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Morphine Induces CD4⁺ T Cell IL-4 Expression through an Adenylyl Cyclase Mechanism Independent of the Protein Kinase A Pathway

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Impaired host defense mechanisms after major operative procedures and trauma are recognized as important factors in the development of infectious complication. Trauma- and sepsis-associated death in the critically ill strongly correlated with impaired T cell function and a deficiency in cellular immunity (1). After human trauma, data are consistent with a shift in CD4⁺ paired T cell function and a deficiency in cellular immunity (1). Impaired immunologic function has been demonstrated in opioid-addicted populations, as evidenced by an increased prevalence of tuberculosis (3). The classical pathway of IFN-γ-dependent activation of macrophages by Th1-type responses is a well-established feature of cellular immunity in response to intracellular pathogens, such as Mycobacterium tuberculosis. An appropriate Th1 immune response is required for the elimination of M. tuberculosis. Th2 differentiation results in diminished CD4⁺ T cell-related IFN-γ synthesis, whereas increasing IL-4 and IL-13 production (4). In this regard, it has been demonstrated that a large increase in IL-4 and IL-13 synthesis correlates with lung damage. This is consistent with Th2 differentiation underlying the efficacy of immunity and contributing to immunopathology (5). Opioids may promote immunodeficiency virus infection by altering the secretion of α and β chemokines (important inhibitory cytokines for the expression of HIV) and at the same time increasing the expression of chemoreceptors CCR5 and CCR3, coreceptors for the virus (6). Increased in vitro replication of CCR5-restricted HIV1 primary isolates in Th2 lymphocytes may correlate with AIDS progression (7). Of interest is the observation that HIV-1 preferentially infects Th2 clones (8). This observation may explain the persistence of HIV virus in Th1-deficient hosts and may represent the link among opioid-induced Th2 differentiation, the course of HIV infection, and opioid addiction (9). Thus, the demonstration that CD4⁺ T cell Th2 differentiation can be induced by chronic opioid agonist treatment in vivo has significant clinical relevance.

Morphine classically operates through cell membrane µ-opioid receptors in the class of G protein-coupled inhibitory receptors leading to inhibition of adenylyl cyclase (G_i) and a consequent reduction in cellular cAMP levels (10–12). A number of investigators have shown, however, that when certain of these inhibitory receptors (e.g., µ-, κ-, and δ-opioid) are chronically activated, there is an increase in cAMP accumulation (10–13). This phenomenon, referred to as adenylyl cyclase superactivation, is thought to be a possible cellular mechanism for the development of opiate tolerance and dependence (11). It has been suggested that such regulation of adenylyl cyclase could be a general means of cellular adaptation to the chronic activation of inhibitory G_{i/o}-coupled receptors (14).

In this investigation we demonstrate that the in vitro phenomenon of morphine-induced Th2 CD4⁺ T cell differentiation previously described also operates in vivo in the mouse. Furthermore, we demonstrate that morphine’s effect on CD4⁺ T cell differentiation operates through a pertussis toxin (PTX)³-sensitive receptor. We

Abbreviations used in this paper: PTX, pertussis toxin; NCS, normal calf serum; p-, phosphorylated; PKA, protein kinase A; PKI, PKA inhibitor.

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show that this receptor results in adenylyl cyclase superactivation and increased cytoplasmic cAMP. Finally, we investigate the cellular transduction mechanism by which morphine-induced cytoplasmic cAMP modulates IL-4 transcription and protein synthesis.

Materials and Methods

Experimental animals

Splenocytes were harvested from naive C57/S129 mice using protocols approved by the animal subjects investigative review board. Animals were housed at the Minneapolis Veterans Affairs Medical Center under institutional animal care and use committee-approved conditions. A maximum of four mice were housed per cage. Food and tap water were available ad libitum. The animal room was maintained on a 12-h light, 12-h dark cycle with constant temperature (72 ± 1°F) and 50% humidity.

In vivo treatment with morphine

C57/S129 were implanted with 1) a placebo pellet, 2) a morphine (75 mg) pellet, or 3) a morphine plus naltrexone pellet for 72 h. The pellets were obtained from the National Institute on Drug Abuse. At the end of the time period, mice were killed by CO2 asphyxiation, followed by cervical dislocation. Spleens were removed aseptically, and single-cell suspensions were prepared by forcing the tissue through a cell strainer with a sterile syringe plunger. Erythrocytes were removed using a RBC lysing buffer (Sigma-Aldrich). Splenocytes were stimulated with plate-bound anti-CD3 and anti-CD28 Abs (5.0 μg/ml; BD Pharmingen) for varying periods depending on the experiment, and supernatant was harvested. The study was conducted with five animals per experimental group and was repeated once.

