Morphine Negatively Regulates Interferon-γ Promoter Activity in Activated Murine T Cells through Two Distinct Cyclic AMP-dependent Pathways

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To explore the mechanism by which morphine promotes the incidence of HIV infection, we evaluated the regulatory role of morphine on the interferon-γ (IFN-γ) promoter in activated T cells from wild type and µ-opioid receptor knockout mice. Our results show that morphine inhibited anti-CD3/CD28-stimulated IFN-γ promoter activity in a dose-dependent manner. Chronic morphine treatment of T cells resulted in increased intracellular cAMP. To evaluate the role of cAMP in morphine’s modulatory function, the effects of dibutyryl cyclic AMP and forskolin were investigated. Both dibutyryl cyclic AMP and forskolin treatment inhibited IFN-γ promoter activity. Treatment with pertussis toxin, but not with a protein kinase A inhibitor, antagonized morphine’s inhibitory effects. Morphine inhibited phosphorylation of ERK1/2 and p38 MAPK; in addition, morphine treatment in the presence of either ERK1/2 or p38 MAPK inhibitor (PD98059 or SB203580) resulted in an additive inhibition of IFN-γ promoter activity. The transcription factor activator protein-1, NF-κB, and nuclear factor of activated T cells (NFAT) were negatively regulated by morphine. Overexpression of NF-κB p65 rescued the inhibitory effect of morphine on IFN-γ promoter activity. However, only when NFATc1 was co-overexpressed with c-fos was the inhibitory effect of morphine on IFN-γ promoter counteracted. The inhibitory effects of morphine were not observed in T cells obtained from µ-opioid receptor knockout mice, suggesting that morphine modulation of IFN-γ promoter activity is mediated through the µ-opioid receptor. In summary, our data indicate that morphine modulation of IFN-γ promoter activity is mediated through two distinct cAMP-dependent pathways, the NF-κB signaling pathway and the ERK1/2, p38 MAPK, AP-1/NFAT pathway.

Chronic opioid abusers have been known to experience higher incidence of infectious diseases. It is now well established that opioid use and abuse promotes human immunodeficiency virus infection and disease progression (1, 2). IFN-γ is a cytokine that modulates all phases of immune processes. It is produced predominantly in activated T cells and large granular lymphocytes as well as natural killer cells (3). IFN-γ plays a central role in host resistance to infection, notably viral infection (4). Previous studies, including our own, have shown that morphine use and abuse results in the inhibition of IFN-γ production and may explain why a higher incidence of human immunodeficiency virus and other viral infections is observed in the drug abuse population (5–8). Although morphine’s inhibitory effects on IFN-γ production are well known, the mechanisms by which morphine causes this suppressive effect have not been fully elucidated.

In neuronal cells, morphine binds to a G protein-coupled µ-opioid receptor to induce analgesia (9, 10). Activation of the µ-opioid receptor leads to regulation of several intracellular effectors, such as modulation of adenylyl cyclase activity (11). Cyclic AMP (cAMP) is a negative regulator of T cell activation. Increased intracellular cAMP concentration is known to inhibit T cell mitogenesis induced by various agonists, including alantogen, interleukin-2, and anti-CD4 antibody (12). Increases in cAMP result in inhibition of T cell receptor/CD3 plus CD28-mediated T cell activation and cytokine production and blockade of cell cycle progression at the G1 phase. Increased cAMP has also been shown to inhibit Ras activation and phosphorylation of the mitogen-activated protein kinase/extracellular signal-regulated kinase downstream target, ERK1/2, and the phosphatidylinositol 3-kinase downstream target, protein kinase B (c-Akt). The signals that regulate cAMP levels after T cell encounter an antigen determine the functional fate toward clonal expansion or repression of primary T cell responses (13). In this study, we investigated whether morphine treatment of murine T cells results in increased adenylyl cyclase activity and a concomitant increase in cAMP levels. In addition, we investigated the role of cAMP and the MAPK pathways in morphine-induced inhibition of IFN-γ promoter activity.

