies were conducted in whole brain ([^{3}H]DAMGO) or morphine and etorphine analgesic ED_{50}s were determined (tail-flick). Morphine tolerance increased linearly with the infusion dose of morphine (ED_{50} shift at highest infusion dose, 4.76). No significant downregulation of μ-receptors in whole brain was observed at the highest morphine treatment dose. Etorphine produced dose-dependent downregulation of μ-opioid receptor density and tolerance (ED_{50} shift at highest infusion dose, 6.97). Downregulation of μ-receptors only occurred at the higher etorphine infusion doses ($\geq$150 μg/kg/day). Unlike morphine tolerance, the magnitude of etorphine tolerance was a nonlinear function of the dose and increased markedly at infusion doses that produced downregulation. These results suggest that μ-opioid receptor downregulation contributes to opioid tolerance in vivo. Therefore, opioid tolerance appears to rely upon both "receptor density-dependent" and " receptor density-independent" mechanisms.

1. Introduction

High intrinsic efficacy opioid analgesics such as etorphine have been shown to be effective in inducing opioid receptor downregulation and dose-dependent tolerance in vivo (e.g., Sehba et al., 1997; Tao et al., 1989; Yoburn et al., 1993). However, previous studies demonstrate that receptor downregulation in vivo is not a requirement for the development of opioid tolerance (Puttfarcken and Cox, 1989; Loh et al., 1988; Tao et al., 1989; Duttaroy and Yoburn, 1995). These results suggest that receptor desensitization, in the absence of downregulation, can play a major role in mediating tolerance. On the other hand, it seems likely that a decrease in receptor density will be expressed as a decrease in agonist potency. In support of this suggestion, previous research demonstrates that doses of etorphine that downregulate μ-opioid receptors produce more tolerance than doses of morphine that do not induce downregulation (Duttaroy and Yoburn, 1995). Furthermore, decreases in μ-opioid receptor availability following administration of irreversible antagonists (Chan et al., 1997; Aceto et al., 1989; Lewis et al., 1989; Comer et al., 1992; Zernig et al., 1994; Burke et al., 1994) or a deficiency of μ-receptors due to genetic factors (Baran et al., 1975; Duttaroy et al., 1999; Moskowitz and Goodman, 1985) have been associated with decreased agonist potency. Finally, chronic opioid antagonist-induced receptor upregulation has been shown to increase the analgesic potency of a wide range of opioid agonists (e.g., Yoburn et al., 1995). Taken together, it is likely that regulation of receptor number, and specifically receptor downregulation, may contribute significantly to the magnitude of tolerance induced by some high intrinsic efficacy opioid agonists.

Formulations of receptor theory (Kenakin, 1997) predict that a reduction in receptor density will be accompanied by a rightward shift in the agonist dose–response function. Nevertheless, it is currently not known if a decrease in μ-opioid receptor density will contribute to the magnitude of tolerance in vivo. In the present study, two relatively μ-opioid receptor selective agonists that differ in their ability to downregulate receptors were used to examine if μ-opioid receptor downregulation is associated with an increase in the magnitude of opioid tolerance in vivo.

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2. Method

2.1. Subjects

Male Swiss–Webster mice (22–40 g) were used throughout the study (Taconic Farms, Germantown, NY). Mice were housed 10–11 per cage with free access to food and water.

2.2. Procedure

In general, mice were chronically treated for 7 days with etorphine HCl or morphine SO_4. Mice treated with etorphine were infused (50–250 μg/kg/day) for 7 days using subcutaneously implanted osmotic pumps (ALZET model no. 2001, Alza, Palo Alto, CA) that delivered pump contents at a rate of 1.0 μl/h. Mice treated with morphine were implanted with an osmotic pump that infused morphine (5–40 mg/kg/day) and a 25-mg morphine pellet for 7 days. One group of mice was implanted with a 25-mg morphine pellet only. All doses were calculated as the base. All control mice were implanted with an inert placebo pellet. At the end of treatment, the pumps and pellets were removed, and, 4 (morphine) or 16 h (etorphine) later, mice were either sacrificed for whole brain binding or tested in a morphine or etorphine cumulative dose–response study. Pumps and pellets were implanted and removed while mice were lightly anesthetized with halothane:oxygen (96:4).

