ELSEVIER

European Journal of Pharmacology 389 (2000) 165–171

ejp

www.elsevier.nl/locate/ejphar

Tolerance to morphine at the μ-opioid receptor differentially induced by cAMP-dependent protein kinase activation and morphine

Zaijie Wang [a,b,*], Wolfgang Sadée [a]

[a] *Departments of Pharmaceutical Chemistry and Biopharmaceutical Sciences, University of California, San Francisco, CA 94143-0446 USA*
[b] *Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724-5050 USA*

Received 16 September 1999; accepted 1 December 1999

Abstract

Human neuroblastoma SH-SY5Y cells express endogenous μ-opioid receptor and develop cellular tolerance to morphine after prolonged (≥ 4 h) treatment with morphine. Treatment with forskolin (25 μM, 12 h), an adenylyl cyclase activator, also desensitized μ-opioid receptor response to morphine (10 μM) by 38% ($P < 0.001$), which was reversed by the cyclic AMP (cAMP) dependent kinase inhibitor *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H8) (100 μM). Treatment with both morphine and forskolin appeared to cause an additive effect in desensitizing μ-opioid receptor. In μ-opioid receptor stably transfected human embryonic kidney 293 (HEK-μ) cells, morphine treatment produced cAMP upregulation, yet failed to induce μ-opioid receptor tolerance. However, treatment with forskolin (25 μM) or 8-bromo-cAMP (1mM) led to profound μ-opioid receptor tolerance, which was reversed by H8. These results demonstrate that cAMP-dependent kinase activation causes μ-opioid receptor tolerance. However, morphine-induced μ-opioid receptor tolerance in SH-SY5Y cells is not mediated by cAMP-dependent kinase activation. In addition, our results indicate that cAMP-upregulation does not necessarily lead to μ-opioid receptor tolerance.

Keywords: μ-Opioid receptor; Tolerance; Desensitization; cAMP-dependent protein kinase; Morphine; *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H8)

1. Introduction

Agonist-induced receptor desensitization is a common phenomenon associated with activation of many neurotransmitter receptors, leading to reduced biological responses during continuous or repeated agonist exposure. Among G-protein coupled receptors, mechanisms of desensitization have been extensively characterized for the β_2-adrenergic receptor. Phosphorylation of β_2-adrenoceptor by the cyclic AMP (cAMP) dependent protein kinase and the G-protein coupled receptor kinase 2 are, among other mechanisms, involved in rapid desensitization of β_2-adrenergic receptor (Freedman and Lefkowitz, 1996).

Opioid receptors belong to the family of G-protein coupled receptors. Activation of all three major (μ, δ, κ) opioid receptor types lead to inhibition of adenylyl cyclase, activation of K^+ channels, as well as inhibition of Ca^{2+} conductance (Loh et al., 1988; Mestek et al., 1995; Koob and Nestler, 1997). Morphine and other clinically used opioids act primarily on the μ-opioid receptor to produce their analgesic and rewarding effects. Tolerance (including attenuated analgesic effect) after repeated administration of opioids is well documented in clinical practice; however, the mechanisms remain to be elucidated. Receptor down-regulation, internalization, and uncoupling from the second messenger system of μ-opioid receptor have all been speculated to contribute to opioid tolerance, yet numerous studies from different laboratories have so far failed to identify consistent changes of receptor numbers or affinity, or G-protein levels after prolonged treatment with morphine (Loh et al., 1988; De Vries et al., 1991; Koob and Nestler, 1997). In addition, unlike the peptide agonist [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), morphine does not internalize μ-opioid receptor (Arden et al., 1995; Keith et al., 1998).

Using a reconstituted in vitro system, activation of cAMP-dependent kinase was shown to disrupt coupling of the μ-opioid receptor to G_i (Harada et al., 1989, 1990). Although morphine acutely inhibits adenylyl cyclase, chronic exposure to morphine results in a compensatory upregulation of adenylyl cyclase activity and cAMP pro-

* Corresponding author. Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724-5050, USA. Tel.: +1-520-626-4940; fax: +1-520-626-2204.
E-mail address: zwang@u.arizona.edu (Z. Wang).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00881-X

duction (Sharma et al., 1975; Yu and Sadée, 1988), which can lead to the activation of cAMP-dependent kinase. Therefore, it is possible that enhanced cAMP-dependent kinase activity, after prolonged morphine treatment, can contribute to μ-opioid receptor tolerance. Phosphorylation of the μ-opioid receptor is enhanced during chronic morphine exposure (Wang et al., 1996). Moreover, activation of cAMP-dependent kinase can phosphorylate and activate cAMP response element binding protein (CREB), which regulates the expression of numerous genes (Koob and Nestler, 1997). Contradicting these results, activation of cAMP-dependent kinase was found to prevent DAMGO-induced μ-opioid receptor desensitization in *Xenopus* oocytes expressing the μ-opioid receptor and a G-protein-activated K^+ channel (Chen and Yu, 1994).