Transcription factors AP-1, NFAT, and NF-κB are thought to play a major role in coordinating transcription of the IFN-γ gene (14–16). DNA footprinting studies show that the AP-1 site at positions −196 to −183 contributes to IFN-γ expression induced by mitogens or treatment with interleukin-18 (17, 18), whereas NFAT and NF-κB proteins bind to positions −278 to −268 (19). In addition, a composite NFAT/AP-1 site at position −160 serves as a CD28 response element for the IFN-γ gene (20).

MORKO, µ-opioid receptor knockout; AP-1, transcription factor activator protein-1; NFAT, nuclear factor of activated T cells; Bl cAMP, dibutyryl-cAMP; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ANOVA, analysis of variance; AC, adenylyl cyclase; PKA, protein kinase A; CREB, cAMP-response element-binding protein.
The focus of this study was to investigate the signal transduction mechanisms involved in morphine-induced inhibition of IFN-γ promoter activity. We first investigated whether morphine-induced inhibition of IFN-γ synthesis is transcriptionally regulated. We next analyzed distinct elements within the IFN-γ promoter that are modulated by morphine treatment and whether overexpression of NF-κB p65, AP-1, NFATc1, and NFATc2 rescued the inhibitory effect on IFN-γ promoter activity. Our results suggest that morphine modulates ERK1/2 and p38 phosphorylation through a cAMP-dependent pathway and regulates the transcription of the IFN-γ gene via the transcription factors NF-κB and AP-1/NFAT.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals—**μ-Opioid receptor knockout mice (Balb/c × C57BL/6 gene background) were produced as previously described by Loh and co-workers (21). Briefly, a Xhol/XbaI fragment, which spans the entire exons 2 and 3, was replaced with a Neo* cassette followed by the ligation of a thymidine kinase expression cassette to the 3′-end of this segment. Wild type mice (C57B6F1/J, Balb/c female × C57BL/6 male) were obtained from Jackson Laboratory. A maximum of four mice were housed per cage. Food and tap water were available *ad libitum.* The animal housing facilities were maintained on a 12h light/dark cycle, with a temperature (72 ± 1°F) and 50% humidity.

**Preparation of Spleen Leukocytes, Enrichment of T Lymphocytes, and Treatment with Various Concentrations of Morphine—**Mice were euthanized by CO₂ asphyxiation. Spleens were removed aseptically, and single-cell suspensions were made by forcing the tissue through a cell strainer with a sterile syringe plunger. Erythrocytes were removed using a red blood cell lysing buffer (Sigma), and the resulting spleen cell strainer with a sterile syringe plunger. Erythrocytes were removed using a red blood cell lysing buffer (Sigma), and the resulting spleen cell preparation was depleted of most B cells and macrophages by a single passage through a sterile nylon wool column (22). Nylon wool-nonadherent spleen cells, T-enriched cells, were used for all experiments described in this study. Spleenic T lymphocytes were treated for 2 h in medium (RPMI 1640, 10% fetal calf serum, glutamine, penicillin/streptomycin) by electroporation (BTX, 300 V, 50 ms, and 1 pulse). In the transfection experiments involving primary lymphoid cells, murine splenic T lymphocytes were harvested and treated with concanavalin A (3 μg/ml) for 18 h prior to transfection to allow uptake of DNA. Spleenic T cells (2 × 10⁶) were transiently transfected with 10 μg of IFN-γ promoter firefly luciferase reporter plasmid (pIFN-γ538; generous gift from Dr. W. M. Weaver, University of Washington, Seattle, WA) according to the manufacturer’s instruction.