2.3. μ-Opioid receptor binding studies

Binding studies were as previously described by Yoburn et al. (1993). Mice ($N=2$ mice/group, for each binding experiment) were sacrificed and 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, centrifuged again and the pellet frozen ($-80°C$) until analysis. The pellets were thawed, resuspended in 50 mM Tris buffer, incubated (30 min at 25°C), centrifuged and finally resuspended in 20–80 volumes of 50 mM phosphate buffer (pH 7.2). An aliquot (200 μl) of homogenate was then assayed in triplicate in tubes containing 0.04–5.0 nM [^{3}H]DAMGO. Nonspecific binding was determined in the presence of 1000 nM levorphanol. Homogenates were incubated for 90 min at 25°C. Incubation was terminated by the addition of ice-cold phosphate buffer and the samples were filtered over glass fiber filters. Filters were washed three times with cold buffer, transferred to vials with scintillation cocktail and then counted in a liquid scintillation analyzer. Cpms were converted to dpms using the external standard method. Specific binding was the difference between binding determined in the absence of cold ligand and the presence of cold ligand. Protein was determined using a microassay technique based on the method of Bradford (1976) using reagent from Bio-Rad (Richmond, CA).

2.4. 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. The intensity of the light was adjusted so that baseline flick latencies determined prior to etorphine or morphine administration were 2–4 s. If a mouse failed to flick by 10 s following etorphine or morphine administration, the test was terminated and mice were defined as analgesic. All testing was conducted in a blind manner.

At either 4 (morphine) or 16 h (etorphine) following removal of the osmotic pump and pellets, mice were tested for analgesia (tail-flick) using a cumulative dose–response protocol (Duttaroy et al., 1997). Mice treated with etorphine were tested in etorphine cumulative dose–response assays, and morphine implanted mice were tested with morphine. All mice in a group ($N=7$ mice/group, for each dose–response experiment) were injected subcutaneously with a starting dose of etorphine (0.25 μg/kg) and tested for analgesia 15 min later. Mice that were not analgesic were given another dose of etorphine within 3 min of testing and then tested for analgesia again 15 min later (cumulative dose range = 0.25–15.25 μg/kg). The morphine dose–response protocol was identical with a starting dose of 0.5 mg/kg and 30 min between injections (cumulative dose range = 0.5–28.5 mg/kg).

2.5. Drugs and reagents

Etorphine hydrochloride, morphine pellets and placebo pellets were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse. Morphine sulfate was obtained from Penick Laboratories (Newark, NJ). Morphine and placebo pellets were wrapped in nylon mesh before subcutaneous implantation in the nape of the neck. Etorphine and morphine were dissolved in 0.9% saline. All reagents were purchased from Sigma (St. Louis, MO) unless noted. [^{3}H]DAMGO was purchased from NEN Life Science Products (Boston, MA).

2.6. Data analysis

Binding data were analyzed by least squares nonlinear regression (Prism version 1.03). All data were best fit by a one-site model. Dose–response data for each experiment were analyzed by probit analysis (Finney, 1973) using a computerized program (Bliss 21, Department of Statistics, University of Edinburgh) that estimated ED_{50}, 95% confidence limits and relative potency. Results from the binding analyses (B_{max}, K_d) and probit analyses (ED_{50}) were analyzed by analysis of variance. The magnitude of tolerance