To study cAMP/cAMP-dependent kinase pathway mediated μ-opioid receptor desensitization, we examined μ-opioid receptor tolerance in human neuroblastoma SH-SY5Y cells, and human embryonic kidney (HEK) 293 cells stably transfected with a cloned μ-opioid receptor (HEK-μ) (Arden et al., 1995). HEK-μ cells express only the cloned μ-opioid receptor. SH-SY5Y cells natively express both μ and δ opioid receptors (ratio ~ 4.5:1). In both cell lines, μ-opioid receptor tolerance can be assessed by the decreased effect of morphine in inhibiting cAMP accumulation. The cAMP-dependent kinase can be activated by treating cells with forskolin, an adenylyl cyclase activator that increases cAMP levels and cAMP-dependent kinase activity (Sibley et al., 1998). More directly, cell permeable cAMP analogs such as 8-bromo-cAMP can activate the cAMP-dependent kinase (Itano et al., 1996; Sibley et al., 1998).

In this study, we report that tolerance to morphine developed in SH-SY5Y cells either by treatment with morphine or by activation of cAMP-dependent kinase, but through different mechanisms. In HEK-μ cells, tolerance occurred after treatment with forskolin or 8-bromo-cAMP, but not with morphine.

2. Materials and methods

2.1. Materials

Human neuroblastoma SH-SY5Y cells were provided by Dr. June L. Biedler of the Sloan–Kettering Institute for Cancer Research (Rye, NY). *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H8) was from Seikagaku America (Rockville, MD). Morphine sulfate was provided by the National Institute on Drug Abuse (Bethesda, MD). All other chemicals were purchased from Sigma (St. Louis, MO). The rat μ-opioid receptor cDNA in vector pRC/CMV was a generous gift from Dr. Lei Yu, and stably transfected into HEK 293 cells (ATCC) with calcium phosphate precipitation method (Arden et al., 1995).

2.2. Cell culture

SH-SY5Y and HEK-μ cells were grown at 37°C in Dulbecco's modified eagle medium (DMEM) (Yu and Sadée, 1988) and DMEM/F12 (1:1) medium (Arden et al., 1995), respectively. Both media were supplemented with 10% fetal calf serum containing 100 μg/ml streptomycin and 100 units/ml penicillin. G418 (200 μg/ml) was added in the HEK-μ cell culture to maintain stable selection. SH-SY5Y cells were pretreated with retinoic acid (5 μM) for 6 days to induce partial neuronal differentiation in all experiments (Yu and Sadée, 1988; Wang et al., 1994).

2.3. cAMP accumulation assay

Cells were treated with morphine (1 μM) and/or a cAMP-dependent kinase activator (25 μM forskolin or 1 mM 8-bromo-cAMP) for 12 h, unless otherwise specified. At the end of treatment, cells were rinsed twice with DMEM medium containing 5% serum and twice more with serum free DMEM medium to remove these compounds. In SH-SY5Y cells, intracellular cAMP accumulation was assayed by incubating cells in 37°C serum free medium containing 1 μM prostaglandin E_1 for 15 min (Yu and Sadée, 1988). Since the prostaglandin E_1 receptor was absent in HEK 293 cells, cAMP accumulation was assayed in the present of 10 μM forskolin for 7 min, unless otherwise indicated. To assess the ability of morphine in inhibiting cAMP levels, morphine sulfate (10 μM) was added into cAMP accumulation assays. Reactions were stopped by adding hydrochloric acid to a final concentration of 0.1 N. Levels of cAMP were determined by the radioimmunoassay (Amersham) (Yu et al., 1990; Wang et al., 1994).

2.4. Data analysis

Inhibition of cAMP accumulation by morphine was expressed in percentage by the following equation:

$$\%\text{Inhibition} = 100\% - \left(\frac{\text{cAMP levels in the presence of morphine}}{\text{cAMP levels in the absence of morphine}}\right) \times 100\%.$$

Morphine EC_{50} values were determined by fitting the data to the logistic function:

$$E = \frac{E_{max} \times C^n}{EC_{50}^n + C^n}$$

(E = % inhibition; E_{max} = maximum % inhibition; C = morphine concentration; n = slope factor; data were fitted with the use of the software KaleidaGraph version 3.0).

Student's *t*-test was used for statistical comparisons.