**Western Blot Analysis of MAPK—**Transfection and Detection of Luciferase Reporter Activity—In the transfection experiments involving primary lymphoid cells, murine splenic T lymphocytes were harvested and treated with concanavalin A (3 μg/ml) for 18 h prior to transfection to allow uptake of DNA. Spleenic T cells (2 × 10⁶) were transiently transfected with 10 μg of IFN-γ promoter firefly luciferase reporter plasmid (pIFN-γ538; generous gift from Dr. W. M. Weaver, University of Washington, Seattle, WA) according to the manufacturer’s instruction. The microtiter plates were then incubated for 24 h at 37°C in a humidified 5% CO₂ incubator. Culture supernatant was analyzed using BOLG2′-immunoassay—Spleen T cells from each mouse were adjusted to a final concentration of 2 × 10⁶ cells/ml in 24-well plates and activated by stimulation with anti-CD3 and anti CD28 (5 μg/ml). The microtiter plates were then incubated for 24 h at 37°C in a humidified 5% CO₂ incubator. Culture supernatant was analyzed using cytokine-specific enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN) according to the manufacturer’s instruction.

**Statistical Analysis—**Results were expressed as mean ± S.E. They were analyzed statistically by the unpaired *t* test between two groups and analysis of variance (ANOVA) among multiple groups. Statistical significance was accepted at *p* < 0.05.

**RESULTS**

**Direct Modulation of the IFN-γ Promoter Is Involved in the Repressive Effect of Morphine on IFN-γ Gene Expression and Protein Production—**Our previous studies with murine splenic and lymph node lymphocytes have shown that morphine inhibited concanavalin A-induced IFN-γ production in *vivo* and *in vitro* (5, 6). In this study, we show that morphine down-regulates anti-CD3- and anti-CD28-stimulated IFN-γ mRNA and protein expression. Spleenic T cells were treated with morphine (100 nM and 10 μM) and then stimulated with anti-CD3 and anti-CD28 (5 μg/ml). Our results show that morphine treatment resulted in a 35% (100 nM morphine treatment) and 75% (10 μM morphine treatment) reduction in IFN-γ message levels when compared with vehicle control in T cells derived from WT mice. This morphine effect was completely abolished in MORKO mice. These results suggest that morphine modulates the IFN-γ synthesis is transcriptionally regulated and is mediated through the μ-opioid receptor. (Fig. 1A).

Experiments were also carried out to investigate the effect of morphine treatment on IFN-γ protein synthesis. Spleenic T cells were treated with morphine for 2 h and then stimulated with anti-CD3 and anti-CD28 (5 μg/ml) for 24 h. Culture supernatant was assayed for IFN-γ protein levels by enzyme-linked immunosorbent assay. Our results show that morphine inhibited IFN-γ protein production in a dose-dependent manner in splenic T cells derived from WT mice. This effect was absent in T cells harvested from MORKO mice (Fig. 1B).

In order to determine whether one of the mechanisms of morphine’s inhibition is a direct interference with the transcriptional activity of the IFN-γ promoter, we initially treated...
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Fig. 1. Effect of morphine on IFN-γ gene expression and protein synthesis stimulated with anti-CD3 and anti-CD28. Primary T lymphocytes from WT and MORKO mice were treated with low and high dose morphine for 2 h and then stimulated with anti-CD3 and anti-CD28 (5 μg/ml) for 6 or 48 h. After 6 h of stimulation with anti-CD3 and anti-CD28, mRNA expression of IFN-γ was determined by reverse transcription-PCR (A). After 48 h of stimulation with anti-CD3 and anti-CD28, the protein expression of IFN-γ in culture supernatant was measured by enzyme-linked immunosorbent assay (B). Reverse transcription-PCR results were quantified using Image-Pro Plus software and presented as a ratio of IFN-γ mRNA/β-2-microglobulin mRNA. Data shown are the mean ± S.E. of three separate experiments. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. *, significance at level p < 0.05; **, significance at level p < 0.01 compared with the control group.

T cells with concanavalin A (3 μg/ml) for 18 h to facilitate DNA uptake and then transiently cotransfected splenic T cells with both pIFN-538 (full-length pIFN-538 promoter) and pRL-TK vector. After electroporation, the cells were rested for 2 h and then treated with vehicle or various doses of morphine for 2 h. Cells were then stimulated with anti-CD3 and anti-CD28 (5 μg/ml). Lysates were harvested after 6 h, assayed for firefly luciferase activity, and corrected for transfection efficiency by normalization with pRL-TK driving Renilla luciferase. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. **, significance at level p < 0.01 compared with the control group. Similar results were obtained in three independent experiments.