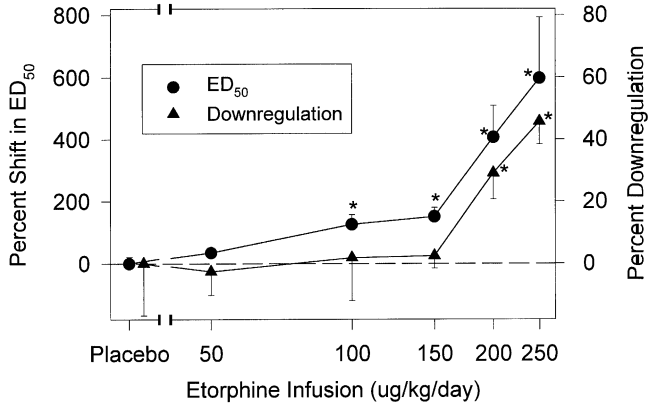

Fig. 1. The effect of etorphine infusion on analgesic potency (tolerance) and μ-opioid receptor density. Etorphine pumps were implanted for 7 days, removed, and, 16 h later, etorphine cumulative dose–response studies (tail-flick) performed or whole brain samples were examined in binding assays. Percent shift in ED_{50} was calculated as: [(treatment ED_{50}/control ED_{50}) × 100] − 100. Each data point represents the mean from three to eight experiments. Control binding (mean ± S.E.M.) was $B_{max} = 160 \pm 27$ fmol/mg protein, $K_d = 1.3$ nM ± 0.1. The control ED_{50} (mean ± S.E.M.) = 1.45 ± 0.30 μg/kg. * Significantly different ($P < .05$) from placebo.

data determined by the probit analyses for morphine and etorphine were fit to a first order polynomial ($y = a + bx$) or an exponential growth function ($y = ae^{kx}$) using Prism version 1.03 and the fits statistically compared using the F test.

3. Results

3.1. Binding

Etorphine (50–250 μg/kg/day) produced a dose-dependent reduction in the density of μ-opioid receptors at 16 h following the removal of the osmotic pump (Fig. 1). The density of μ-opioid receptors was significantly reduced for the 200–250 μg/kg/day dosing groups relative to the control. To determine if morphine treatment affected μ-receptor density, binding following the highest treatment dose of morphine (25 mg pellet + 40 mg/kg/day infusion) was determined. In contrast to etorphine, there was no significant morphine-induced change in μ-receptor density in three experiments (mean $B_{max} \pm$ S.E.M. = 190 ± 40 and 177 ± 38 fmol/mg protein, mean $K_d \pm$ S.E.M. = 1.2 nM ± 0.1 and 1.4 nM ± 0.1, for the placebo and morphine groups, respectively).

3.2. Tolerance

Etorphine infusion (50–250 μg/kg/day) for 7 days produced biphasic dose-dependent tolerance (Fig. 1). All doses above 100 μg/kg/day produced significant tolerance. Higher

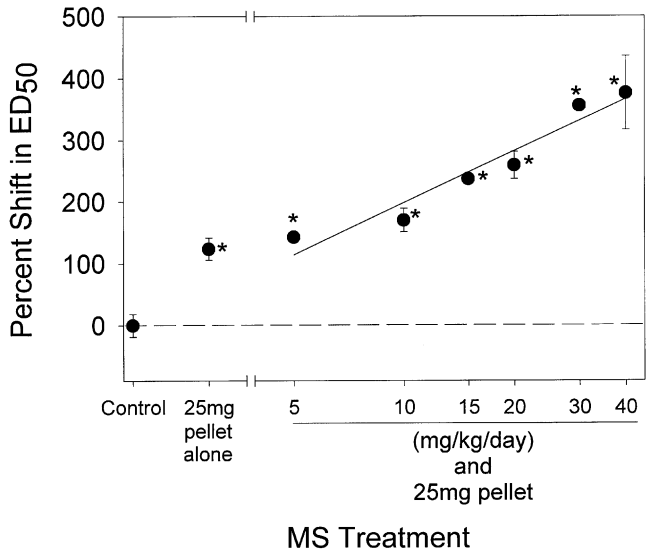

Fig. 2. The effect of morphine treatment on analgesic potency (tolerance). Morphine pumps and pellets were implanted for 7 days, removed, and, 4 h later, a morphine cumulative dose–response study (tail-flick) was conducted. Percent shift in ED_{50} was calculated as: [(treatment ED_{50}/control ED_{50}) × 100] − 100. The control ED_{50} (mean ± S.E.M.) = 3.10 ± 0.55 mg/kg. Each data point represents the mean of three to four experiments, except for 5, 15 and 30 mg, which are a single experiment. * Significantly different ($P < .05$) from placebo.

doses of etorphine (200–250 μg/kg/day) produced a nonlinear increase in tolerance. A nonlinear exponential growth function ($y = ae^{kx}$) fit the data (magnitude of tolerance vs. dose) significantly better than the straight line ($P < .05$). Morphine treatment also produced dose-dependent increases in ED_{50} values (Fig. 2). All morphine doses caused a significant degree of tolerance relative to the control, although unlike etorphine, tolerance was linearly related to treatment doses. A straight line fit the data significantly better than the nonlinear growth function ($P < .05$)