3. Results

3.1. Tolerance to morphine after treatment with morphine in SH-SY5Y cells

Upon prolonged exposure to morphine, SH-SY5Y cells develop a moderate degree tolerance to morphine while the cAMP second messenger system is upregulated (Sharma et al., 1975; Yu et al., 1990; Wang et al., 1994). To determine the time course of the development of tolerance and cAMP upregulation, SH-SY5Y cells were pretreated with 1 μM morphine for 0–24 h. At the end of incubation, cells were thoroughly washed to remove morphine. At 1 μM, morphine was able to induce full cAMP upregulation and μ-opioid receptor tolerance, while the washout procedure ensured removal of morphine to a pharmacologically insignificant level (Wang et al., 1999). Pretreatment of SH-SY5Y cells with morphine for 20 min or less caused a small, but significant decrease of prostaglandin E_1-stimulated cAMP accumulation (Fig. 1), which was not reversed by naloxone (data not shown), ruling out the presence of residual morphine. While the reason for this initial decrease is not clear (Yu et al., 1990; Wang et al., 1994), a similar phenomenon has been reported by others in perfused brain tissue (Parolaro et al., 1990). Levels of cAMP, however, recovered after 30 min incubation. When morphine treatment lasted for 4–24 h, an increased cAMP level (cAMP upregulation) was observed, which peaked at 12 h with a 75 ± 3% (mean ± S.E.M., $n = 6$) increase over the control ($P < 0.001$). To assess the inhibitory effect of morphine on cAMP levels, fresh morphine (10 μM) was added to half of the samples in the cAMP accumulation assays to reach a full inhibition response (Yu and Sadée, 1988). Inhibition by morphine was significantly attenuated from 62 ± 1% (mean ± S.E.M., $n = 12$) to 47 ± 2% (mean ± S.E.M., $n = 6$) ($P < 0.001$) after 4 h incubation with morphine (1 μM), indicating the development of detectable tolerance to morphine in SH-SY5Y cells (Fig. 1). In the previous studies, we found morphine treatment also shifted EC_{50} 3–4 fold higher (Yu and Sadée, 1988; Yu et al., 1990).

3.2. Tolerance to morphine induced by forskolin in SH-SY5Y cells

We next tested if activation of cAMP-dependent kinase by forskolin would desensitize μ-opioid receptor. After pretreatment with forskolin (25 μM) for 12 h, SH-SY5Y cells notably lost sensitivity to morphine, as morphine produced a maximum inhibition of 38 ± 2% (mean ± S.E.M., $n = 6$) with forskolin treatment vs. 62 ± 2% (mean ± S.E.M., $n = 6$) in control cells ($P < 0.001$) (Fig. 2), indicating the development of μ-opioid receptor tolerance to morphine. Moreover, forskolin induced greater μ-opioid receptor desensitization than morphine did ($P < 0.01$). Forskolin-induced tolerance was blocked by the cAMP-dependent kinase inhibitor H8 (100 μM), which re-sensitized μ-opioid receptor response to morphine to 52 ± 5% (mean ± S.E.M., $n = 4$) inhibition on cAMP accumulation ($P < 0.05$ vs. treatment with forskolin alone, $P > 0.05$ vs. control). Therefore, activation of cAMP-dependent kinase led to μ-opioid receptor tolerance to morphine.

If morphine-induced μ-opioid receptor tolerance occurred by the same mechanism as that induced by forskolin, then addition of high concentrations of morphine and forskolin would not produce any additional effect. However, co-treatment with forskolin (25 μM) and morphine (1 μM) led to further loss of μ-opioid receptor sensitivity to morphine, producing 21 ± 4% (mean ± S.E.M., $n = 6$) inhibition on cAMP accumulation by 10 μM morphine ($P < 0.001$ vs. control, $P < 0.001$ vs. either forskolin or

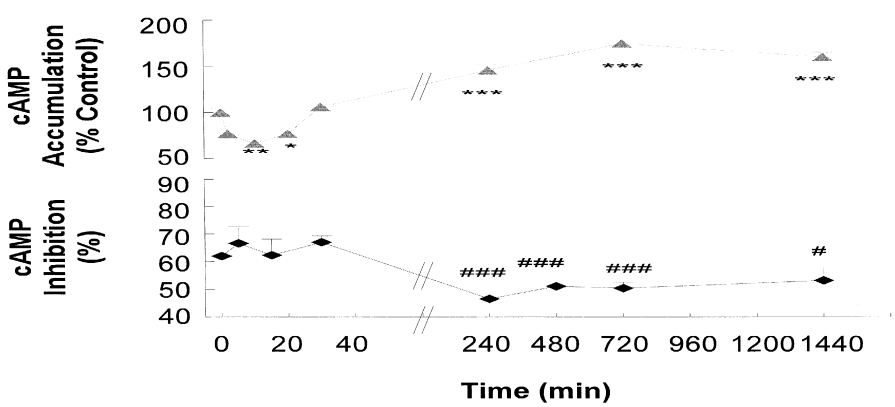

Fig. 1. Time course of the development of cAMP upregulation and morphine tolerance in SH-SY5Y cells. SH-SY5Y cells were pretreated with medium containing 1 μM morphine for 0–24 h. Prostaglandin E_1-stimulated (1 μM) cAMP accumulation was assayed, after washing away morphine, in the absence of morphine to obtain data for the construction of the ''cAMP upregulation'' curve (-▲-). The cAMP level before treatment was 2150 ± 100 pmol/mg protein/15 min (mean ± S.E.M.). To obtain the ''cAMP inhibition'' curve (-◆-), cAMP accumulation assays were performed in the presence of 10 μM morphine. Values are mean ± S.E.M. ($n = 6$–12). Asterisks indicate significant differences from cAMP levels before morphine-pretreatment: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; #'s indicate significant differences from cAMP inhibition before morphine-pretreatment: $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$.