Fig. 2. Effect of morphine on IFN-γ promoter activity stimulated with anti-CD3 and anti-CD28. Primary T lymphocytes from WT and MORKO mice were transfected with 10 μg of the pIFN-538 firefly luciferase reporter plasmid and co-transfected with 1 μg of pRL-TK plasmid driving Renilla luciferase as described under “Experimental Procedures.” After 2 h of electroporation, cells were treated with vehicle or morphine (10 nM to 10 μM) for 2 h prior to stimulation with anti-CD3 and anti-CD28 (5 μg/ml). Lysates were harvested after 6 h and assayed for firefly and Renilla luciferase activity using a dual luciferase reporter kit. Data (mean ± S.E.) are presented as relative light units after correction for transfection efficiency by normalization with pRL-TK driving Renilla luciferase. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. **, significance at level p < 0.01 compared with the control group. Similar results were obtained in three independent experiments.

FIG. 1. Effect of morphine on IFN-γ gene expression and protein synthesis stimulated with anti-CD3 and anti-CD28. Primary T lymphocytes from WT and MORKO mice were treated with low and high dose morphine (1 nM to 10 μM) for 6 or 48 h. After 6 h of stimulation with anti-CD3 and anti-CD28, mRNA expression of IFN-γ was determined by reverse transcription-PCR (A). After 48 h of stimulation with anti-CD3 and anti-CD28, the protein expression of IFN-γ in culture supernatant was measured by enzyme-linked immunosorbent assay (B). Reverse transcription-PCR results were quantified using Image-Pro Plus software and presented as a ratio of IFN-γ mRNA/β-2-microglobulin mRNA. Data shown are the mean ± S.E. of three separate experiments. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. *, significance at level p < 0.05; **, significance at level p < 0.01 compared with the control group.

Phenomen a was not observed in T cells harvested from MORKO mice (Fig. 2).

Cyclic AMP, but Not PKA Is Involved in the Inhibitory Effect of Morphine on IFN-γ Promoter Activity—Adenylyl cyclase (AC) is the typical effector of the μ-opioid receptor. Acute stimulation of opioid receptors leads to inhibition of AC activity. Chronic stimulation of the μ-opioid receptor leads to AC supersensitization, which is completely abolished by pertussis toxin (PTX) pretreatment. Downstream of AC, cAMP also plays an important role in the physiology of opioid use and abuse. Although the role of AC supersensitization in the pathophysiology of drug dependence is well recognized, the intracellular signal transduction pathway responsible for the onset of immunosuppression is still unknown. In the present study, T-cells were treated with low and high doses of morphine for greater than 5 h and then stimulated with anti-CD3 and anti-CD28. Intracellular cAMP was then determined by immunobosassay. Both low and high dose chronic morphine treatment resulted in a significant increase in intracellular cAMP in WT splenic T cells. This effect was abolished by PTX (Fig. 3A).

To determine the potential involvement of an increase in cAMP in regard to the inhibition of IFN-γ, we used forskolin, an activator of adenylate cyclase, to increase intracellular cAMP. As shown in Fig. 3B, forskolin treatment significantly inhibited IFN-γ production in WT splenic T cells, implicating cAMP in these effects. PTX completely abolished the inhibition of IFN-γ production by morphine but not that of forskolin.

To investigate the involvement of the cAMP-PKA pathway in the inhibitory effect of morphine on IFN-γ promoter activity, we tested the effect of dibutyryl-cAMP (Bt2cAMP), a cell-permeable cAMP analogue, on IFN-γ promoter activity and the ability of myristoylated protein kinase inhibitor amide (Biomol, Plymouth Meeting, PA), a selective cell-permeable PKA inhibitor, to overcome the inhibitory effect of morphine. Bt2cAMP (0.1–10 μM) added to the cultures strongly inhibited IFN-γ promoter activity in a dose-dependent manner (Fig. 3C). However, the addition of the PKA inhibitor (1 μg/ml) did not alter morphine-induced inhibition of IFN-γ promoter activity (Fig. 3D). Mentioned previously, cAMP is a negative regulator of T

