

# Role of cAMP-Dependent Protein Kinase (PKA) in Opioid Agonist-Induced $\mu$ -Opioid Receptor Downregulation and Tolerance in Mice

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**ABSTRACT** Studies suggest that acute and chronic opioids can regulate the cAMP-dependent protein kinase (PKA) signaling pathway and that changes in this pathway may be involved in opioid tolerance. In the present study, we examined the role of cAMP-PKA on  $\mu$ -opioid receptor downregulation and tolerance in mice. Mice were injected intracerebroventricular (i.c.v.) and intrathecal (i.t.) once a day with an antisense oligodeoxynucleotide directed at the mRNA for the  $\alpha$  catalytic subunit of mouse PKA. Controls were treated with saline or a mismatch oligodeoxynucleotide. On day 2 of treatment, mice were implanted s.c. with a 25-mg morphine pellet and an osmotic minipump infusing morphine (40 mg/kg/day) for 3 days. Other mice were implanted with an osmotic minipump infusing etorphine (125, 250  $\mu$ g/kg/day) for 2 days. Control mice were implanted s.c. with inert placebo pellets. At the end of treatment, pumps and pellets were removed and mice tested for morphine or etorphine analgesia. Other mice were sacrificed and  $\mu$ -opioid receptor binding assays conducted in whole brain. Both infusion doses of etorphine produced significant tolerance ( $ED_{50}$  shift = 3.6 and 6.3-fold). The higher etorphine infusion produced downregulation of  $\mu$ -receptor density ( $\approx 30\%$ ) while the lower infusion dose of etorphine did not. Morphine treatment also produced significant tolerance in mice ( $ED_{50}$  shift = 4.5-fold), but no receptor downregulation. Antisense to PKA partially blocked tolerance induced by the higher dose of etorphine, but had no effect on receptor downregulation. On the other hand, antisense to PKA completely blocked tolerance induced by morphine and the lower infusion dose of etorphine. The mismatch oligodeoxynucleotide had no effect on any measure. These results suggest that PKA has a limited role in opioid agonist-induced receptor downregulation. However, the partial block of tolerance for the high infusion dose of etorphine and the complete block of tolerance for morphine and the low infusion dose of etorphine suggests that PKA may play a critical role in tolerance that is "receptor-regulation-independent." **Synapse 38:322–327, 2000.** © 2000 Wiley-Liss, Inc.

## INTRODUCTION

Chronic treatment with opioid agonists has been shown to produce both tolerance and opioid receptor regulation (Carter and Medzihradsky, 1993; Law et al., 1983; Werling et al., 1989; Tao et al., 1998). Studies have shown that in addition to producing tolerance and receptor downregulation, opioid agonists with high intrinsic efficacy (e.g., etorphine) are effective in regulating receptor gene expression (Duttaroy and Yoburn, 2000; Kim et al., 1995; Sehba et al., 1997). Conversely, lower intrinsic efficacy agonists (e.g., morphine) pro-

duce substantial tolerance in the absence of regulation of receptor density or mRNA (Buzas et al., 1996; Castelli et al., 1997; Yoburn et al., 1993). Overall, the fact that both etorphine and morphine produce tolerance, while only etorphine regulates opioid receptors, raises the possibility that tolerance may be mediated by both

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receptor desensitization (i.e., receptor-regulation-independent) and receptor downregulation.

Recent *in vitro* and *in vivo* reports have focused on the possible mechanisms that might account for differential trafficking of opioid receptors following treatment with high and low intrinsic efficacy opioids (Burford et al., 1998; Keith et al., 1996, 1998; Sternini et al., 1996; Whistler and von Zastrow, 1998; Yu et al., 1997). These studies suggest that etorphine is more efficient than morphine in inducing  $\mu$ -opioid receptor internalization, and perhaps downregulation, because of recruitment of G-protein coupled receptor kinase-2 (GRK-2) and  $\beta$ -arrestin to the agonist-occupied receptor (Whistler and von Zastrow, 1998; Zhang et al., 1998). This suggestion is based on findings which show that when GRK-2 and/or  $\beta$ -arrestin are overexpressed, morphine is capable of inducing  $\mu$ -opioid receptor internalization (Whistler and von Zastrow, 1998; Zhang et al., 1998).

