µ-Opioid receptor downregulation contributes to opioid tolerance in vivo

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Received 9 November 2000; received in revised form 23 January 2001; accepted 19 February 2001

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 ([³H]DAMGO) or morphine and etorphine analgesic ED₅₀ values were determined (tail-flick). Morphine tolerance increased linearly with the infusion dose of morphine (ED₅₀ 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 (ED₅₀ shift at highest infusion dose, 6.97). Downregulation of µ-receptors only occurred at the higher etorphine infusion doses (≥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. © 2001 Elsevier Science Inc. All rights reserved.

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 (Puttfarken 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 (Kennek, 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.
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 SO4. 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 [3H]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. Cpm’s were converted to dpm 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 techni-que 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 (Dutta-ray 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. [3H]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 EDS50, 95% confidence limits and relative potency. Results from the binding analyses (Bmax, KD) and probit analyses (ED50) were analyzed by analysis of variance. The magnitude of tolerance
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 ($y = ae^{bx}$) using Prism version 1.03 and the fits statistically compared using the $F$ test.

3. Results

3.1. Binding

Eorphine (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 $B_{\text{max}} \pm \text{S.E.M.} = 190 \pm 40$ and $177 \pm 38$ fmol/mg protein, mean $K_d \pm \text{S.E.M.} = 1.2$ nM ± 0.1 and 1.4 nM ± 0.1, for the placebo and morphine groups, respectively).

3.2. Tolerance

Eorphine 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
doses of etorphine (200–250 μg/day) produced a non-
linear increase in tolerance. A nonlinear exponential growth
function ($y = ae^{bx}$) 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 down-
regulation 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 downregu-
lation may be important in enhancing the magnitude of
opioid tolerance by high intrinsic efficacy agonists. Con-
sistent 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 downregula-
tion in opioid tolerance in an intact animal model has not
been explored. Therefore, in the present study, we deter-
mined if downregulation of $\mu$-opioid receptors contributes
to opioid analgesic tolerance in vivo.

The results of the present experiments demonstrate that
chronic treatment with both morphine and etorphine pro-
duces dose-dependent analgesic tolerance in vivo, although
the magnitude of tolerance produced by etorphine ($\approx 7$-
fold) exceeded that produced by morphine ($\approx 5$-fold).
Furthermore, the magnitude of etorphine tolerance increased
nonlinearly with etorphine infusion doses that significantly
downregulated $\mu$-receptors (200–250 μg/kg/day). In con-
trast, 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 $\mu$-opioid receptor down-
regulation 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-inde-
dendent” and “receptor density-dependent” mechanisms.

Receptor desensitization and changes in receptor signal-
ing 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; Ter-
williger et al., 1994) by cAMP-dependent protein kinase
(PKA) (Nestler and Tallman, 1988), PKC (Basbaum, 1995)
and $\beta$-adrenergic receptor kinase (Terrylliger 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 downregu-
lation. 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 $\mu$-receptor
downregulation, there was a marked increase in the magni-
tude of tolerance at etorphine infusion doses that induced a
decrease in $\mu$-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-indepen-
dent” mechanisms. Thus, our results agree with previous
suggestions that tolerance is likely a multifactor process
involving varying degrees of desensitization and down-
regulation depending on the characteristic of the opioid
agonist used to induce tolerance (e.g., Zadina et al., 1993).

To our knowledge, the contribution of $\mu$-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.

Acknowledgments

The authors wish to thank Drs. A. Duttaroy and S. Shah
for patience and guidance throughout the course of these
experiments. Of course, Dr. M.T. Turnock provided indirect
support and her efforts are always appreciated.

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