ELSEVIER

European Journal of Pharmacology 371 (1999) 1–9

ejp

www.elsevier.nl/locate/ejphar

3-Isobutyl-1-methylxanthine inhibits basal μ-opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice

Zaijie Wang [a,b,*], Edward J. Bilsky [b], Danxin Wang [a], Frank Porreca [b], Wolfgang Sadée [a]

[a] *Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California, San Francisco, CA, USA*
[b] *Department of Pharmacology, University of Arizona College of Medicine, P.O. Box 245050 Tucson, AZ, 85724-5050, USA*

Received 15 February 1999; accepted 23 February 1999

Abstract

Phosphorylation of the μ-opioid receptor may play a role in opioid tolerance and dependence. 3-Isobutyl-1-methylxanthine (IBMX) was found to inhibit basal μ-opioid receptor phosphorylation ($IC_{50} \leq 10$ μM) either upon acute treatment or after 8 h pre-treatment in HEK293 cells transfected with the μ-opioid receptor. In mice made acutely tolerant to and dependent on morphine, IBMX (30–100 nmol, i.c.v.) significantly attenuated the naloxone-induced withdrawal jumping and partially reversed morphine antinociceptive tolerance. IBMX also blocked changes to μ-opioid receptor signaling associated with chronic morphine treatment, specifically, the inverse agonist effect elicited by naloxone, in which naloxone paradoxically elevated the cAMP levels in cells previously exposed to morphine for ≥ 12 h. These results suggest a new effect of IBMX in inhibiting basal μ-opioid receptor phosphorylation, and provide additional evidence for the involvement of receptor phosphorylation in the development of opioid tolerance and dependence.

Keywords: Opioid receptor; Tolerance; Dependence; Phosphorylation; Inverse agonist

1. Introduction

Multiple opioid receptors mediate the effects of opioid drugs. The μ-opioid receptor is primarily responsible for their analgesic and rewarding effects. Repeated uses of opioid agonists such as morphine can lead to tolerance and dependence. Biochemically, both tolerance and dependence are found to associate with an upregulated cAMP second messenger system, resulting in elevated cAMP levels upon agonist removal (Yu et al., 1990; Nestler, 1992; Avidor-Reiss et al., 1995). Thus, prolonged morphine treatment of cells expressing μ-opioid receptors, e.g., human neuroblastoma SH-SY5Y, causes a subsequent cAMP overshoot upon agonist removal and a moderate degree of tolerance (Yu and Sadée, 1988). Moreover, in these morphine-treated cells, the μ-opioid receptor appears to be converted into a state uniquely sensitive to some opioid antagonists, which is indicated by direct effect of naloxone on cAMP, causing a significant increase in cAMP level (naloxone induced cAMP overshoot) on top of the cAMP upregulation (spontaneous cAMP overshoot) (Wang et al., 1994a; Wang et al., 1994b). The direct action of naloxone at the μ-opioid receptor in dependent tissue, opposite to that of an agonist, suggested an inverse agonist effect (i.e., negative antagonism) at a basally (i.e., constitutively) active μ-opioid receptor. Such unique action of naloxone is consistent with its high potency in eliciting withdrawal in morphine-dependent animals (Way et al., 1969; Bilsky et al., 1996). The inverse agonism is only found in μ-opioid receptor-expressing cells that have been prolongly exposed to morphine (Wang et al., 1994b). Therefore, it could represent a salient feature of opioid tolerance and dependence.

The molecular mechanism underlying the naloxone-induced cAMP overshoot is not clear. It appears to involve phosphorylation since it was prevented by the protein kinase inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) in SH-SY5Y cells (Wang et al., 1994b). Development of tolerance in SH-SY5Y cells was also

* Corresponding author. Department of Pharmacology, University of Arizona, College of Medicine, P.O. Box 245050, Tucson, AZ 85724-5050, USA.

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00131-4

blocked by H7 (Wang et al., 1994b; Elliott et al., 1997). Moreover, H7 reversed morphine antinociceptive tolerance in mice (Bilsky et al., 1996) and rats (Narita et al., 1994). In mice made acutely dependent on morphine, H7 significantly suppressed naloxone-precipitated withdrawal jumping (Wang et al., 1994b; Bilsky et al., 1996). These results suggested that protein phosphorylation contributes to the expression of morphine tolerance and dependence.

To study if the H7-sensitive protein kinase phosphorylates the μ-opioid receptor, we have developed a phosphorylation assay (Arden et al., 1995; Wang et al., 1996). The μ-opioid receptor was found to be phosphorylated at a basal rate, which was enhanced by morphine. Basal μ-opioid receptor phosphorylation has been confirmed to occur in other cells with heterologous or native receptor expression (Patterson et al., 1995; Zhang et al., 1996). H7 was indeed found to inhibit basal μ-opioid receptor phosphorylation (Wang et al., 1996).

It will be useful to search for other inhibitors of basal μ-opioid receptor phosphorylation, which can be subsequently tested for their effects in vivo. This not only affords additional tests for the role of μ-opioid receptor phosphorylation in the development of opioid tolerance and dependence but may also provide useful agents to disrupt opioid dependence and tolerance. 3-Isobutyl-1-methylxanthine (IBMX), a cAMP phosphodiesterase inhibitor, prevents the naloxone-induced cAMP overshoot. This would suggest that either the inverse naloxone effect involves cAMP phosphodiesterase, or IBMX has a direct effect on μ-opioid receptor phosphorylation. We test here if IBMX affects basal μ-opioid receptor phosphorylation and morphine tolerance and dependence.

2. Materials and methods

2.1. Materials

IBMX was purchased from Sigma (St. Louis, MO). The rat μ-opioid receptor cDNA in vector pRC/CMV (Chen et al., 1993) was provided by Dr. Lei Yu and tagged with the epitope EYMPME (EE), immediately after the first Methionine (EE-μ) (Arden et al., 1995). Morphine sulfate, naloxone, and [^{3}H]diprenorphine were obtained from the National Institute on Drug Abuse (Rockville, MD). All other chemicals were from common commercial sources.