The fact that low intrinsic efficacy agonists such as morphine can induce tolerance in the absence of receptor regulation suggests that signal transduction pathways other than GRKs or  $\beta$ -arrestin may be involved. A particularly likely candidate that might mediate morphine tolerance is the cAMP-PKA pathway. It is well established that opioids can regulate the cAMP-PKA pathway and that chronic opioid agonist treatment is associated with increased activity of this pathway (e.g., Nestler and Tallman, 1988). Although cell culture studies indicate that activation of the cAMP-PKA pathway can decrease  $\delta$  opioid receptor (DOR) density and mRNA (Buzas et al., 1997; Jenab and Inturrisi, 1997), it is not known if this pathway plays a role in opioid receptor downregulation *in vivo*.

In the present study, we examined the role of PKA in tolerance and  $\mu$ -opioid receptor regulation *in vivo* using an antisense approach. We initially anticipated that antisense to PKA would reduce opioid tolerance to both morphine and etorphine. However, given the recent data implicating GRKs in  $\mu$ -opioid receptor internalization, it was hypothesized that antisense to PKA would not affect etorphine-induced  $\mu$ -opioid receptor downregulation. Furthermore, it was predicted that morphine tolerance which is not associated with receptor regulation would be more substantially reduced than etorphine-induced tolerance, since it seemed likely that etorphine-induced downregulation of  $\mu$ -opioid receptors would contribute to the magnitude of tolerance.

## MATERIALS AND METHODS

### Subjects

Male Swiss Webster mice (20–35 g) (Taconic Farms, Germantown, NY) were used throughout. Mice were housed 6–8/cage and were used only once.

### Oligodeoxynucleotide and opioid agonist treatment

Oligodeoxynucleotides (ODNs) were synthesized by Midland Certified Reagents (Midland, TX). The antisense ODN was targeted to the  $\alpha$  isoform for catalytic subunit of mouse PKA (5'-GGC GGC GGC GTT GCC CAT-3') complementary to nucleotides 187–204 from the ATG starting site of the mRNA (GB#M12303). A mismatch ODN was also synthesized in which four sets of bases were reversed (note underline: 5'-GCG GCG GGC TGT CGC CAT-3'). The ODNs were dissolved in saline and injected *i.c.v.* unilaterally (30  $\mu$ g / 4  $\mu$ l) and *i.t.* (30  $\mu$ g / 2  $\mu$ l) once a day. Controls were injected with saline.

On day 2 of ODN treatment, an osmotic minipump (Model #2001, Alza Pharmaceuticals, Palo Alto, CA) infusing etorphine (125 or 250  $\mu$ g/kg/day) was implanted *s.c.* Other mice were implanted *s.c.* with both an osmotic pump infusing morphine (40 mg/kg/day) and a 25 mg morphine implant pellet. Controls were implanted with inert placebo pellets. The placebo and morphine implant pellets were obtained from the Research Technology Branch of NIDA. ODN and control injections continued once per day during morphine and etorphine treatment. After 48 h of etorphine treatment or 72 h of morphine treatment, the pumps and pellets were removed and 16 h (etorphine) or 4 h (morphine) later, mice were tested for etorphine or morphine antinociception (see below), or sacrificed for whole brain binding assays (see below). It should be noted that the morphine and etorphine treatment duration differed. Morphine-treated animals received 72 h of drug exposure, while etorphine-treated animals were infused for 48 h. These dosing protocols are based on pilot studies that demonstrated robust tolerance for both drugs and receptor downregulation in etorphine-treated mice. All pellet and pump implantation and removals, as well as ODN injections, were performed while mice were lightly anesthetized with halothane:oxygen (4:96).