In vitro treatment of splenocytes and enrichment of CD4+ T lymphocytes

To simulate the conditions of in vivo morphine treatment in vitro, splenocytes were pretreated for 2 h with medium (RPMI 1640 plus 10% normal calf serum (NCS)) containing either vehicle or morphine (National Institute on Drug Abuse). Cells were then stimulated with plate-bound anti-CD3 and anti-CD28 Abs (5.0 μg/ml; BD Pharmingen) for 72 h. To minimize T cell activation during purification, splenocytes were depleted of most B cells and macrophages by a single passage through a sterile nylon-wool column. Nylon-wool-nonadherent splenic T-enriched cells were then negatively selected using an anti-CD8+ T cell affinity column. This purification protocol achieved 97% purity in CD4+ T cells when analyzed by FACS. Splenic-derived CD4+ enriched T cells were pretreated for 2 h with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (National Institute on Drug Abuse) and then stimulated with plate-bound anti-CD3 and anti-CD28 Abs for varying periods of time depending on the experiment. The morphine concentration was maintained in the treatment arm throughout all manipulations to prevent opioid withdrawal. Experiments were performed in quadruplicate and were repeated twice.

PTX treatment of cells

Splenocytes were pretreated with either vehicle or PTX (100 ng/ml) for 18 h, then treated with either morphine (200 ng/ml) or vehicle for 2 h, and then activated with anti-CD3 and anti-CD28 plate-bound Abs (5 μg/ml) for 72 h to induce differentiation of T cells. CD4+ T cells were isolated as described and restimulated with anti-CD3 and anti-CD28 plate-bound Abs for varying periods of time depending on the experiment. The morphine concentration was maintained in the treatment arm throughout all manipulations to prevent opioid withdrawal. Experiments were performed in quadruplicate and were repeated twice.

Cytokine protein assay

Supernatant IFN-γ and IL-4 immunoreactive protein concentrations were measured using sandwich ELISA kits (R&D Systems). In experiments in which kinase inhibitors were used, splenocytes were pretreated with vehicle, the protein kinase A (PKA) inhibitor H89 (0.5 and 1 μM) and myristoylated PKA inhibitor (PKI) (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or the p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). Cells were activated with anti-CD3 and anti-CD28 plate-bound Abs for 72 h, and CD4+ T cells were prepared. CD4+ T cells were purified and transfected with an IL-4 promoter construct as previously described (1). A control reporter vector pRL-TK containing the Renilla luciferase gene downstream of the thymidine kinase promoter was used as the internal standard along with pLucIL-1; 1 × 105 cells were used, and identical transfection conditions were performed using with DMRIE-C reagent (Invitrogen Life Technologies) as described previously (1). The transfected cells were allowed to recover for 4 h, then cultured with either vehicle or morphine plus H89, PKI (14–22) amide, PD98059, or SB203580 at the same concentration as before and restimulated with anti-CD3 and anti-CD28 for 12 h. Luciferase assays were performed using the dual luciferase reporter assay (Promega). The luciferase activity was normalized by determining the ratio of firefly/Renilla luciferase activity. Experiments were performed in triplicate.

Western immunoblot analysis for activated CREB (p-CREB)

Splenocytes were treated with morphine (100 and 200 ng/ml) or vehicle for 2 h before stimulation with anti-CD3 and anti-CD28 for 72 h. Splenocytes were washed and restimulated with plate-bound anti-CD3 and anti-CD28 Ab in the presence of morphine for 20 min and lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl supplemented with protease inhibitor mixture). After one freeze/thaw cycle, lysates were centrifuged. The protein concentration was determined by Bradford assay (Bio-Rad) using BSA as the standard. Five micrograms of protein sample was subjected to SDS-PAGE. Proteins were then transferred to an ECL nitrocellulose membrane (Amersham Biosciences) by electroblotting. Non-specific binding was reduced by incubating membranes in Superblock (Pierce) for 1 h. Membranes were incubated overnight at 4°C in primary Ab, total or active ERK1/2 (Cell Signaling Technology), and p38 MAPK or p-CREB (BioSource International). The immunoblots were washed and then incubated with HRP-conjugated anti-rabbit secondary Ab and visualized using Hyperfilm ECL (Amersham Biosciences). Western immunoblot analysis was conducted according to standard procedures using Psoralen Chemiluminescence Detection Substrate (Pierce).