2.2. Cell culture

The plasmids encoding μ- and EE-μ-opioid receptors were transfected into HEK293 cells, and stably transfected clonal cell lines (HEK-μ and HEK-EE-μ) were established as described (Arden et al., 1995). Cells were maintained in Dulbecco's modified eagle medium (DMEM)/F-12 (50%/50%) medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum containing 100 μg/ml streptomycin and 100 units/ml penicillin. G418 (200 μg/ml) was added in HEK-μ and HEK-EE-μ cultures to maintain stable selection. Fresh cells were thawed and plated every 6–8 weeks from a same frozen stock to maintain constant receptor expression at $\sim 5 \times 10^5$ receptor sites per cell.

2.3. Receptor binding and cAMP accumulation assays

Receptor expression was examined with [^{3}H]diprenorphine (2 nM final concentration) in intact cells as described (Arden et al., 1995). The cAMP accumulation assays were performed in washed cell monolayers by incubating HEK-μ or HEK-EE-μ cells in serum-free DMEM medium at 37°C for 7 min in the presence of 10 μM forskolin, which was determined to be the optimum condition in HEK293 cells (Wang and Sadée, manuscript submitted). The amount of cAMP was determined by a radioimmunoassay as described previously (Wang et al., 1994b). Student's *t*-test was used for statistical comparisons.

2.4. Morphine pre-treatment and washing protocol

Cells were pre-treated with 1 μM morphine and/or IBMX (10–500 μM) for 12 h. Immediately before the cAMP accumulation assay, cells were washed twice with DMEM medium containing 5% serum and twice more with serum-free DMEM medium, requiring 10 min for effective agonist removal. To assess the efficiency of removing morphine from the pre-treatment, HEK293 and HEK-μ cells were also incubated with various concentrations of [^{3}H]morphine (NEN Dupont, Boston, MA) for 0–12 h, and washed twice with DMEM medium containing 5% serum and twice more with serum-free DMEM medium. Some cells were incubated in serum-free DMEM medium in the presence of 10 μM forskolin at 37°C for 7 min. [^{3}H] in both cell pellets and media were measured by a scintillation counter.

2.5. Phosphorylation and immunoprecipitation of the epitope-tagged μ-opioid receptor

HEK-EE-μ cells were pre-treated with morphine (1 μM) or IBMX (10–500 μM) as indicated, and phosphorylation of EE-μ was determined in washed, permeabilized cells as described (Arden et al., 1995; Wang et al., 1996). Briefly, digitonin-permeabilized cells were labeled with 0.2 mCi [γ-^{32}P]ATP (200 μM, 0.5 ml) in phosphate-free medium for 15 min in the presence or absence of morphine (10 μM) or IBMX (10–500 μM). Labeled cells were washed and homogenized in 5 mM Tris buffer (pH 7.4) containing 100 nM diprenorphine, phosphatase and pro-

tease inhibitors (Arden et al., 1995). The 30,000 × *g* membrane pellet was collected and solubilized in 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in the presence of 100 nM diprenorphine, and phosphatase and protease inhibitors. Solubilized EE-μ was immunoprecipitated with anti-EE monoclonal antibody, subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and autoradiographed. The phosphorylated band was quantified by scanning densitometry. The identity of EE-μ band at ~65 kDa, which was absent in untransfected HEK293 cells, was confirmed by Western blotting using monoclonal antibody against the epitope tag (Arden et al., 1995). No [^{32}P]-labeled bands appeared at ~65 kDa in solubilized, immunoprecipitated membrane proteins made from untransfected HEK293 cells.

2.6. Morphine tolerance and naloxone-induced withdrawal in mice

Experiments in mice were performed exactly as described previously for testing the effect of H7 (Wang et al., 1994b; Bilsky et al., 1996), and according to the guidelines and protocols approved by the University of Arizona Institutional Animal Care and Use Committee. Male ICR mice weighing 20–30 g (Harlan Industries, Cleveland, OH), in groups of 10 each, were used in all experiments. Antinociception was determined using the warm water (55°C) tail-flick test after intracerebroventricular (i.c.v.) or subcutaneous (s.c.) injection of morphine sulfate. To test if IBMX (60–100 nmol) altered morphine antinociception, it was injected (i.c.v.) 30 min before the administration of morphine. Mice were also made acutely tolerant to morphine by a single dose of morphine sulfate (100 mg/kg, s.c.). At 4.5 h later, either saline or IBMX (100 nmol) was injected (i.c.v.) into mice. After 30 min (5 h after the initial dose of morphine), the tail-flick test was performed. Tolerance to morphine was apparent from the decrease in morphine-mediated antinociception. Following the same treatment with morphine sulfate (100 mg/kg, s.c.), acute dependence was documented 4 h later by naloxone-induced withdrawal jumps when the mice were challenged with increasing doses of naloxone given intraperitoneally (i.p.). To test the effect of IBMX on morphine dependence, morphine-treated mice were given (i.c.v.) equal volumes of IBMX or saline 30 min before the withdrawal test.

3. Results

3.1. Coupling of the cloned μ-opioid receptor to adenylyl cyclase and the naloxone-induced cAMP overshoot in HEK-μ cells

Morphine inhibited forskolin-stimulated cAMP accumulation in HEK-μ cells (Fig. 1A) with an EC_{50} ~ 30 nM, while the maximum inhibition was 76 ± 6% ($n = 20$). Potency and efficacy of cAMP inhibition by morphine were similar in HEK-EE-μ cells (data not shown). After HEK-μ cells were treated with morphine (1 μM) for 12 h, followed by washout of the drug, freshly added morphine still produced maximum inhibition of 85 ± 3% ($n = 15$), and did not alter the EC_{50} value (Fig. 1B). Lack of tolerance may suggest substantial spare receptors available in the transfected cells because of relatively high receptor expression levels (Chavkin and Goldstein, 1984).