### 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 were 2–4 sec. Baseline flicks were determined prior to *s.c.* morphine or etorphine injection. If a mouse failed to flick by 10 sec following etorphine or morphine administration, the test was terminated and a latency of 10 sec was recorded. Mice that had a latency of 10 sec were defined as analgesic. Mice were tested for analgesia 30 min following morphine or 15 min following etorphine administration. Experimenters evaluating analgesia were blinded with respect to the treatment group of each animal.

### Dose-response protocol

A cumulative dose-response protocol was used for all studies. All mice in a treatment group (6–8/group) received s.c. injection with a starting dose of morphine (0.5 mg/kg) or etorphine (0.5  $\mu$ g/kg) and the analgesia test was conducted 30 min following morphine or 15 min following etorphine administration. Mice that were not analgesic were given a second dose within 5 min of testing and then tested for analgesia again 30 or 15 min later. This cumulative dosing was continued until 100% of the mice were analgesic (dose ranges are 0.5–23.5 mg/kg and 0.5–11.0  $\mu$ g/kg for morphine and etorphine, respectively). The doses used in the cumulative dose-response protocol were based on previous studies (Duttaray and Yoburn, 1995).

### Mouse whole-brain $\mu$ -opioid receptor saturation binding assay

Mice ( $n = 2$ /treatment) were sacrificed following treatment and whole brain was rapidly removed, weighed, and then homogenized in 35 ml ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were then centrifuged at 15,000 rpm for 15 min, the supernatant discarded, and the pellet resuspended in buffer, centrifuged again, and the pellet frozen ( $-80^{\circ}\text{C}$ ) until analysis. The pellets were thawed, resuspended in Tris buffer, incubated (30 min at  $25^{\circ}\text{C}$ ), centrifuged, and finally resuspended in 50 mM phosphate buffer (pH 7.2). An aliquot (200  $\mu$ l) of homogenate was assayed in triplicate in tubes containing 0.03–5.0 nM [ $^3\text{H}$ ]DAMGO ( $\mu$  ligand, New England Nuclear, Boston, MA). Non-specific binding was determined in the presence of 1000 nM levorphanol. Homogenates were incubated for 90 min at  $25^{\circ}\text{C}$ . Incubation was terminated by the addition of ice-cold phosphate buffer and the samples were filtered over GF/B glass fiber filters (Brandel, Gaithersburg, MD). Filters were washed three times with cold buffer, transferred to vials, scintillation cocktail added, and then counted. Counts per minute (CPMs) were converted to disintegration per minute (DPMs) using the external standard method. Protein was determined using a microassay technique based on the method of Bradford (1976) using reagent purchased from BIO-RAD (Richmond, CA).

### Data analysis

All data are presented as means  $\pm$  SEM unless noted otherwise. Quantal dose-response data were analyzed using Probit analysis that estimated  $\text{ED}_{50}$ s (95% CL) and relative potencies. The shift in the  $\text{ED}_{50}$  was determined by dividing the  $\text{ED}_{50}$  for the treated groups by the  $\text{ED}_{50}$  for the control group.  $B_{\text{max}}$ s and  $K_{\text{d}}$ s were determined using nonlinear regression (Prism ver. 1.03, Graphpad Software, San Diego, CA). In all cases binding data were fit best by a one-site model. Significant differences ( $P < 0.05$ ) were determined by ANOVA with post-hoc tests.