4. Discussion

High intrinsic efficacy opioid agonists have been shown to produce tolerance and downregulation of opioid receptor density in both cell culture and in vivo models (Whistler and von Zastrow, 1998; Pak et al., 1999; Yoburn et al., 1993; Tao et al., 1989). Although opioid receptor downregulation is not a requirement for tolerance (Loh et al., 1988; Tao et al., 1989; Duttaroy and Yoburn, 1995; Law and Loh, 1999), the role that downregulation of opioid receptors plays in the functional effects of opioids is not known. Evidence suggests that opioid receptor downregulation may be important in enhancing the magnitude of opioid tolerance by high intrinsic efficacy agonists. Consistent with this suggestion are reports demonstrating that changes in opioid receptor density directly impact opioid potency. Specifically, pharmacologically induced increases and decreases in opioid receptor density and genetically based receptor deficient mice display corresponding changes in agonist potency (Loh et al., 1998; Kitanaka et al., 1998; Chan et al., 1995, 1997; Paronis and Holtzman, 1991; Yoburn et al., 1986, 1995; Duttaroy et al., 1999). However, the specific role of agonist-induced downregulation in opioid tolerance in an intact animal model has not been explored. Therefore, in the present study, we determined if downregulation of μ-opioid receptors contributes to opioid analgesic tolerance in vivo.

The results of the present experiments demonstrate that chronic treatment with both morphine and etorphine produces dose-dependent analgesic tolerance in vivo, although the magnitude of tolerance produced by etorphine (≈7-fold) exceeded that produced by morphine (≈5-fold). Furthermore, the magnitude of etorphine tolerance increased nonlinearly with etorphine infusion doses that significantly downregulated μ-receptors (200–250 μg/kg/day). In contrast, morphine tolerance increased linearly with morphine infusion doses and no downregulation was observed at the highest morphine treatment dose (25 mg pellet and 40 mg/kg/day pump). This latter finding confirms the reports of other investigators that morphine does not downregulate opioid receptors in vivo and that μ-opioid receptor downregulation is not required for tolerance (e.g., Nishino et al., 1990; Yoburn et al., 1990, 1993). However, the nonlinearity of the etorphine tolerance function support our suggestion that opioid tolerance relies on both "receptor density-independent" and "receptor density-dependent" mechanisms.

Receptor desensitization and changes in receptor signaling pathways in the absence of changes in receptor density constitute "receptor density-independent" mechanisms of tolerance. Several candidates have been proposed that might mediate opioid tolerance in the absence of changes in opioid receptor density, including regulation of cAMP and adenylyl cyclase (Sharma et al., 1975; Chakrabarti et al., 1998) and receptor phosphorylation (Nestler and Tallman, 1988; Terwilliger et al., 1994) by cAMP-dependent protein kinase (PKA) (Nestler and Tallman, 1988), PKC (Basbaum, 1995) and β-adrenergic receptor kinase (Terwilliger et al., 1994). In a recent study, Shen et al. (2000) have shown that antisense knockdown of PKA differentially interferes with opioid tolerance that is independent of receptor downregulation. Taken together with the present results using the lower dose etorphine infusions and morphine treatment, it is clear that there are mechanisms of opioid tolerance that do not require changes in receptor density.

Although opioid tolerance does not require μ-receptor downregulation, there was a marked increase in the magnitude of tolerance at etorphine infusion doses that induced a decrease in μ-opioid receptor density. Consistent with drug receptor theory that predicts "receptor density-dependent" mechanisms should contribute to tolerance (Kenakin, 1997), decreases in receptor density appeared to produce a loss in potency that was additive with "receptor density-independent" mechanisms. Thus, our results agree with previous suggestions that tolerance is likely a multifactor process involving varying degrees of desensitization and downregulation depending on the characteristic of the opioid agonist used to induce tolerance (e.g., Zadina et al., 1993).

To our knowledge, the contribution of μ-opioid receptor downregulation to tolerance in vivo has not been previously established. The present findings indicate that there is a role for downregulation in the manifestation of tolerance in vivo. In summary, although downregulation is not necessary for opioid tolerance, it appears to be a determinant of the magnitude of tolerance in vivo.

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