After morphine pre-treatment, a small stimulatory response was seen in several experiments when low concentrations of morphine (1–10 nM) were added (Fig. 1B). While the stimulatory response was reproducible, the magnitude was very variable. Therefore, it was not pursued further in this study.

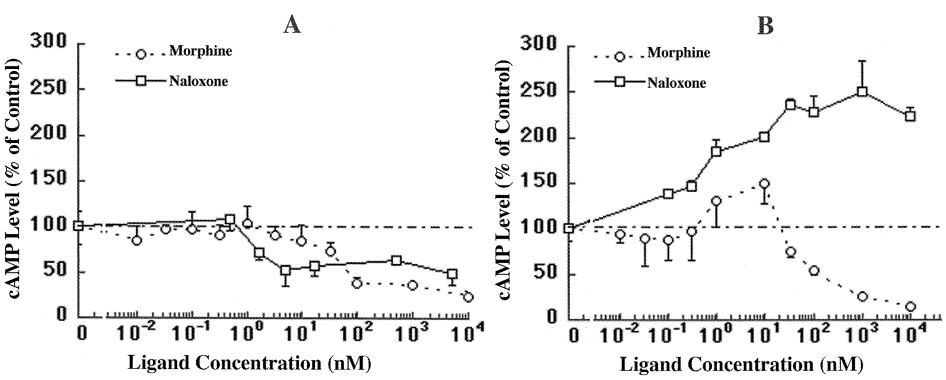

Fig. 1. Dose–response curves of morphine and naloxone in affecting forskolin-stimulated cAMP accumulation in HEK-μ cells (A) before and (B) after morphine pre-treatment. HEK-μ cells were either not treated or pre-treated with 1 μM morphine for 12 h. Cells were washed four times before the cAMP accumulation assays, which were performed by incubating cells in DMEM medium at 37°C for 7 min in the present of 10 μM forskolin and morphine or naloxone at the indicated concentrations. The cAMP levels were normalized to corresponding drug-free control levels in naive or morphine-pre-treated cells. After pre-treatment, control cAMP levels were approximately two-fold higher than those in untreated cells, representing the cAMP upregulation (see Fig. 2). Data represent the mean ± SD of several experiments ($n \geq 6$).

Surprisingly, naloxone also decreased cAMP levels in untreated HEK-μ cells with an $EC_{50} \sim 1$ nM and maximum inhibition of 51 ± 12% (Fig. 1A). This is consistent with the ability of naloxone to reduce cAMP levels in untreated SH-SY5Y cells (Wang et al., 1994b). Naloxone may not be a pure antagonist of the μ-receptor. We have recently found that naloxone acts as a partial agonist of the μ-receptor coupling to Gi and Go in untreated HEK-μ cell membranes (Burford et al., 1997).

As previously observed in SH-SY5Y cells (Wang et al., 1994b) and in Chinese hamster ovary cells stably transfected with μ-opioid receptor (CHO-μ) (Avidor-Reiss et al., 1995), pre-treatment with 1 μM morphine led to a substantial spontaneous cAMP overshoot in HEK-μ cells upon washout of morphine (Fig. 2). Moreover, addition of naloxone to the thoroughly washed cells caused an additional increase of cAMP levels in HEK-μ cells (Fig. 1B and Fig. 2) with an $EC_{50} \sim 1$ nM (Fig. 1B), in agreement with the naloxone-induced cAMP overshoot observed in SH-SY5Y cells (Wang et al., 1994b). The spontaneous cAMP overshoot and the naloxone-induced cAMP overshoot were similarly observed in HEK-EE-μ cells (data not shown). However, in preliminary experiments using CHO-μ cells, we failed to observe a significant inverse naloxone effect under the same conditions (data not shown), which may highlight biochemical differences among cell lines with regard to cAMP second messenger regulation.

3.2. Efficiency of removing morphine after pre-treatment

The interpretation of the naloxone-induced cAMP overshoot as an inverse agonist effect requires an effect by an antagonist in the absence of any agonist. We addressed the efficiency of removing morphine by incubating cells directly with [^{3}H]morphine. After 12 h incubation, over 99.7% of [^{3}H] activity was removed from the HEK-μ cells by the washing procedure used throughout this study. HEK-μ cells did not retain more tracer than non-transfected HEK-293 cells, and [^{3}H] retained was unaffected by the co-incubation of excessive unlabeled morphine (1 μM). These results demonstrated that the residual tracer was not selectively bound to the μ-opioid receptor.

In order to act at the receptor, morphine must redistribute to the cell surface. Only 0.1% of the originally added tracer was recovered in the medium at the end of 7 min incubation, which amounted to 1 nM morphine if there was 1 μM drug in the pre-treatment discounting degradation or impurities of the tracer. At this concentration, morphine did not inhibit cAMP accumulation under the assay conditions used in this study (Fig. 1B). Therefore, the washing procedure effectively removed morphine from the cells. A similar washing procedure was also found sufficient to remove residual morphine in CHO-μ cells (Avidor-Reiss et al., 1995).