morphine treatment alone). Therefore, the combination of these two compounds produced an additive effect in desensitizing μ-opioid receptor: 66% desensitization (loss of ability in inhibiting cAMP accumulation by 10 μM morphine) vs. 17% desensitization by morphine and 38% desensitization by forskolin. These results suggested that μ-opioid receptor tolerance after treatment with morphine or forskolin took place via different mechanisms.

3.3. Coupling and tolerance of μ-opioid receptor expressed in HEK-μ cells

Tolerance of μ-opioid receptor was further studied in HEK-μ cells expressing a cloned rat μ-opioid receptor (Chen et al., 1993). The adenylyl cyclase activity in HEK 293 can be stimulated by forskolin. Addition of 10, 50 or 100 μM forskolin resulted in 30-, 160- and 333-fold increases in cAMP levels, respectively (Fig. 3A). The time course of forskolin-stimulated cAMP accumulation was studied by incubating HEK-μ cells with 10 μM forskolin at 37°C for 0–10 min before the reactions were stopped by HCl. Intracellular cAMP levels increased within minutes after the addition of forskolin, and peaked around 7 min in cells either pretreated or untreated with morphine (Fig. 3B). The transfected μ-opioid receptor was functionally coupled to adenylyl cyclase, as morphine (10 μM) produced 68%, 76% and 90% inhibition on cAMP accumulation in the presence of 10, 50 and 100 μM forskolin, respectively (Fig. 4). Like in SH-SY5Y cells, pretreatment with morphine resulted in cAMP upregulation in HEK-μ cells, ranging from 75% to 200% increase (Fig. 3B). In contrast to the results in SH-SY5Y cells, pretreatment of HEK-μ cells with 1 μM morphine for 12 h did not cause any loss of effect by morphine (Figs. 4 and 5). On the contrary, we repeatedly saw enhanced maximum effect of morphine after pretreatment with morphine (Fig. 4). EC_{50} of morphine remained unchanged (29 ± 2 vs. 29 ± 6 nM

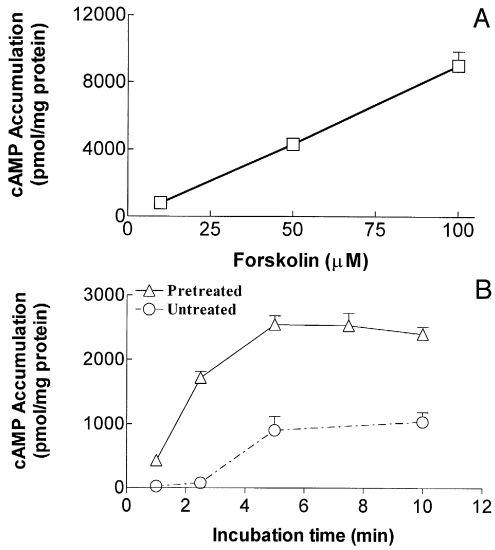

Fig. 3. Concentration-dependent (A) and time course (B) of stimulation of cAMP accumulation by forskolin in HEK-μ cells. (A) Naïve HEK-μ cells were incubated with 10–100 μM forskolin at 37°C for 7 min. (B) Naïve (-○-) or morphine (1 μM, 12 h) (-Δ-) pretreated cells were washed four times and incubated with 10 μM forskolin in serum-free DMEM medium at 37°C for 1–10 min in the absence of any phosphodiesterase inhibitor. The reactions were stopped by adding HCl to a final concentration of 0.1 N. cAMP levels were determined by the radioimmunoassay. Values are mean ± S.E.M. ($n = 6$).

before treatment) (Fig. 5). Lack of morphine-tolerance was not due to insufficient morphine concentration or time of pretreatment, nor was it affected by the presence of phosphodiesterase inhibitors in the assay (data not shown).

Morphine at low concentrations (1 nM in control; 1 and 10 nM in pretreated cells) actually exhibited stimulatory effect on cAMP accumulation. Excitatory effects by vari-

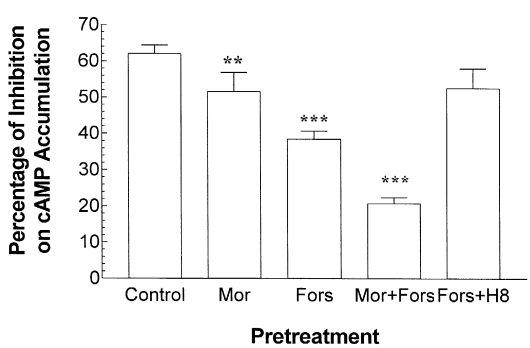

Fig. 2. Effect of morphine and/or forskolin on morphine-mediated cAMP inhibition. SH-SY5Y cells were pretreated for 12 h with medium (Control), or media containing 1 μM morphine (Mor) and/or 25 μM forskolin (Fors), or medium containing 25 μM forskolin and 100 μM H8 (Fors + H8). Prostaglandin E_1-stimulated cAMP accumulation was assayed in the absence or presence of 10 μM morphine after washing away these compounds. Values are mean ± S.E.M. ($n = 4$–6). The asterisks indicate significant differences from control: $^{**}P < 0.01$; $^{***}P < 0.001$.