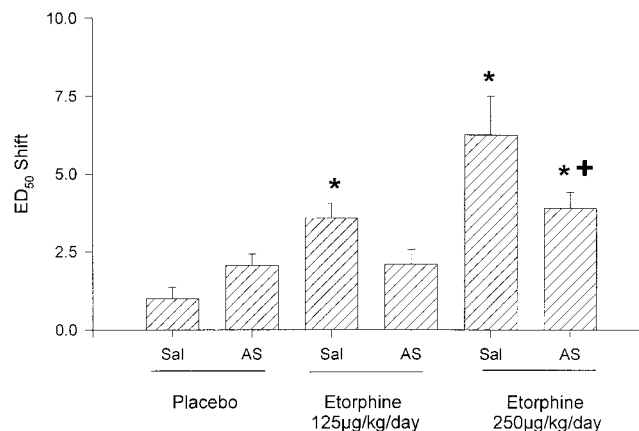


Fig. 1. The effects of PKA antisense on etorphine-induced tolerance in mice. Mice received i.c.v. and i.t. injection with antisense ODN once a day. Control mice received an equal volume of saline (for details, see Methods). On day 2, mice were implanted with s.c. osmotic minipumps infusing etorphine (125 or 250  $\mu$ g/kg/day) for 2 days. Controls received placebo pellets. At the end of treatment, pumps and pellets were removed and 16 h later, mice were tested for etorphine analgesia. The  $\text{ED}_{50}$  shift was calculated as the  $\text{ED}_{50}$  for the treated groups divided by the  $\text{ED}_{50}$  for the placebo-saline group. Data are mean  $\pm$  SEM from 3–4 independent experiments. \*Significantly different from saline-placebo group ( $P < 0.05$ ). †Significantly different from saline-etorphine group ( $P < 0.05$ ). AS = antisense, Sal = saline. Etorphine  $\text{ED}_{50}$ s = saline-placebo:  $0.91 \pm 0.19$   $\mu$ g/kg; antisense-placebo:  $1.89 \pm 0.35$   $\mu$ g/kg; saline-etorphine (125  $\mu$ g/kg):  $3.27 \pm 0.71$   $\mu$ g/kg; saline-etorphine (250  $\mu$ g/kg):  $6.93 \pm 1.39$   $\mu$ g/kg; antisense-etorphine (125  $\mu$ g/kg):  $1.92 \pm 0.54$   $\mu$ g/kg; antisense-etorphine (250  $\mu$ g/kg):  $4.33 \pm 0.80$   $\mu$ g/kg.

## RESULTS

There were no significant differences among the baseline tail flick latencies for any treatment prior to dose-response testing (range = 1.8–2.1 sec). Both infusion doses of etorphine (125, 250  $\mu$ g/kg/day) produced significant tolerance ( $P < 0.05$ ) to etorphine-induced analgesia in mice ( $\text{ED}_{50}$  shifts = 3.6, 6.3-fold, 125, 250  $\mu$ g/kg/day, respectively, Fig. 1). Antisense to PKA partially, but significantly ( $P < 0.05$ ) decreased etorphine-induced tolerance in the high infusion dose group by 38%. Conversely, antisense to PKA completely blocked tolerance induced by the low infusion dose of etorphine (Fig. 1). Antisense to PKA had no significant effect on etorphine potency in placebo-treated mice.

Chronic morphine treatment significantly ( $P < 0.05$ ) reduced morphine potency ( $\text{ED}_{50}$  shift = 4.5-fold, Fig. 2). Antisense alone had no significant effect on morphine potency. Antisense completely ( $P < 0.05$ ) blocked morphine tolerance in mice. To determine if the effects of antisense on tolerance were specific to the PKA antisense sequence, mice were tested for the effects of a mismatch ODN. The mismatch had no effect on etorphine potency or tolerance induced by an etorphine (250  $\mu$ g/kg/day) infusion (Fig. 3). Since the mismatch ODN had no effect on etorphine-induced effects, we did not examine morphine.