3.3. Effects of IBMX on the naloxone-induced cAMP overshoot in HEK-μ cells

Addition of IBMX to the cAMP assay at 500 μM, a concentration commonly employed to suppress cAMP phosphodiesterase, increased cAMP levels in HEK-μ cells by two-fold ($P < 0.001$) (Fig. 2 'IBMX Post-treat'), as expected from its action as a phosphodiesterase inhibitor. IBMX did not suppress the spontaneous cAMP overshoot observed after agonist removal; however, it abolished the inverse naloxone effect on cAMP accumulation. The

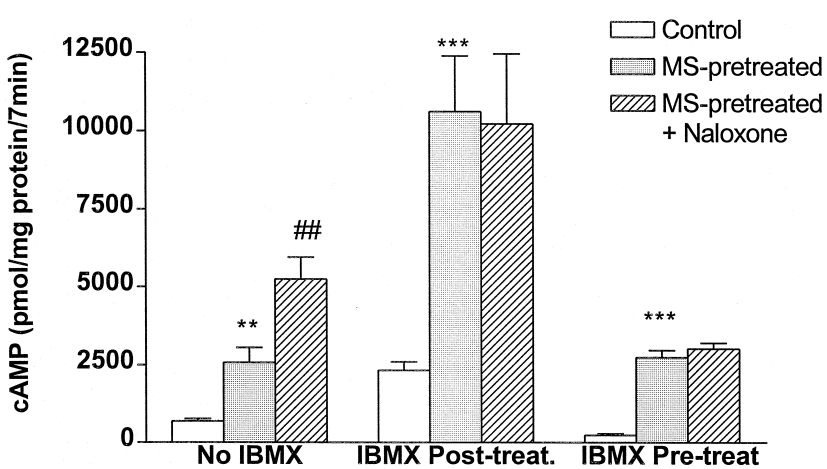

Fig. 2. Effects of IBMX on the spontaneous cAMP overshoot and the naloxone-induced cAMP overshoot. HEK-μ cells were either not treated (Control) or pre-treated with 1 μM morphine sulfate for 12 h (MS-pre-treated), and washed four times before assays. The cAMP accumulation assays were performed in the absence ('Control' and 'MS-pre-treated') or presence (MS-pre-treated + naloxone) of 10 μM naloxone. IBMX (500 μM) was present in cAMP accumulation assays (IBMX Post-treat), or present only in the 12 h pre-treatment (IBMX Pre-treat), or not present (No IBMX). Both morphine and IBMX were removed before cAMP assays. $N = 8$ for each column. $^{**}P < 0.01$, $^{***}P < 0.001$ ('MS-pre-treated' vs. 'Control'); $^{\#\#}P < 0.01$ ('MS-pre-treated + naloxone' vs. 'MS-pre-treated').

naloxone-induced cAMP overshoot was also abolished in the presence of 100 μM IBMX (data not shown), while 10 μM IBMX reduced the effect of naloxone from 82 ± 16% to 51 ± 8% ($P < 0.01$, $n = 6$) above the spontaneous cAMP overshoot. Therefore, IBMX dose-dependently suppressed the inverse naloxone effect on cAMP accumulation.

When IBMX (500 μM) was added to the pre-treatment together with 1 μM morphine, it did not elevate cAMP levels after both drugs were removed. On the contrary, we saw a decrease of cAMP levels after 8 h incubation with 500 μM IBMX ($P < 0.001$). IBMX did not alter the spontaneous cAMP overshoot. However, it blocked the naloxone-induced cAMP overshoot (Fig. 2, 'IBMX Pre-treat'). Full blockade effect was observed after pre-treatment with 10 and 100 μM IBMX (data not shown). It is important to note that the cAMP accumulation assays were performed after rigorous washes to remove both morphine and IBMX, and cAMP levels were not increased; therefore, it is unlikely that inhibition of phosphodiesterase contributed to the abolition of the naloxone-induced cAMP overshoot after IBMX pre-treatment.

3.4. Effect of IBMX on basal μ-opioid receptor phosphorylation in HEK-EE-μ cells

The μ-opioid receptor was phosphorylated at a basal rate, which was further enhanced by morphine (10 μM, 15 min). Moreover, basal μ-opioid receptor phosphorylation was increased after prolonged (8 h) treatment with morphine (1 μM), representing a feature associated with chronic morphine effects (Fig. 3, also see Arden et al., 1995; Wang et al., 1996). We determined the ability of IBMX to inhibit basal μ-opioid receptor phosphorylation when it was added into the phosphorylation labeling medium and incubated with cells for 15 min. IBMX inhibited basal μ-opioid receptor phosphorylation in a dose-dependent fashion. IBMX at 10 μM reduced basal μ-opioid receptor phosphorylation by 60%, whereas higher doses (100 μM and 500 μM) suppressed phosphorylation by 90% (Fig. 3).

We next pre-treated cells with IBMX for 8 h and subsequently measured receptor phosphorylation after removing IBMX. Pre-treatment with 10 μM and 100 μM IBMX for 8 h reduced basal μ-opioid receptor phosphory-