RT-PCR analysis of IL-4 and GATA 3 mRNA

CD4+ T cell total RNA was extracted by cell lysis in guanidinium isothiocyanate, followed by phenol acid extraction. RNA (1.0 μg) was reverse transcribed for 90 min at 42°C with 200 U of superscript Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) using random hexamers. Five microliters of cDNA was added to each PCR, and amplification was performed with the oligonucleotide primers specific for mouse IL-4, GATA3, or β2-microglobulin (BD Clontech) as described previously (1).

Adenylyl cyclase activity and cAMP assay

Adenylyl cyclase assay was performed in triplicate as previously described (17). Splenocytes were cultured in 24-well plates (250,000 cells/well) with either morphine or vehicle and were stimulated with anti-CD3 and anti-CD28 plate-bound Ab for 72 h. CD4+ T cells were isolated as described above, treated with vehicle or morphine, and restimulated with anti-CD3 and anti-CD28 plate-bound Ab for 2 h, and the cytoplasmic cAMP concentration was measured as described below. In experiments in which forskolin was included, cAMP was determined with 0.5 mM/well DMEM containing HEPES (pH 7.4), 0.1 mg/ml BSA, 1-methyl-3-isobutylxanthine (100 μM), ZK 62711 (250 μM), and forskolin (1.0 μM) and treated for 10 min at 37°C. To determine cAMP in the cells, medium was removed, and the reaction was terminated with 1 ml of 2.5% perchloric acid. After 15 min with perchloric acid at 4°C, the supernatant was neutralized with 100 μl of a mixture of 3.8 M KOH and 0.16 M K2CO3. Aliquots of the supernatant were assayed for cAMP using a competitive cAMP immunoassay kit (Sigma-Aldrich).

Murine IL-4 promoter-reporter constructs and transfections

Splenocytes were pretreated with vehicle, the PKA inhibitor H89 (0.5 and 1 μM) and myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or the p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). Cells were activated with anti-CD3 and anti-CD28 plate-bound Abs for 72 h, and CD4+ T cells were prepared. CD4+ T cells were purified and transfected with an IL-4 promoter construct as previously described (1). A control reporter vector pRL-TK containing the Renilla luciferase gene downstream of the thymidine kinase promoter was used as the internal standard along with pLucIL-1; 1 × 105 cells were used, and identical transfection conditions were performed using with DMRIE-C reagent (Invitrogen Life Technologies) as described previously (1). The transfected cells were allowed to recover for 4 h, then treated with either vehicle or morphine plus H89, PKI (14–22) amide, PD98059, or SB203580 at the same concentration as before and restimulated with anti-CD3 and anti-CD28 for 12 h. Luciferase assays were performed using the dual luciferase reporter assay (Promega). The luciferase activity was normalized by determining the ratio of firefly/Renilla luciferase activity. Experiments were performed in triplicate.

Western immunoblot analysis for activated MAPK and phosphorlyated CREB (p-CREB)

Splenocytes were treated with morphine (100 and 200 ng/ml) or vehicle for 2 h before stimulation with anti-CD3 and anti-CD28 for 72 h. Splenocytes were washed and restimulated with plate-bound anti-CD3 and anti-CD28 Ab in the presence of morphine for 20 min and lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl supplemented with protease inhibitor mixture). After one freeze/thaw cycle, lysates were centrifuged. The protein concentration was determined by Bradford assay (Bio-Rad) using BSA as the standard. Five micrograms of protein sample was subjected to SDS-PAGE. Proteins were then transferred to an ECL nitrocellulose membrane (Amersham Biosciences) by electroblotting. Non-specific binding was reduced by incubating membranes in Superblock (Pierce) for 1 h. Membranes were incubated overnight at 4°C in primary Ab, total or active ERK1/2 (Cell Signaling Technology), and p38 MAPK or p-CREB (BioSource International). The immunoblots were washed and then incubated with HRP-conjugated anti-rabbit secondary Ab and visualized using Hyperfilm ECL (Amersham Biosciences). Western immunoblot analysis was conducted according to standard procedures using Psoralen Chemiluminescence Detection Substrate (Pierce).
Results

Chronic morphine treatment in vivo induces CD4 T cell Th2 differentiation in mice