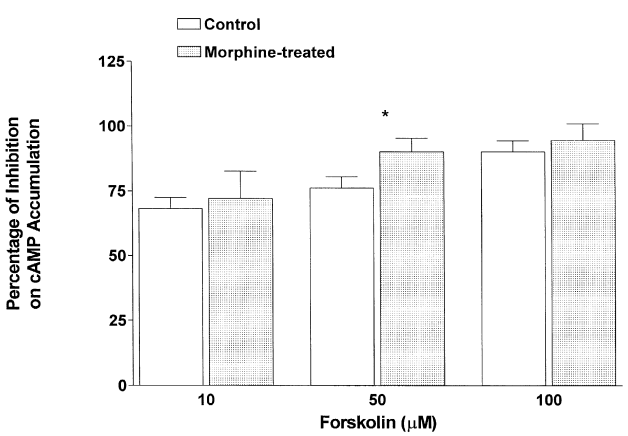

Fig. 4. Inhibition on cAMP accumulation by morphine (10 μM) in control and morphine-pretreated HEK-μ cells in the presence of 10, 50 and 100 μM forskolin. Forskolin-stimulated cAMP accumulation was assayed in HEK-μ cells before (open column) and after (dotted column) morphine pretreatment (1 μM, 12 h). Refer to Fig. 3 for the control cAMP levels at each forskolin concentration. Values are mean ± S.E.M ($n = 4$–6). The asterisks indicate significant differences from control: $^{*}P < 0.05$.

ous opioids at certain (usually low) concentrations have been reported previously, which were thought to occur through the coupling of μ-opioid receptor to Gs pathway (Shen and Crain, 1994; Wang and Gintzler, 1994).

3.4. Tolerance to morphine induced by cAMP-dependent kinase activation in HEK-μ cells

To test whether cAMP-dependent kinase activation still causes μ-opioid receptor tolerance to morphine in HEK-μ cells, forskolin (25 μM, 12 h) was employed to activate the cAMP-dependent kinase in these cells (Sibley et al., 1998). Forskolin-stimulated (10 μM) cAMP level was 56% lower after pretreatment with forskolin (25 μM, 12 h) (344 ± 78 pmol/mg protein/7 min after treatment vs. 778 ± 183 pmol/mg protein/7 min before treatment, $P < 0.01$), suggesting the reduced response of adenylyl cyclase to acute forskolin after pretreatment. Nevertheless, even with reduced stimulation, 10 μM forskolin still gave us sufficiently high cAMP levels for measurement. After 12 h forskolin (25 μM) treatment, effect of 10 μM morphine in inhibiting cAMP accumulation was greatly reduced from $68 \pm 3\%$ (mean ± S.E.M., $n = 6$) to $30 \pm 3\%$ (mean ± S.E.M., $n = 6$) ($P < 0.001$) (Fig. 5), indicating the development of μ-opioid receptor tolerance. However, the EC_{50} was reduced from 29 ± 4 nM before treatment to 8 ± 2 nM after treatment (Fig. 5). The latter was probably due to the stimulatory effect of morphine at low concentrations, which was absent after pretreatment with forskolin.

Forskolin-induced μ-opioid receptor tolerance to morphine in HEK-μ cells was completely reversed by co-treatment with 100 μM H8 (Fig. 6) ($P < 0.01$ vs. forskolin treatment alone; not significantly different from the control).

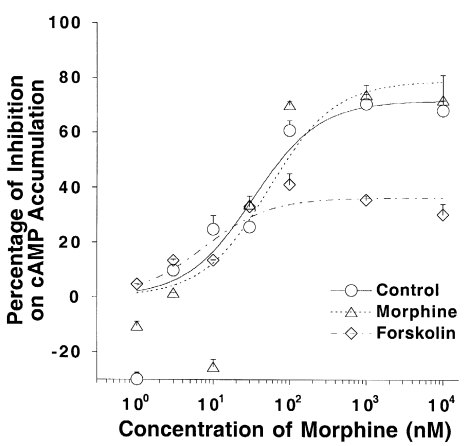

Fig. 5. Dose–response curves of morphine in inhibiting cAMP accumulation before and after treatment with morphine or forskolin. HEK-μ cells were pretreated with 1 μM morphine or 25 μM forskolin for 12 h, followed by washing out of these compounds before the cells were incubated for cAMP accumulation assays in the presence of 10 μM forskolin and increasing concentrations of morphine at 37°C for 7 min. Values are mean ± S.E.M. ($n = 6$). The curves were fitted with logistic function: $E = E_{max} \times C^n/(C^n + EC_{50}^n)$, which was best done when n is 1. Points below 0% inhibition (stimulatory effect) were plotted, but were not used for fitting.