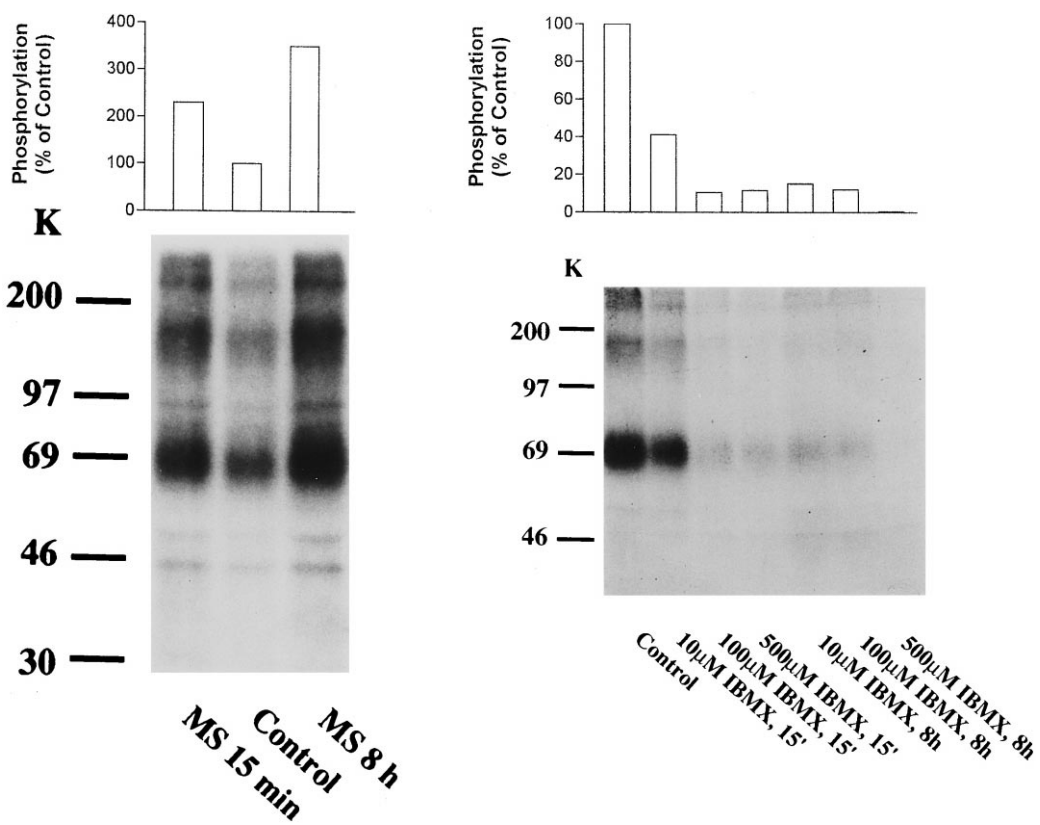

Fig. 3. Effects of morphine and IBMX on basal μ-opioid receptor phosphorylation. HEK-EE-μ cells were washed with phosphate-free DMEM medium five times, permeabilized with digitonin, and incubated with [γ-^{32}P]ATP for 15 min. The [^{32}P]-labeled band at ~65 kDa represents the μ-opioid receptor as confirmed by Western blotting. (Left panel) Basal μ-opioid receptor phosphorylation (Control) was stimulated in the presence of 10 μM morphine (MS, 15 min) or after 8 h pre-treatment with 1 μM morphine (MS 8 h). (Right panel) Effect of IBMX on basal μ-opioid receptor phosphorylation. IBMX was added either directly into the phosphorylation labeling buffer (IBMX, 15 min) or in the 8 h pre-treatment (IBMX, 8 h). Final IBMX concentrations ranged from 10 μM to 500 μM as indicated. Insert bar-graphs show relative phosphorylation intensities quantified by scanning densitometry.

lation by 85% and 90%, respectively. After HEK-EE-μ cells were treated with 500 μM IBMX for 8 h, basal μ-opioid receptor phosphorylation was no longer detectable (Fig. 3). Therefore, IBMX pre-treatment dose-dependently reduced basal μ-opioid receptor phosphorylation.

3.5. Effect of IBMX on morphine antinociception, antinociceptive tolerance to morphine, and naloxone-induced withdrawal jumping

The effect of IBMX on morphine antinociception was assessed in mice using the tail-flick test. When IBMX was given i.c.v. 30 min before the s.c. injection of morphine sulfate, there was no measurable change in morphine antinociception dose–response curve (Fig. 4A). The calculated morphine sulfate EC_{50} values (95% confidence limit) are 8.5 (6.5–11) mg/kg in naive mice and 7.5 (4.5–12) mg/kg in mice given 100 nmol IBMX. To exclude the possibility that differential distribution of the two drugs might account for this negative result, morphine and IBMX were given concomitantly by the i.c.v. route (Fig. 4B), IBMX at doses of 60 and 100 nmol did not affect morphine antinociception (Fig. 4B). The calculated ED_{50} values (95% confidence limit) are 3.3 (2.4–4.7) mg/kg for morphine sulfate, 3.1 (1.6–5.9) mg/kg for morphine sulfate plus 60 nmol IBMX, and 2.9 (1.7–5.1) mg/kg for morphine sulfate plus 100 nmol IBMX. Therefore, IBMX given i.c.v. had no detectable effect on the antinociceptive effects of morphine given either s.c. or i.c.v. in the tail-flick assay.

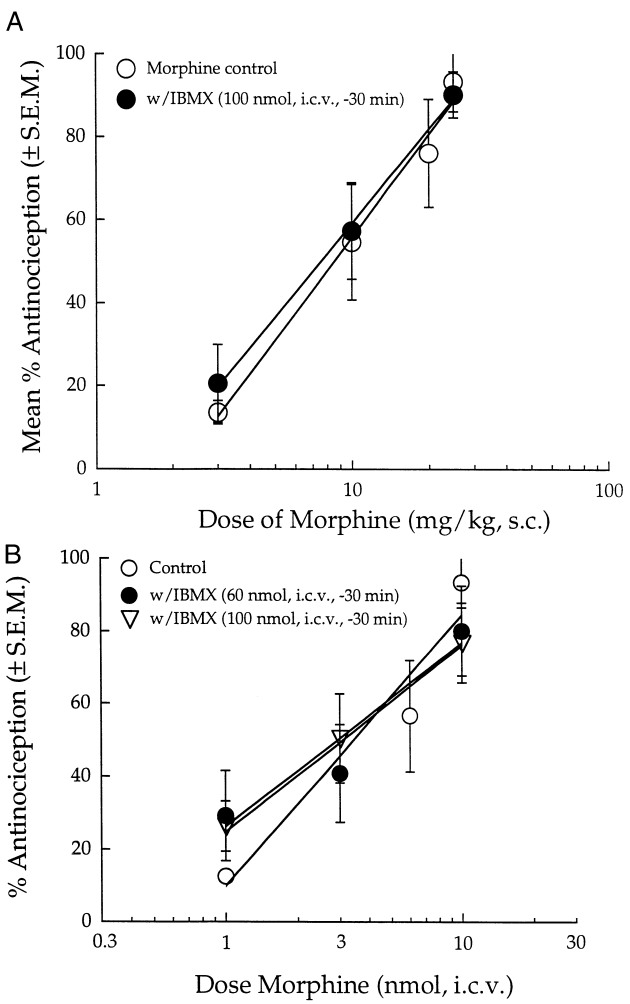

Fig. 4. Effects of IBMX on morphine antinociception in mice. Morphine was given (A) s.c. or (B) i.c.v. Morphine antinociception (– ○ –) was tested using the warm water (55°C) tail-flick assay. IBMX (60 nmol or 100 nmol, i.c.v.) was given 30 min before the test (– ● – and - ▽ -). Separate groups of mice were used for each dose–response curve. $N = 10$ for each point.