Changes in whole mouse brain DAMGO binding were determined following morphine and etorphine treatment. The high infusion dose of etorphine (250

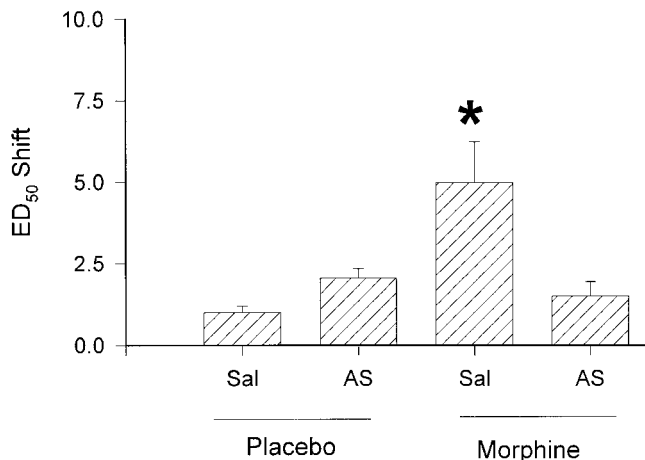


Fig. 2. The effects of PKA antisense on morphine tolerance in mice. Mice received i.c.v. and i.t. injection with antisense ODN once a day. Control mice received an equal volume of saline (for details, see Methods). On day 2, mice were implanted s.c. with a 25-mg morphine pellet and an osmotic minipump infusing morphine (40 mg/kg/day) for 3 days. Controls received placebo pellets. At the end of treatment, pumps and pellets were removed and 4 h later mice were tested for morphine analgesia. AS = antisense, Sal = saline. The ED<sub>50</sub> shift was calculated as the ED<sub>50</sub> for the treated groups divided by the ED<sub>50</sub> for the placebo-saline group. Data are mean ± SEM from three independent experiments. \*Significantly different from saline-placebo group ( $P < 0.05$ ). Morphine ED<sub>50</sub>s = saline-placebo:  $2.16 \pm 0.38$ ; antisense-placebo:  $3.96 \pm 0.76$ ; saline-morphine:  $9.21 \pm 2.14$ ; antisense-morphine:  $3.87 \pm 1.14$  mg/kg.

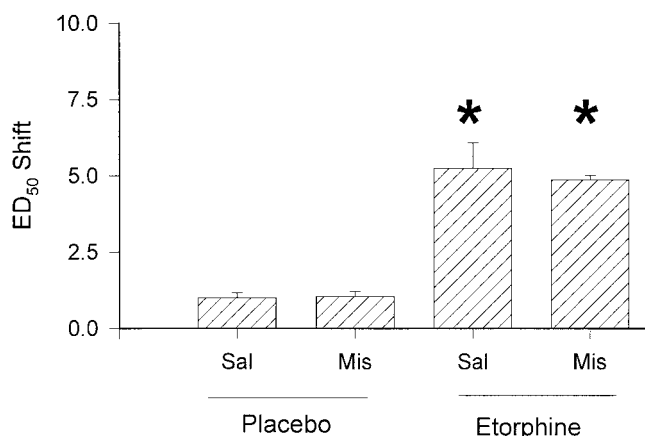


Fig. 3. The effects of PKA mismatch on etorphine-induced tolerance in mice. Mice received i.c.v. and i.t. injection with a mismatch ODN once a day. Control mice received equal volume of saline (for details, see Methods). On day 2, mice were implanted with s.c. osmotic minipumps infusing etorphine (250  $\mu$ g/kg/day) for 2 days. Controls received placebo pellets. At the end of treatment, pumps and pellets were removed and 16 h later mice were tested for etorphine analgesia. Mis = mismatch, Sal = saline. The ED<sub>50</sub> shift was calculated as the ED<sub>50</sub> for the treated groups divided by the ED<sub>50</sub> for the placebo-saline group. Data are mean ± SEM from 3–6 independent experiments. \*Significantly different from saline-placebo group ( $P < 0.05$ ). Etorphine ED<sub>50</sub>s = saline-placebo:  $1.0 \pm 0.19$   $\mu$ g/kg; mismatch-placebo:  $1.01 \pm 0.17$   $\mu$ g/kg; saline-etorphine:  $5.1 \pm 0.82$   $\mu$ g/kg; mismatch-etorphine:  $4.7 \pm 1.4$   $\mu$ g/kg.