FIG. 2. Effect of morphine on IFN-γ promoter activity stimulated with anti-CD3 and anti-CD28. Primary T lymphocytes from WT and MORKO mice were transfected with 10 μg of the pIFN-538 firefly luciferase reporter plasmid and co-transfected with 1 μg of pRL-TK plasmid driving Renilla luciferase as described under “Experimental Procedures.” After 2 h of electroporation, cells were treated with vehicle or morphine (10 nM to 10 μM) for 2 h prior to stimulation with anti-CD3 and anti-CD28 (5 μg/ml). Lysates were harvested after 6 h and assayed for firefly and Renilla luciferase activity using a dual luciferase reporter kit. Data (mean ± S.E.) are presented as relative light units after correction for transfection efficiency by normalization with pRL-TK driving Renilla luciferase. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. **, significance at level p < 0.01 compared with the control group. Similar results were obtained in three independent experiments.
FIG. 3. Role of cAMP in the regulation of IFN-γ promoter activity by morphine. A, morphine treatment results in an increase in intracellular cAMP. Splenic T lymphocytes from WT mice were pretreated with PTX (100 ng/ml; hatched bar) or vehicle (open bar) for 18 h and then treated with morphine or vehicle and stimulated with anti-CD3 and anti-CD28 for 24 h. Cell lysate was obtained by adding 0.1 M HCl to the cells. The levels of intracellular cAMP were determined by competitive enzyme immunoassay. B, forskolin increases intracellular cAMP and inhibits IFN-γ synthesis. Primary T lymphocytes from WT mice were pretreated with PTX (100 ng/ml) or vehicle for 18 h and then treated with either forskolin or morphine and stimulated with anti-CD3 and anti-CD28 for 24 h. The data shown are the mean ± S.E. of three experiments. C, Bt,cAMP inhibited IFN-γ promoter activity in a dose-dependent manner. After electroporation, T cells were treated with vehicle or Bt,cAMP (0.1–10 μM) for 2 h and then stimulated with anti-CD3 and anti-CD28 (5 μg/ml). Lysates were harvested after 6 h of stimulation. Luciferase activity was measured with a luminometer using a Dual Luciferase Reporter System. Values are the mean ± S.E. of three experiments. D, PKA inhibitor did not abolish the inhibitory effect of morphine on IFN-γ promoter activity. T cells were cultured in presence of protein kinase A inhibitor (PKAI) (1 μg/ml) or vehicle for 24 h post-transfection and then were treated with morphine (10 nM and 1 μM) or vehicle for 2 h prior to stimulation with anti-CD3 and anti-CD28 (5 μg/ml). Lysates were harvested after 6 h of stimulation. Luciferase activity was measured with a luminometer using a dual luciferase reporter system. Values are the mean ± S.E. of three experiments. Statistical differences were determined by a factorial ANOVA followed by an unpaired t test. *,p < 0.05; **,p < 0.01 compared with the control group.
cell activation, as made evident by previous studies demonstrating that cAMP inhibits T lymphocyte activation, and is associated with down-regulation of two parallel MAPK pathways (12). Our current studies expand these findings by suggesting that morphine inhibits IFN-γ promoter activity through a cAMP-dependent but PKA-independent pathway.

ERK1/2 and p38 MAPK Pathways Are Involved in the Modulation of the IFN-γ Promoter by Morphine—To determine whether ERK1/2 and p38 MAPK pathways are involved in the signal transduction mechanisms by which the μ-opioid receptor modulates the IFN-γ promoter, the effects of ERK1/2 and p38 MAPK inhibitors (PD98059 and SB20358) on IFN-γ promoter activity were investigated. Low dose SB20358 (1 μM) or PD98059 (2 μM) was sufficient to inhibit ~50% of the IFN-γ promoter activity stimulated by anti-CD3 and anti-CD28. The addition of 100 nM morphine to SB20358-treated splenic T cells inhibited the IFN-γ promoter activity by 75%, and PD98059-treated splenic T cells inhibited the IFN-γ promoter activity by 74.6%. Morphine in the presence of either PD98059 or SB20358 resulted in a additive inhibition of IFN-γ promoter activity (Fig. 4A). Likewise, low dose SB20358 (1 μM) or PD98059 (2 μM) treatment also inhibited IFN-γ protein production stimulated by anti-CD3 and anti-CD28. The addition of 100 nM morphine to SB20358- or PD98059-treated T cells resulted in an additive inhibition of IFN-γ protein production (Fig. 4B). These results suggest that p38 and ERK1/2 MAPK may represent potential intracellular pathways by which morphine negatively regulates IFN-γ promoter activity.