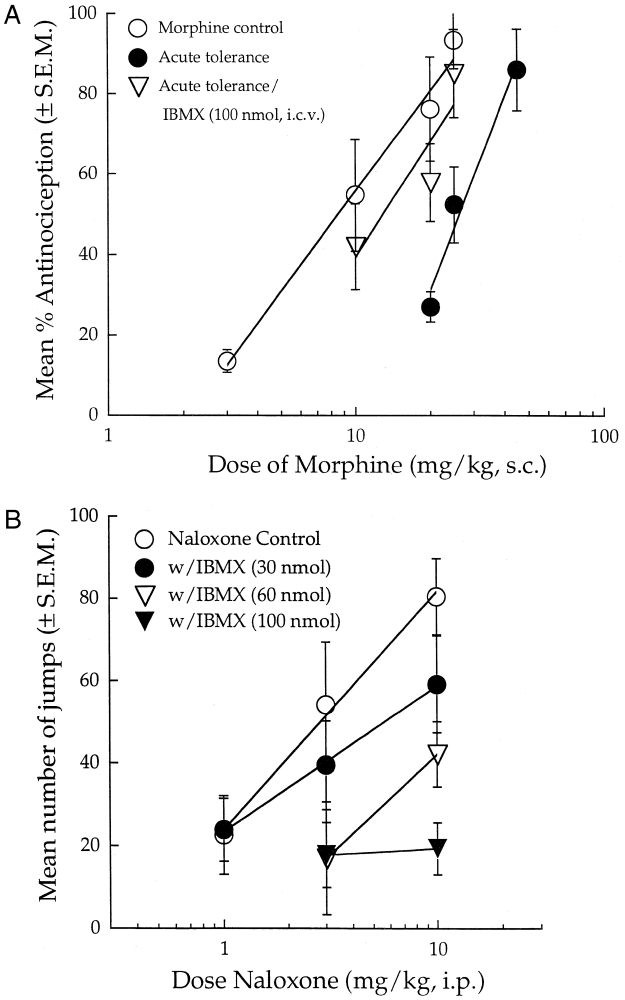

Fig. 5. Effects of IBMX on (A) morphine antinociception tolerance and (B) morphine dependence. (A) To induce tolerance, mice were injected with morphine sulfate (100 mg/kg, s.c.). A morphine (s.c.) antinociception dose–response curve was constructed 5 h later (– ● –) and compared to that in naive mice received a placebo injection (– ○ –). IBMX (100 nmol) was given i.c.v. 30 min before the antinociception assay (- ▽ -). (B) Mice were made dependent on morphine by a single subcutaneous injection of morphine sulfate (100 mg/kg, s.c.). Naloxone was given i.p. 4 h later. IBMX at the indicated doses was injected i.c.v. 30 min before the administration of naloxone.

The antinociceptive tolerance to morphine was tested 5 h after a single s.c. injection of 100 mg/kg morphine sulfate. The regimen led to a 2.5-fold rightward shift of the dose–response curve for morphine antinociception (Fig. 5A) with a calculated morphine sulfate EC_{50} value (95% confidence limit) of 24 (19–30) mg/kg compared with those in naive mice: 8.5 (6.5–11) mg/kg. When given 30 min before the tail-flick test, IBMX (100 nmol, i.c.v.) largely reversed this shift of the dose–response curve with a calculated morphine sulfate EC_{50} value (95% confidence limit) of 15 (13–18) mg/kg, indicating that IBMX reduced morphine antinociceptive tolerance.

Naloxone (i.p.) precipitated withdrawal in morphine-treated mice as evidenced by animal jumps. This effect peaked at 4 h after the initial dose of morphine sulfate (100 mg/kg, s.c.) (Bilsky et al., 1996), and was naloxone dose-dependent (Fig. 5B). IBMX, given i.c.v. 30 min before the injection of naloxone, significantly reduced the number of withdrawal jumps in a dose-dependent fashion, with the largest effects seen at 100 nmol.

4. Discussion

The protein kinase inhibitor H7 blocked the development of tolerance to morphine in SH-SY5Y cells (Wang et al., 1994b; Elliott et al., 1997), and prevented the naloxone-induced cAMP overshoot in morphine pre-treated SH-SY5Y cells (Wang et al., 1994b). It was also found to potently inhibit μ-opioid receptor phosphorylation (Wang et al., 1996). Moreover, H7 reversed morphine tolerance and dependence in vivo (Narita et al., 1994; Wang et al., 1994b; Bilsky et al., 1996). These results led us to hypothesize that μ-opioid receptor phosphorylation is important for the development of opioid tolerance and dependence.

In the present study, we further tested the hypothesis in HEK-μ cells. The stably expressed μ-opioid receptor was able to response to the opioid receptor agonist morphine and led to a profound inhibition of cAMP accumulation. Moreover, morphine-pre-treatment was able to induce cAMP upregulation. Using [^{3}H]morphine, we demonstrated that morphine could be functionally washed out after pre-treatment. However, in these morphine-pre-treated, thoroughly washed cells, naloxone was able to cause a substantial increase of intracellular cAMP levels, suggesting an inverse agonist effect by naloxone. Since the μ-opioid receptor was the only opioid receptor transfected into these HEK293 cells, and the inverse naloxone effect was absence in untransfected HEK293 cells, we concluded that the inverse naloxone effect was mediated by the μ-opioid receptor.