We had previously shown that chronic in vitro treatment of human PBMCs and murine splenocytes results in Th2 differentiation (1). To determine the effect of chronic in vivo morphine treatment on Th2 differentiation, mice were implanted with 1) two placebo pellets, 2) a placebo pellet and a morphine pellet, or 3) a morphine pellet and a naltrexone pellet. Seventy-two hours after pellet implantations, splenocytes were harvested, and single-cell suspensions were made and activated with plate-bound anti-CD3 and anti-CD28 Abs for 24 h. Morphine treatment resulted in a significant increase in anti-CD3/anti-CD28 Ab-induced splenocyte IL-4 protein synthesis compared with that in the placebo group (Fig. 1). Naltrexone pellet implantation along with morphine resulted in a significant reduction in the morphine-induced increase in IL-4 protein synthesis (Fig. 1), indicating that the effect of morphine was mediated through an opioid receptor. In contrast, morphine treatment resulted in a significant decrease in anti-CD3/anti-CD28 Ab-induced splenocyte IFN-γ protein synthesis (data not shown). These results are consistent with previous in vitro studies demonstrating that chronic morphine treatment induces CD4+ T cell Th2 differentiation (1).

Chronic morphine treatment in vitro increases CD4+ T cell IL-4 supernatant protein production, IL-4 mRNA expression, and GATA-3 mRNA expression through a PTX-sensitive receptor

To determine whether the effect of chronic morphine treatment was mediated by the Gαi/o subset of G protein-coupled receptors, murine splenocytes were pretreated with PTX or vehicle and then treated with either vehicle (saline) or morphine. Cells were activated for 72 h with plate-bound anti-CD3/CD28 Ab to induce Th2 differentiation. CD4+ T cells were harvested and restimulated in the presence of the various treatments, culture supernatant was assayed for IL-4 protein synthesis, and cells were harvested for total RNA extraction. Results show that morphine treatment significantly increased anti-CD3/anti-CD28 Ab-induced CD4+ T cell IL-4 protein synthesis, CD4+ T cell IL-4 mRNA expression, and GATA3 mRNA expression compared with all other experimental groups (Figs. 2 and 3). PTX pretreatment significantly inhibited morphine-induced CD4+ T cell supernatant IL-4 protein synthesis, CD4+ T cell IL-4 mRNA expression, and GATA3 mRNA expression (Figs. 2 and 3).

Chronic morphine treatment in vitro increases CD4+ T cell anti-CD3/anti-CD28 Ab-induced cytoplasmic cAMP level

To determine whether chronic morphine treatment increases cytosolic cAMP levels, murine splenocytes were treated in vitro with either morphine or vehicle and activated for 72 h to induce Th2 differentiation. CD4+ T cells were isolated and restimulated with plate-bound anti-CD3/anti-CD28 Ab for the determination of cytoplasmic cAMP. As shown in Fig. 4, chronic morphine treatment increased the anti-CD3/anti-CD28 Ab-induced cytoplasmic cAMP level in a concentration-dependent manner (Fig. 4).

Chronic morphine treatment in vitro increases CD4+ T cell forskolin-stimulated cytoplasmic cAMP level through a PTX-sensitive receptor

To determine whether chronic morphine treatment results in super-activation of adenyl cyclase activity, murine splenocytes were pretreated in vitro with either vehicle or PTX for 18 h and then treated with either vehicle or morphine for 2 h before activation with plate-bound anti-CD3/CD28 Ab for 72 h to induce Th2 differentiation. CD4+ T cells were isolated and stimulated with forskolin in the presence of either vehicle or morphine. Chronic morphine treatment increased the forskolin-induced cytoplasmic cAMP concentration, suggesting that it involved activation of adenyl cyclase activity. Pretreatment with PTX resulted in a significant decrease in the morphine-induced, forskolin-stimulated cytoplasmic cAMP concentration. These results suggest that morphine treatment results in increased cytosolic cAMP accumulation through activation of the adenyl cyclase system, and these effects are mediated through a Gαi/o-coupled receptor mechanism (Fig. 5).
Chronic morphine treatment in vitro induces anti-CD3/anti-CD28 Ab activation of p38 MAPK, ERK1/2 MAPK, and PKA activity

To determine whether p38 and ERK1/2 MAPK or the PKA signaling cascade are involved in the induction of IL-4 by morphine, the effects of chronic morphine treatment on PKA, p38, and ERK1/2 activity were analyzed using Western immunoblot analysis. Splenocytes were pretreated with either morphine or vehicle and activated for 72 h to induce Th2 differentiation. CD4+ T cells were harvested and restimulated for 6 h in the presence of vehicle or morphine, and cell lysates were subjected to Western immunoblot analysis. As shown in Fig. 6, morphine treatment resulted in an increase in both p-p38 and p-ERK1/2 MAPK. To determine whether PKA was similarly activated by morphine, phosphorylation of CREB was used as a marker. Chronic morphine treatment resulted in an increase in p-CREB (Fig. 6).