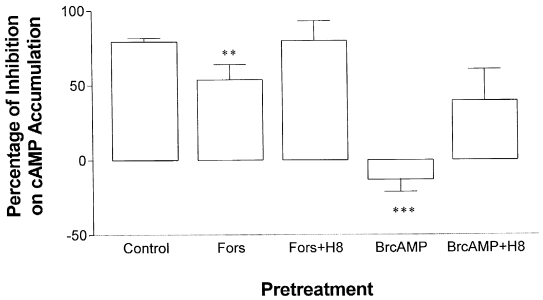

Fig. 6. Effect of forskolin and 8-bromo-cAMP on morphine-mediated (1 μM) cAMP inhibition. HEK-μ cells were pretreated for 12 h with DMEM medium (Control), or media containing 25 μM forskolin (Fors), both 25 μM forskolin and 100 μM H8 (Fors + H8), 1 mM 8-bromo-cAMP (BrcAMP), or both 1 mM 8-bromo-cAMP and 100 μM H8 (BrcAMP + H8). Forskolin-stimulated (10 μM) cAMP accumulation were assayed after washing out these compounds. The control cAMP level in the absence of morphine was 807 ± 82 pmol/mg protein/7 min (mean ± S.E.M.). All values are mean ± S.E.M. ($n = 4–6$). The asterisks indicate significant differences from the control: $^{**}P < 0.01$; $^{***}P < 0.001$.

HEK-μ cells were also treated with the cell-permeable cAMP-dependent kinase activator, 8-bromo-cAMP (1 mM, 12 h), which abolished the effect of morphine in inhibiting cAMP accumulation ($P < 0.001$). The response to morphine was partially restored by H8 (100 μM).

4. Discussion

Exposure of SH-SY5Y cells to μ-opioid receptor agonist morphine led to reduced ability of subsequent morphine to inhibit adenylyl cyclase activity and cAMP accumulation. Unlike the rapid desensitization seen with other G-protein coupled receptors including the δ-opioid receptor (Freedman and Lefkowitz, 1996), μ-opioid receptor tolerance to morphine developed over 4 h in SH-SY5Y cells. The delayed on-set of tolerance paralleled to the development of tolerance in vivo (Grumbach et al., 1974). Interestingly, treatment with forskolin or 8-bromo-cAMP led to μ-opioid receptor tolerance to morphine, which was blocked by co-pretreatment with H8, a non-selective cAMP-dependent kinase inhibitor (IC_{50} at 10 μM). These data suggested a new pathway for μ-opioid receptor tolerance, namely the cAMP/cAMP-dependent kinase-mediated μ-opioid receptor tolerance. Such mechanism has been proposed for G-protein coupled receptors coupled with Gs, which positively regulates the adenylyl cyclase and cAMP levels, and may be acting as a negative feedback mechanism (Sibley et al., 1998).

The next question was if cAMP-mediated μ-opioid receptor tolerance contributed to opioid-induced μ-opioid receptor tolerance. To address this question, one can test whether morphine-induced μ-opioid receptor tolerance is blocked by cAMP-dependent kinase inhibitors. Few

cAMP-dependent kinase inhibitors available are both selective and cell-permeable. *N*-[2-((*p*-bromocinnamyl)-amino)ethyl]-5-isoquinolinesulfonamide (H89) is often used for this purpose; however, achieving both full cAMP-dependent kinase inhibition and selectivity has been a concern. Moreover, H89 may interfere with binding and function of G-protein coupled receptors (Penn et al., 1999). Alternatively, the problem can be addressed by simultaneously activating the receptor and cAMP/cAMP-dependent kinase pathways. If a common mechanism were shared, no additive effect would be observed. Surprisingly, co-treatment with forskolin and morphine, both at concentrations to produce maximum effects, caused additive effect on μ-opioid receptor tolerance. This result suggested that cAMP/cAMP-dependent kinase pathway mediated μ-opioid receptor tolerance did not contribute to morphine-induced μ-opioid receptor tolerance. To test this further, we studied μ-opioid receptor tolerance in HEK-μ cells, in which we found striking differences in μ-opioid receptor tolerance induced by cAMP/cAMP-dependent kinase activation and morphine treatment. Tolerance to morphine was induced after activation of cAMP-dependent kinase by forskolin or 8-bromo-cAMP, yet morphine did not induce μ-opioid receptor tolerance in HEK-μ cells.