The mechanism underlying the inverse naloxone effect is less understood. IBMX, a cAMP phosphodiesterase inhibitor commonly used in adenylyl cyclase assays to prevent cAMP degradation (Morgan et al., 1993), abolished the inverse naloxone effect. The inverse naloxone effect was also absent in HEK-μ cells after the cells were treated with both IBMX and morphine. These results indicate that IBMX can suppress the inverse naloxone effect. The similarity of IBMX and H7 in preventing the inverse naloxone effect led us to test and discover that IBMX inhibits basal μ-opioid receptor phosphorylation. Basal μ-opioid receptor phosphorylation was also inhibited after 8 h treatment with IBMX. Other than inhibition of cAMP phosphodiesterase ($IC_{50} \sim 5$ μM) (Parsons et al., 1988), IBMX has been shown to act as an antagonist at adenosine receptors ($Ki = 50$ μM) (Snyder et al., 1981), inhibit sustained voltage-dependent Ca^{2+} currents ($Ki = 1.25$ mM) (Simasko and Yan, 1993) and inhibit Gi proteins at high doses (Parsons et al., 1988). Moreover, IBMX has effects in vivo that are difficult to reconcile by considering only its currently known biochemical effects (Holtzman, 1989; Nicholson et al., 1991). Whether inhibition of a protein kinase can account for some of these effects remains to be seen.

The identity of IBMX-sensitive protein kinase is not clear. The phosphorylation assay presented here was optimized to measure the protein kinase activity responsible for basal μ-opioid receptor phosphorylation. The kinase involved in basal μ-opioid receptor phosphorylation is Ca^{2+}-dependent, unaffected directly by high Na^+ ion concentrations or selective cAMP-dependent protein kinase and protein kinase C inhibitors (Wang et al., 1996). Therefore, these kinases are unlikely to play roles in the effect of IBMX. It was recently reported that μ-opioid receptors in isolated Neuro2A cell membranes can be phosphorylated when reconstituted with a catalytic subunit of the cAMP-dependent protein kinase, it is not known if the same phosphorylation event occurs in intact Neuro2A cells (Chakrabarti et al., 1998). However, it remains a possibility that IBMX may indirectly reduce μ-opioid receptor phosphorylation via phosphodiesterase inhibition and raised cAMP levels, or by affecting a member of phosphorylation cycle. As a purine analog, and thus an analog of ATP, it is possible that IBMX inhibits kinases other than the one(s) involved in basal μ-opioid receptor phosphorylation. In a separate study, however, we found that IBMX at 500 μM failed to inhibit the G protein-coupled receptor kinase 2 (GRK2 or βARK1) in assays using bovine rhodopsin as a substrate (Kassack et al., manuscript in preparation). This is also consistent with the suggestion that basal μ-opioid receptor phosphorylation is not mediated by GRK2 (Wang et al., 1996).

When given i.c.v., mice tolerated up to 100 nmol IBMX without visible adverse effects, and IBMX did not interfere with morphine antinociception. Others have also reported that IBMX (i.c.v.) did not affect morphine (i.c.v.) antinociception in mice (Suh et al., 1995). However, IBMX partially reversed morphine tolerance and effectively suppressed naloxone-induced withdrawal jumps. Therefore, IBMX selectively blocked chronic effects of morphine without affecting its antinociception.

Without considering inhibition of μ-opioid receptor phosphorylation, one would have expected high dose of IBMX to exacerbate withdrawal symptoms because it increases cAMP levels by blocking cAMP phosphodiesterase. Indeed, IBMX administered peripherally to rats was previously shown to cause 'quasi-narcotic withdrawal syndrome' upon naloxone challenge (Holtzman, 1989). Moreover, it was demonstrated that IBMX (s.c., 1 h before testing) intensified the naloxone-induced withdrawal syndrome in morphine-dependent rats (Collier and Francis, 1975). These results were attributed to a possible rise in cAMP levels of target neurons achieved by large doses of IBMX. We did not observe any 'quasi-narcotic withdrawal syndrome' nor did IBMX enhance the naloxone-induced withdrawal signs. These differences may be due to the different experimental animals and morphine-dependence models used, or may suggest that the cAMP levels were less disturbed in the present study. If the latter is true, then we might be observing the effect of IBMX less or little dependent on its effect on phosphodiesterase and resultant cAMP levels. Nonetheless, the potential role of cAMP phosphodiesterase in opioid tolerance and dependence requires further investigation. There is evidence that cAMP phosphodiesterase can be regulated by the μ-opioid receptor (Law and Loh, 1993), and a similar pathway has also been suggested for the μ-opioid receptor (Yu and Sadée, 1988).

In summary, our results demonstrate that IBMX inhibits basal μ-opioid receptor phosphorylation. This previously unknown effect of IBMX may account for some of its behavioral effects. This study provides additional support for the role of μ-opioid receptor phosphorylation in the development of opioid tolerance and dependence. IBMX and H7 represent two agents of drastically different pharmacological properties and chemical structures; however, both inhibited basal μ-opioid receptor phosphorylation, prevented the inverse naloxone effect in morphine-pretreated cells, suppressed morphine tolerance and dependence. In contrast, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H8), a close congener of H7, failed to affect basal μ-opioid receptor phosphorylation, inverse naloxone effect or morphine tolerance and dependence (Wang et al., 1994b). These suggest some degree of correlation among basal μ-opioid receptor phosphorylation, the inverse naloxone effect, and morphine dependence and tolerance in vivo. Since high-throughput assays can be designed to screen agents for their affect on the inverse naloxone effect, this study presents a new approach to search for agents that could potentially modulate opioid tolerance and dependence.

Acknowledgements

This work was supported by National Institute on Drug Abuse grant DA04166. Z.W. is a recipient of NIH training grant, GM07175.