To further determine the potential involvement of p38 and ERK1/2 MAPK cascades in the negative regulation of IFN-γ promoter activity by morphine, the effects of morphine on p38 and ERK1/2 activity were analyzed by Western blot analysis. As shown in Fig. 5A, p38 phosphorylation levels were significantly decreased in morphine-treated splenic T cells derived from WT mice. This effect was not observed in T cells derived from MORKO mice. Chronic treatment with morphine also resulted in significant inhibition of ERK1/2 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 phosphorylation in T cells derived from WT mice (Fig. 5B).

Morphine Decreases Nuclear Expression of c-fos and c-jun—The downstream targets of ERK1/2 and p38 MAPK in the nucleus are ELK-1 and ATF, which activate c-fos and c-jun. To address the question of whether c-fos and c-jun act as the nuclear mediators, mediating the inhibition of IFN-γ promoter activity induced by morphine, the relative levels of c-fos and c-jun mRNA expression were measured by reverse transcription-PCR. As shown in Fig. 6, morphine induced a dose-dependent decrease in c-fos and c-jun mRNA. These results suggest that c-fos and c-jun may act as the transcriptional factors that link the intermediary signals from ERK1/2 and p38 MAPK to the IFN-γ promoter.

Morphine Down-regulates AP-1, NF-κB, NFAT, and Overexpression of NF-κB p65, and Overexpression of NF-κB p65, and Co-overexpression of NFATc1 with c-fos Rescues the Inhibitory Effect of Morphine on IFN-γ Promoter Activity—To investigate the effect of morphine on IFN-γ transcription is the result of a decrease in DNA-protein binding to cognate DNA elements, we next evaluated the effect of morphine on nuclear protein binding to several regulatory elements in the IFN-γ promoter, including AP-1, NF-κB, and NFAT. DNA binding and transcriptional activities were determined by an electrophoretic mobility shift assay. The current study showed that the binding activities of AP-1, NF-κB, and NFAT were down-regulated by morphine (Fig. 7A).

To further determine whether these transcriptional factors were directly involved in the inhibitory effect of morphine on the IFN-γ promoter, we co-transfected NFATc1, NFATc2, NF-κB p65, and c-fos expression plasmids with IFN-γ promoter ligated to firefly luciferase reporter plasmid. As shown in Fig. 7B, overexpression of NF-κB p65, NFATc1, NFATc2, or c-fos significantly increased anti-CD3/anti-CD28-induced IFN-γ promoter activity. Overexpression of NF-κB p65 significantly rescued morphine-induced inhibition of IFN-γ promoter activity. Co-overexpression of NFATc1, NFATc2, or c-fos alone did not rescue morphine’s effect. However, it was interesting to observe that co-overexpression of c-fos with NFATc1 significantly reversed the inhibitory effect of morphine on IFN-γ promoter activity (Fig. 7, B and C).

Overexpression of NF-κB p65 Reverses the Inhibitory Effect of Bt2cAMP on IFN-γ Promoter Activity—To address the question
of whether a cAMP-dependent NF-κB pathway was involved in the inhibitory effect of morphine on IFN-γ promoter activity, we investigated the effect of NF-κB overexpression on Bt$_2$cAMP-induced IFN-γ promoter activity. As illustrated in Fig. 8, NF-κB p65 overexpression rescued the inhibition of IFN-γ promoter activity induced by Bt$_2$cAMP.