The reason for the lack of μ-opioid receptor tolerance in HEK-μ cells is unclear. Lack of tolerance was also reported in μ-opioid receptor transfected Chinese hamster ovary (CHO) cells (Avidor-Reiss et al., 1995). When the same cloned receptor was co-expressed with a K^+ channel in *Xenopus* oocytes, μ-opioid receptor tolerance developed as measured by reduced-K^+ conductance (Chen and Yu, 1994). Opioid tolerance also occurred in murine neuroblastoma neuro2A cells and human epidermoid carcinoma A431 cells stably transfected with the μ-opioid receptor gene (Chakrabarti et al., 1995; Ammer and Schulz, 1997).

Phosphorylation of β_2-adrenoceptor (and many other Gs-coupled receptors) by cAMP-dependent kinase is thought to be involved mainly in heterologous receptor desensitization (Freedman and Lefkowitz, 1996). This is in agreement with our finding that the cAMP-dependent kinase was not involved in morphine-induced μ-opioid receptor tolerance. It was further supported by other experiments, both in vivo and in vitro, that cAMP-dependent kinase-mediated phosphorylation was found not to be involved in homologous opioid tolerance, although μ-opioid receptor phosphorylation in general is important for the development of μ-opioid receptor tolerance (Wang et al., 1994; Mestek et al., 1995; Narita et al., 1995; Bilsky et al., 1996).

It is generally accepted that there is little, if any, cross-tolerance between the different subtypes of opioid receptors. Nevertheless, there is evidence for the interaction between μ-opioid receptor and other G-protein coupled receptors such as α_2-adrenergic receptor (Aley and Levine, 1997). Morphine tolerance was also modulated by NMDA receptor activity (Trujillo and Akil, 1991). Whether any of these interactions is mediated by the cAMP-dependent kinase remains to be tested. When reconstituted with a purified catalytic subunit of the cAMP-dependent kinase, μ-opioid receptor in isolated membranes was able to undergo cAMP-dependent kinase-mediated phosphorylation (Bernstein and Welch, 1998; Chakrabarti et al., 1998).

The upregulated cAMP levels after chronic opioid treatment have been proposed as biochemical mediators underlying chronic effects of opioids including tolerance (Sharma et al., 1975; Yu et al., 1990; Wang et al., 1994). However, in HEK-μ cells, cAMP upregulation resulting from morphine treatment was not associated with the development of morphine tolerance, suggesting that morphine-induced cAMP-upregulation did not necessary lead to μ-opioid receptor tolerance. Similar results were reported by others in the rat brain slices (De Vries et al., 1991) and cultured neurons (Van Vliet et al., 1991) that prior morphine exposure did not lead to μ-opioid receptor tolerance albeit cAMP upregulation.

In summary, we reported here that elevated cAMP levels and cAMP-dependent kinase activation, without prior treatment with any opioids, can effectively lead to μ-opioid receptor tolerance to morphine. However, cAMP/cAMP-dependent kinase-mediated μ-opioid receptor tolerance did not participate in the development of morphine tolerance.

Acknowledgements

This work was supported by The National Institute on Drug Abuse grant DA04166. Z.W. was supported in part by The National Institute of Health grant GM07175.

References

Aley, K.O., Levine, J.D., 1997. Multiple receptors involved in peripheral alpha 2, mu, and A1 antinociception, tolerance, and withdrawal. J. Neurosci. 17, 735–744.

Ammer, H., Schulz, R., 1997. Chronic morphine treatment increases stimulatory beta-2 adrenoceptor signaling in A431 cells stably expressing the mu-opioid receptor. J. Pharmacol. Exp. Ther. 280, 512–520.

Arden, J.R., Segredo, V., Wang, Z., Lameh, J., Sadée, W., 1995. Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-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. Adenylylcyclase supersensitization in mu-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. J. Biol. Chem. 270, 29732–29738.

Bernstein, M.A., Welch, S.P., 1998. mu-Opioid receptor downregulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. Brain Res. Mol. Brain Res. 55, 237–242.

Bilsky, E.J., Bernstein, R.N., Wang, Z., Sadée, W., Porreca, F., 1996. Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 and the protein kinase inhibitors H7 and H8 on acute morphine

dependence and antinociceptive tolerance in mice. J. Pharmacol. Exp. Ther. 277, 484–490.

Chakrabarti, S., Law, P.Y., Loh, H.H., 1995. Neuroblastoma Neuro2A cells stably expressing a cloned mu-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. Brain Res. Mol. Brain Res. 30, 269–278.

Chakrabarti, S., Law, P.Y., Loh, H.H., 1998. Distinct differences between morphine- and [D-Ala^2, *N*-MePhe^4,Gly-ol^5]-enkephalin-mu-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. J. Neurochem. 71, 231–239.

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

Chen, Y., Yu, L., 1994. Differential regulation by cAMP-dependent protein kinase and protein kinase C of the mu-opioid receptor coupling to a G-protein-activated K^+ channel. J. Biol. Chem. 269, 7839–7842.