References

Arden, J.R., Segredo, V., Wang, Z., Lameh, J., Sadée, W., 1995. Phosphorylation and agonist specific intracellular trafficking of an epitope-tagged μ-opioid receptor expressed in HEK 293 cells. J. Neurochem. 65, 1636–1645.

Avidor-Reiss, T., Bayewitch, M., Levy, R., Matus-Leibovitch, N., Nevo, I., Vogel, Z., 1995. Adenylyl cyclase supersensitization in mu-opioid receptor-transfected chinese hamster ovary cells following chronic opioid treatment. J. Biol. Chem. 270, 29732–29738.

Bilsky, E.J., Bernstein, R.N., Wang, Z., Sadée, W., Porreca, F., 1996. Effect of naloxone and CTAP and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. J. Pharmacol. Exp. Ther. 277, 484–490.

Burford, N.T., Wang, D., Sadée, W., 1997. Coupling of μ opioid receptors to multiple G proteins: enhanced activity at Gi3 and agonistic properties of naloxone. Abstract, International Narcotic Research Conference.

Chen, Y., Mestek, A., Liu, J., Hurley, J.A., Yu, L., 1993. Molecular cloning and functional expression of a μ-opioid receptor from rat brain. Mol. Pharmacol. 44, 8–12.

Chakrabarti, S., Law, P.Y., Loh, H.H., 1998. Distinct differences between morphine- and [D-Ala2,*N*-MePhe4,Gly-ol5]- enkephalin-mu-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. J. Neurochem. 7, 231–239.

Chavkin, C., Goldstein, A., 1984. Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. Proc. Natl. Acad. Sci. U.S.A. 81, 7253–7257.

Collier, H.O.J., Francis, D.L., 1975. Morphine abstinence is associated with increased brain cyclic AMP. Nature 253, 159–162.

Elliott, J., Guo, L., Traynor, J.R., 1997. Tolerance to mu-opioid agonists in human neuroblastoma SH-SY5Y cells as determined by changes in guanosine-5′-*O*-(3-[^{35}S]-thio)triphosphate binding. Br. J. Pharmacol. 121, 1422–1428.

Holtzman, S.G., 1989. Phosphodiesterase inhibitors potentiate opiate-antagonist discrimination by morphine-dependent rats. Pharmacol. Biochem. Behav. 33, 875–879.

Law, P.Y., Loh, H.H., 1993. δ-opioid receptor activates cAMP phosphodiesterase activities in neuroblastoma × glioma NG108-15 hybrid cells. Mol. Pharmacol. 43, 684–693.

Morgan, A.J., Murray, K.J., Challiss, R.A., 1993. Comparison of the effect of isobutylmethylxanthine and phosphodiesterase-selective inhibitors on cAMP levels in SH-SY5Y neuroblastoma cells. Biochem. Pharmacol. 45, 2373–2380.

Narita, M., Feng, Y., Makimura, M., Hoskins, B., Ho, I.K., 1994. A protein kinase inhibitor, H-7, inhibits the development of tolerance to opioid antinociception. Eur. J. Pharmacol. 271, 543–545.

Nestler, E., 1992. Molecular mechanism of drug addiction. J. Neurosci. 12, 2439–2450.

Nicholson, D., Reid, A., Sawynok, J., 1991. Effects of forskolin and phosphodiesterase inhibitors on spinal antinociception by morphine. Pharmacol. Biochem. Behav. 38, 753–758.

Parsons, W.J., Ramkumar, V., Stiles, G.L., 1988. Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, Gi. Mol. Pharmacol. 34, 37–41.

Patterson, T.A., Appleyard, S.M., Chavkin, C., 1995. Opioid receptors are basely phosphorylated in guinea pig brain slices by PKC and PKA. Abstracts, Soc. Neurosci. 21, 1356.

Simasko, S.M., Yan, S., 1993. 3-Isobutyl-1-methylxanthine inhibits sustained calcium current independently of cyclic AMP in neuronal and endocrine cells. Mol. Pharmacol. 44, 622–627.

Snyder, S.H., Katims, J.J., Annau, Z., Bruns, R.F., Daly, J.W., 1981. Adenosine receptors and behavioral actions of methylxanthines. Proc. Natl. Acad. Sci. U.S.A. 78, 3260–3264.

Suh, H.-W., Sim, Y.-B., Choi, Y.-S., Song, D.-K., Kim, Y.-H., 1995. Differential effects of 3-isobutyl-1-methylxanthine injected intrathecally or intracerebroventricularly on antinociception induced by opi-

oids administrated intracerebroventricularly in the mouse. Neuropeptide 28, 357–363.

Wang, Z., Arden, J.R., Segredo, V., Sadée, W., 1994a. Agonist induced constitutive activation of the μ receptor by phosphorylation. Regul. Pept. 54, 323–324.

Wang, Z., Bilsky, E.J., Porreca, F., Sadée, W., 1994b. Constitutive μ opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci. PL 54, 339–350.

Wang, Z., Arden, J., Sadée, W., 1996. Basal phosphorylation of μ opioid receptor is agonist modulated and Ca^{2+}-dependent. FEBS Lett. 387, 53–57.

Way, E.L., Loh, H.H., Shen, F., 1969. Simultaneous quantitative assessment of morphine tolerance and physical dependence. J. Pharmacol. Exp. Ther. 167, 1–8.

Yu, V., Sadée, W., 1988. Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in differentiated human neuroblastoma cells. J. Pharmacol. Exp. Ther. 245, 350–355.

Yu, V., Eiger, S., Duan, D.S., Lameh, J., Sadée, W., 1990. Regulation of cyclic AMP by the mu-opioid receptor in human neuroblastoma SH-SY5Y cells. J. Neurochem. 55, 1390–1396.

Zhang, L., Yu, L., Machin, S., Weight, F.F., Uhl, G.R., Wang, J.B., 1996. Differential μ opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J. Biol. Chem. 271, 11449–11454.