$\mu$ g/kg/day, Fig. 4) produced significant ( $P < 0.05$ ) downregulation ( $\approx 30\%$ ), whereas the low dose of etorphine (125  $\mu$ g/kg/day, Fig. 5) and morphine (Fig. 6) did not. High dose etorphine produced  $\mu$ -opioid receptor down-

regulation without changing affinity in whole mouse brain (range of K<sub>d</sub> = 0.9–1.1 nM). Etorphine-induced downregulation was not affected by antisense or mismatch treatment (Fig. 4). Neither antisense nor mismatch had any effect on receptor density or affinity (range of K<sub>d</sub> = 0.9–1.1 nM) for any group.

## DISCUSSION

It is generally accepted that chronic opioid agonist treatment that produces tolerance will increase the activity of the cAMP-PKA pathway (Chakrabarti et al., 1998; Duman et al., 1988; Nestler and Tallman, 1988). These findings have raised the possibility that regulation of the cAMP-PKA pathway may play an important role in tolerance. Although the  $\mu$ -opioid agonist morphine has been shown to produce substantial tolerance, it does not downregulate receptor density (e.g., Yoburn et al., 1993). These data suggest that receptor downregulation is not required for tolerance. However, several cellular and in vivo studies have confirmed that high intrinsic efficacy opioids can induce both tolerance and receptor downregulation. To date, it is not clear if the cAMP-PKA pathway was involved in receptor downregulation.

In the present study, we used an antisense strategy to knockdown PKA in order to examine the participation of PKA in chronic opioid tolerance in vivo. Our results confirm that both morphine and etorphine produce robust tolerance to the analgesic effects of each drug and that high-dose etorphine can downregulate  $\mu$ -opioid receptors in vivo (e.g., Yoburn et al., 1993; Duttaroy and Yoburn, 1995). In addition, the present study supports previous reports demonstrating that morphine produces tolerance in the absence of receptor downregulation (e.g., Yoburn et al., 1993).

Although antisense directed towards PKA had no effect on acute morphine or etorphine analgesic potency, it significantly reduced the tolerance produced by both drugs. Morphine and low-dose etorphine (125  $\mu$ g/kg/day)-induced tolerance were abolished by antisense to PKA, whereas tolerance induced by the high-dose etorphine (250  $\mu$ g/kg/day) tolerance was only partially blocked. The partial block of etorphine tolerance coincided with the failure of antisense towards PKA to affect etorphine-induced  $\mu$ -opioid receptor downregulation in vivo. Taken together, the complete block of morphine and low dose etorphine induced tolerance, and the partial block of high dose etorphine induced tolerance suggest that PKA plays a role in tolerance to both agonists. However, the residual tolerance to high dose of etorphine in antisense-treated mice may be due to receptor downregulation, which is likely to be mediated by a process independent of PKA.

Although receptor theory predicts that receptor downregulation will contribute to tolerance (e.g., Kenakin 1997), numerous studies both in cell culture and in vivo have documented that receptor downregulation is not required for the expression of opioid toler-