FIG. 5. Effect of morphine treatment on ERK1/2 or p38 MAPK phosphorylation. Primary mouse T lymphocytes were treated with low and high dose morphine for 2 h and then stimulated with anti-CD3 and anti-CD28 for 20 min. Proteins were extracted, and activation of ERK1/2 and p38 MAPK was examined using Western blot analysis. To verify quality of loading, the blots were reprobed with anti-tubulin antibody.

DISCUSSION

In this study, we report that morphine inhibits the transcriptional activity of the IFN-γ promoter, mapped some of the responsive elements, and examined molecular events and signal transduction pathways involved in the regulation of IFN-γ production by morphine following anti-CD3 and anti-CD28 activation. Our results indicate that morphine can directly modulate the IFN-γ promoter and alter IFN-γ gene expression and protein synthesis.

AC is the typical effector of the μ-opioid receptor. Acute stimulation of opioid receptors leads to inhibition of AC activity. Chronic stimulation of the μ-opioid receptor leads to AC supersensitization, and PTX pretreatment completely abolishes AC supersensitization. Downstream from AC, cAMP has been shown to play an important role in the physiology of opioid use and abuse. Although the role of AC supersensitization in the pathophysiology of drug dependence is well recognized, the intracellular signal transduction pathway responsible for the onset of immunosuppression is still unknown (24). Up-regulation of the cAMP pathway in response to chronic opiate administration was first demonstrated in neuroblastoma × glioma cells in culture (25) and later shown to occur in specific brain regions in vivo (26). In T cells, cAMP elevation has been shown to inhibit T cell receptor/CD3 plus CD28-mediated T cell activation (27, 28), cytokine production, and progression of cell cycle (13). In the present study, we show that treatment with low and high dose chronic morphine results in a significant increase in intracellular cAMP in WT splenic T cells. This effect was abolished by PTX pretreatment. To investigate the role of cAMP in morphine-induced inhibition of IFN-γ promoter activity, the effects of forskolin, a cAMP-elevating agent, and Bt$_2$cAMP, a cell-permeable cAMP analogue, were studied in activated murine T cells. Our results show that treatment with both forskolin and Bt$_2$cAMP resulted in a significant inhibition in IFN-γ production. This suggests that morphine-induced increase in adenylyl cyclase activity with a concomitant increase in intracellular cAMP may be a potential mechanism by which morphine treatment inhibits anti-CD3/CD28-activated IFN-γ promoter activity.

Although the role of cAMP in cell proliferation and cytokine production are well accepted, downstream mediators of cAMP involved in these functions are still not clearly elucidated. Both
PKA-dependent and PKA-independent pathways have been implicated in cAMP-mediated effects (29). Whereas the growth-inhibitory effects are PKA-dependent, the cAMP effects on cell proliferation are PKA-independent. Our study showed that myristoylated protein kinase inhibitor amide, a selective cell-permeable PKA inhibitor, did not antagonize the inhibitory effect of morphine on IFN-$\gamma$ promoter activity. The PKA-dependent mechanism that has been implicated in activation of IFN-$\gamma$ promoter activity acts through phosphorylation of cAMP-response element-binding proteins (CREBs) (30, 31). Overexpression of CREB in Jurkat cells caused marked inhibition of prox. IFN-$\gamma$, dist. IFN-$\gamma$, and IFN-$\gamma$ promoter-directed transcriptional activities. In addition, overexpression of CREB inhibited the production of IFN-$\gamma$ (32). However, our studies show that chronic morphine treatment did not result in CREB phosphorylation or CREB binding to the IFN-$\gamma$ promoter element (data not shown). Based on these observations, we speculate that morphine regulation of IFN-$\gamma$ promoter activity,