De Vries, T.J., Van Vliet, B.J., Hogenboom, F., Wardeh, G., Van der Laan, J.W., Mulder, A.H., Schoffelmeer, A.N., 1991. Effect of chronic prenatal morphine treatment of mu-opioid receptor-regulated adenylate cyclase activity and neurotransmitter release in rat brain slices. Eur. J. Pharmacol. 208, 97–104.

Freedman, N.J., Lefkowitz, R.J., 1996. Desensitization of G-protein-coupled receptors. Recent Prog. Horm. Res. 51, 319–351.

Grumbach, L., Shelofsky, M., Boston, J.E. Jr., 1974. The kinetics of physical dependence to morphine in the rat. Pharmacology 11, 38–51.

Harada, H., Ueda, H., Katada, T., Ui, M., Satoh, M., 1990. Phosphorylated mu-opioid receptor purified from rat brains lacks functional coupling with G_{il}, a GTP-binding protein in reconstituted lipid vesicles. Neurosci. Lett. 113, 47–49.

Harada, H., Ueda, H., Wada, Y., Katada, T., Ui, M., Satoh, M., 1989. Phosphorylation of mu-opioid receptors — a putative mechanism of selective uncoupling of receptor-G_i interaction, measured with low-Km GTPase and nucleotide-sensitive agonist binding. Neurosci. Lett. 100, 221–226.

Itano, Y., Ito, A., Uehara, T., Nomura, Y., 1996. Regulation of Bcl-2 protein expression in human neuroblastoma SH-SY5Y cells: positive and negative effects of protein kinases C and A, respectively. J. Neurochem. 67, 131–137.

Keith, D.E., Anton, B., Murray, S.R., Zaki, P.A., Chu, P.C., Lissin, D.V., Monteillet-Agius, G., Stewart, P.L., Evans, C.J., von Zastrow, M., 1998. mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol. Pharmacol. 53, 377–384.

Koob, G.F., Nestler, E.J., 1997. The neurobiology of drug addiction. J. Neuropsychiatry Clin. Neurosci. 9, 482–497.

Loh, H.H., Tao, P.L., Smith, A.P., 1988. Role of receptor regulation in opioid tolerance mechanisms. Synapse 2, 457–462.

Mestek, A., Hurley, J.H., Bye, L.S., Campbell, A.D., Chen, Y., Tian, M., Liu, J., Schulman, H., Yu, L., 1995. The human mu-opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. J. Neurosci. 15, 2396–2406.

Narita, M., Mizoguchi, H., Tseng, L.F., 1995. Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered mu-opioid receptor agonist in the mouse. Eur. J. Pharmacol. 280, R1–R3.

Parolaro, D., Patrini, G., Giagnoni, G., Massi, P., Groppetti, A., Parenti, M., 1990. Pertussis toxin inhibits morphine analgesia and prevents opiate dependence. Pharmacol. Biochem. Behav. 35, 137–141.

Penn, R.B., Parent, J.L., Pronin, A.N., Panettieri, R.A. Jr., Benovic, J.L., 1999. Pharmacological inhibition of protein kinases in intact cells: antagonism of beta adrenergic receptor ligand binding by H89 reveals limitations of usefulness. J. Pharmacol. Exp. Ther. 288, 428–437.

Sharma, S.K., Klee, W.A., Nirenberg, M., 1975. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc. Natl. Acad. Sci. USA 72, 3092–3096.

Shen, K.F., Crain, S.M., 1994. Antagonists at excitatory opioid receptors on sensory neurons in culture increase potency and specificity of opiate analgesics and attenuate development of tolerance/dependence. Brain Res. 636, 286–297.

Sibley, D.R., Ventura, A.L., Jiang, D., Mak, C., 1998. Regulation of the D_1 dopamine receptor through cAMP-mediated pathways. Adv. Pharmacol. 42, 447–450.

Trujillo, K.A., Akil, H., 1991. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science 251, 85–87.

Van Vliet, B.J., De Vries, T.J., Wardeh, G., Mulder, A.H., Schoffelmeer, A.N., 1991. mu-Opioid receptor-regulated adenylate cyclase activity in primary cultures of rat striatal neurons upon chronic morphine exposure. Eur. J. Pharmacol. 208, 105–111.

Wang, L., Gintzler, A.R., 1994. Bimodal opioid regulation of cyclic AMP formation: implications for positive and negative coupling of opiate receptors to adenylyl cyclase. J. Neurochem. 63, 1726–1730.

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

Wang, Z., Bilsky, E.J., Porreca, F., Sadée, W., 1994. Constitutive mu-opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci. 54, L339–L350.

Wang, Z., Bilsky, E.J., Wang, D., Porreca, F., Sadée, W., 1999. 3-Isobutyl-1-methylxanthine inhibits basal mu-opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice. Eur. J. Pharmacol. 371, 1–9.

Yu, V.C., 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.

Yu, V.C., 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.