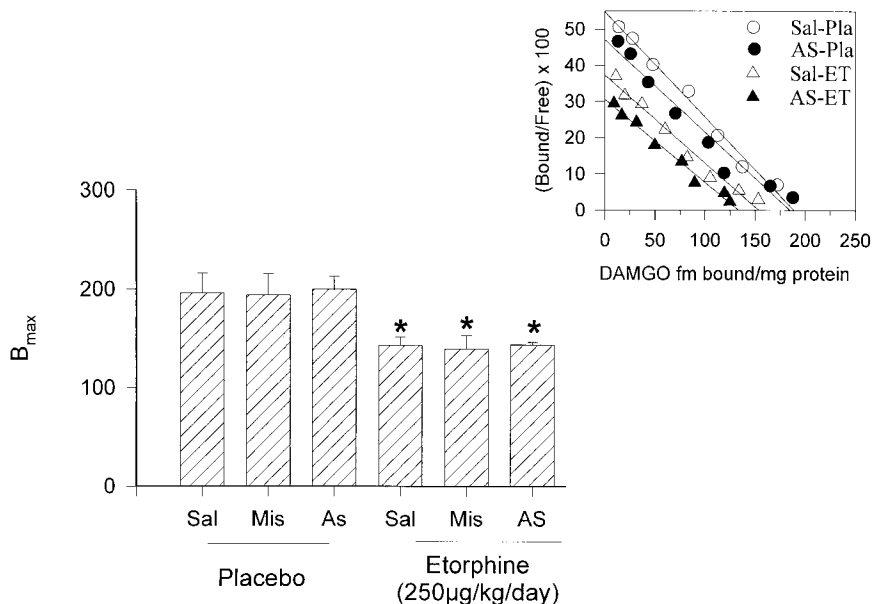


Fig. 4. The effects of PKA antisense on  $\mu$ -opioid receptor downregulation by high-dose etorphine (250  $\mu$ g/kg/day) in mice. Mice were treated as described for Figure 1, except that at the end of treatment mice were sacrificed and whole brain was removed for  $\mu$ -opioid receptor binding assays. Data are mean  $\pm$  SEM from five independent experiments ( $B_{MAX}$  = femtomoles/mg protein). \*Significantly different from saline-placebo group ( $P < 0.05$ ). Inset: Scatchard plot from a representative experiment. AS = antisense, Mis = mismatch, Sal = saline, Et = etorphine, Pla = placebo.

ance (Law et al., 1983; Roth et al., 1995; Yoburn et al., 1993). While most opioid agonists can induce desensitization and phosphorylation of receptors (e.g., Yu et al., 1997; Zhang et al., 1996), only high intrinsic efficacy opioid agonists such as etorphine can produce receptor internalization and downregulation in cell culture and whole animal studies (Keith et al., 1996, 1998; Sternini et al., 1996; Whistler and von Zastrow 1998; Yoburn et al., 1993). These results suggest that common and divergent mechanisms are activated when opioids of different intrinsic efficacy occupy the  $\mu$ -opioid receptor.

Although studies have suggested the contribution of several kinase systems (e.g., PKC, MAP kinases; Kramer and Simon, 1999; Narita, 1995), it is generally believed that the cAMP-PKA pathway is activated by chronic morphine in vivo (for review, see Nestler and Aghajanian, 1997). Therefore, we anticipated that mor-

phine tolerance would be reduced by antisense to PKA. On the other hand, recent cell culture data strongly suggests that receptor density regulation may specifically involve GRKs (Whistler and von Zastrow, 1998; Zhang et al., 1998). Thus, it seemed likely that antisense knockdown of PKA would not impact on opioid agonist-induced receptor downregulation. As such, the failure of PKA antisense to modify etorphine-induced downregulation was not unexpected and supports cell culture studies that indicate a role for GRKs in receptor regulation and suggests that PKA is involved in "receptor-regulation-independent" tolerance. In support of this suggestion is the fact that antisense to PKA only partially blocked etorphine tolerance and that this may be attributed to  $\mu$ -opioid receptor downregulation.

In summary, the present results demonstrate that PKA plays a role in opioid tolerance in vivo. However, PKA does not appear to mediate opioid agonist-induced