**Fig. 7.** Morphine modulates IFN-$\gamma$ promoter activity through NF-$\kappa$B and NFAT/AP-1 sites. A, effect of morphine on AP-1, NF-$\kappa$B, and NFAT binding using an electrophoretic mobility shift assay. Primary T lymphocytes from WT mice were treated with vehicle (lane 1), low dose (lane 2), or high dose (lane 3) morphine for 5 h and then stimulated with anti-CD3 and anti-CD28 for 30 min. Binding activities of three nuclear protein transcriptional factors were analyzed using electrophoretic mobility shift analysis. B, effect of NF-$\kappa$B and NFAT/AP-1 overexpression on the inhibitory effect of morphine on IFN-$\gamma$ promoter activity. Splenic T cells were transiently co-transfected with pIFN-538 firefly luciferase reporter plasmid (10 $\mu$g) and 5 $\mu$g of transcriptional factor plasmids (NF-$\kappa$B p65, NFATc1, NFATc2, and Fos) by electroporation. Morphine treatment and anti-CD3 and CD28 stimulation followed as described under "Experimental Procedures." The data are presented as mean ± S.E. for three independent experiments. C, relative activity of IFN-$\gamma$ promoter. To compare the ability of overexpression of the transcriptional factors to counteract morphine's inhibitory effect, the promoter activity of the treatment groups was calculated as a percentage of vehicle treatment based on the IFN-$\gamma$ promoter relative activity of the vehicle treatment groups being 100%. **, significant at level $p < 0.01$.**
subsequent to cAMP elevation, is independent of the cAMP-PKA-CREB signaling pathway.

Optimal activation of T cells requires effective occupancy of both the antigen-specific T cell receptor and a second co-receptor, such as CD28 (33). In this study, we investigated the effect of morphine on IFN-γ promoter activity using both anti-CD3 and anti-CD28 stimulation in primary murine T cells. Previous studies have shown that morphine had an effect similar to that of "mitogens" and increased the activated state of ERK1 and ERK2 in human lymphocytes (34) and cord blood CD34+CD38+ cells (35). Stimulation of the μ-opioid receptor by the specific agonist, DAMGO (Tyr-γ-Ala-Gly-(me)Phe-Gly-ol) induced the phosphorylation and activation of Akt and p70s6k in HEK 293 stably transfected with murine μ-opioid receptor (36, 37). However, our studies show that in contrast to morphine's effect on resting cells, chronic morphine treatment of T cells activated by CD3/CD28 coligation inhibited phosphorylation and activation of ERK1/2 and p38 MAPK. This suggests that the effect of morphine on ERK1/2 may be tissue-dependent, and the immunomodulatory effects of morphine may be regulated in a different manner in primary activated T cells versus transformed cell lines. Furthermore, our results show that ERK1/2 and p38 MAPK inhibitors (SB203580 and PD98058) significantly inhibited IFN-γ promoter activity, and morphine pretreatment resulted in an additive inhibition. Since cAMP has been implicated in inhibition of anti-CD3/CD28-induced Ras activation and phosphorylation of ERK1/2 (27, 28), we hypothesize that morphine-induced inhibition of ERK1/2 and p38 MAPK phosphorylation is mediated through a cAMP-dependent pathway.

One of the targets for MAPK activation in mammalian cells is the transcription factor AP-1. AP-1 is composed of members of the Fos and Jun family of DNA-binding proteins (38, 39). The MAPK signaling pathways regulate AP-1 activity both by increasing the abundance of the AP-1 components and by directly stimulating their activity (40). ERK family

**Fig. 8.** Effect of NF-κB overexpression on Bt2cAMP-induced inhibition of IFN-γ promoter activity. Cells were transfected with pIFN538 or co-transfected with pIFN538 and NF-κB p65 as described under "Experimental Procedures." T cells were treated with either Bt2cAMP (1 or 10 μM) or vehicle for 2 h. The IFN-γ promoter activity was monitored by a dual luciferase report assay system. Results are an average of three independent experiments. *, significant at level \( p < 0.05; **, \) significant at level \( p < 0.01.\)

**Fig. 9.** Hypothetical model of the mechanisms involved in morphine-mediated inhibition of anti-CD3/CD28-induced IFN-γ promoter activity.
morphine-induced inhibition. These results are in agreement with the binding to the AP-1 site alone was not the mechanism for the inhibitory effect. These results suggest that inhibition of cAMP leads to a decrease in NF-

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