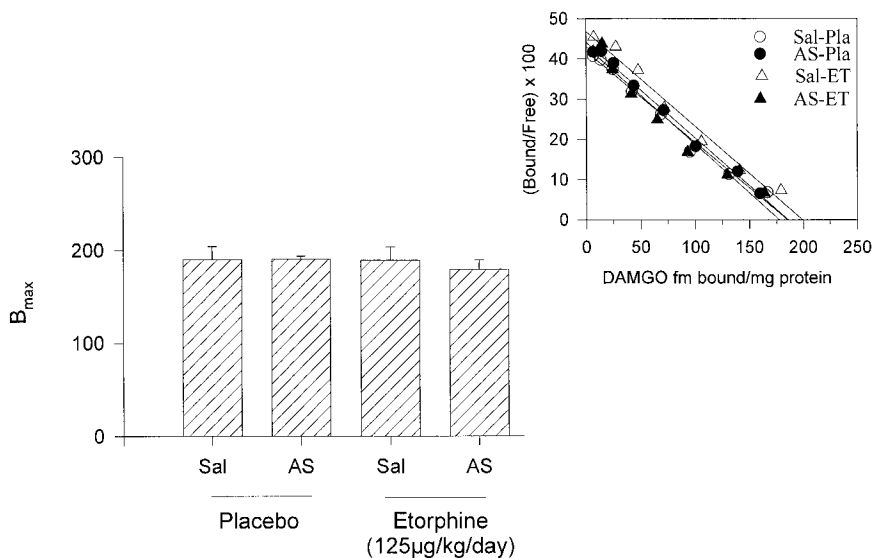
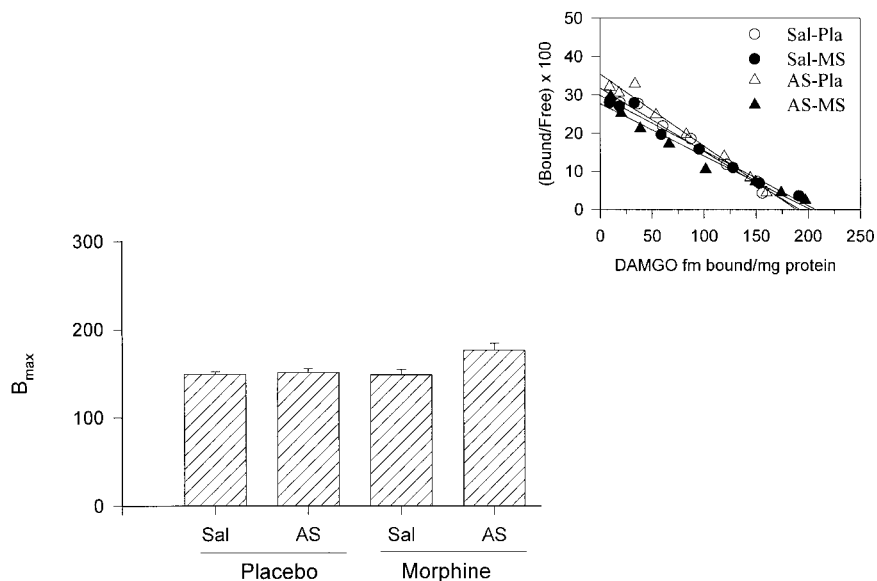


Fig. 5. The effects of PKA antisense on  $\mu$ -opioid receptor downregulation by low-dose etorphine (125  $\mu$ g/kg/day) in mice. Mice were treated as described in Figure 1, except that at the end of treatment mice were sacrificed and whole brain was removed for  $\mu$ -opioid receptor binding assays. Data are mean  $\pm$  SEM from three independent experiments ( $B_{MAX}$  = femtomoles/mg protein). Inset: Scatchard plot from a representative experiment. AS = antisense, Sal = saline, Et = etorphine, Pla = placebo.

Fig. 6. The effects of PKA antisense on  $\mu$ -opioid receptor density in morphine-tolerant mice. Mice were treated as described in Methods; at the end of treatment mice were sacrificed and whole brain was removed for receptor binding assays. Data are mean  $\pm$  SEM from three independent experiments ( $B_{\text{MAX}}$  = femtomoles/mg protein). Inset: Scatchard plot from a representative experiment. AS = antisense, Sal = saline, MS = morphine, Pla = Placebo.



receptor downregulation. The results support suggestions that multiple signaling pathways play a role in opioid tolerance.

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