Chronic morphine treatment in vitro induces anti-CD3/anti-CD28 Ab-stimulated IL-4 promoter activity through a p38 MAPK-dependent, but PKA- and ERK1/2-independent, mechanism

To determine whether all three intracellular signal transduction mechanisms modulate chronic morphine-induced IL-4 transcription, splenocytes were pretreated with vehicle, the PKA inhibitor H89 (0.5 and 1 μM) or myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 nM), or p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h. Forskolin-induced cAMP was determined by replacing the medium with DMEM containing HEPES (pH 7.4), 0.1 mg/ml BSA, 1-methyl-3-isobutylxanthine (100 μM), ZK 62711 (250 μM), and forskolin (1.0 μM) for 10 min at 37°C. cAMP in the cells was determined as described. Each group was run in quadruplicate, and the experiments were repeated twice. Each bar represents the mean of 12 determinations ± SE. *, Significance (p < 0.004) compared with vehicle treatment groups.

FIGURE 3. The effect of chronic morphine treatment on anti-CD3/anti-CD28 Ab-stimulated IL-4 and GATA-3 mRNA accumulation. Naive splenocytes were treated as described in Fig. 3. IL-4 mRNA or GATA-3 transcript levels were determined using RT-PCR. β2-Microglobulin was used as an unregulated control to correct for equality of loading. Splenocytes were treated with 200 ng/ml morphine (MS). Veh, vehicle.

FIGURE 4. The effect of chronic morphine treatment on anti-CD3/anti-CD28 Ab-stimulated IL-4 promoter activity through a p38 MAPK-dependent, but PKA- and ERK1/2-independent, mechanism

To determine whether all three intracellular signal transduction mechanisms modulate chronic morphine-induced IL-4 transcription, splenocytes were pretreated with vehicle, the PKA inhibitor H89 (0.5 and 1 μM) or myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). Cells were activated to induce Th2 differentiation. CD4+ T lymphocytes were harvested and transfected with pIL-4-firefly luciferase reporter plasmid and

FIGURE 5. The effect of chronic morphine (MS) treatment in vitro on forskolin-induced cytoplasmic cAMP levels in anti-CD3/anti-CD28 Ab-stimulated CD4+ T cells. Splenocytes were treated as described above. CD4+ T cells were isolated as described in Materials and Methods, were treated with vehicle (Veh) or morphine (100 and 200 ng/ml), and restimulated with anti-CD3 and anti-CD28 plate-bound Ab for 2 h. Forskolin-induced cAMP was determined by replacing the medium with DMEM containing HEPES (pH 7.4), 0.1 mg/ml BSA, 1-methyl-3-isobutylxanthine (100 μM), ZK 62711 (250 μM), and forskolin (1.0 μM) for 10 min at 37°C. cAMP in the cells was determined as described. Each group was run in quadruplicate, and the experiments were repeated twice. Each bar represents the mean of 12 determinations ± SE. *, Significance (p < 0.004) compared with vehicle treatment groups.

FIGURE 6. Effect of morphine treatment on ERK1/2 or p38 MAPK and CREB phosphorylation. Splenocytes were treated as described above. CD4+ T cells were isolated as described in Materials and Methods, treated with vehicle or morphine (100 and 200 ng/ml), and restimulated with anti-CD3 and anti-CD28 plate-bound Ab for 20 min. Proteins were extracted, and activation of CREB, ERK1/2, and p38 MAPK was examined using Western immunoblot analysis. To verify equality of loading, the immunoblots were reprobed with anti-tubulin Ab.
pRL-TK plasmid driving Renilla luciferase as described in Materials and Methods. Results show that treatment with the PKA inhibitors H89 and PKI (14–22) had no effect on chronic morphine-induced CD4+ T cell IL-4 promoter activity at any of the concentrations tested (Fig. 7A). Similarly, pretreatment with two different concentrations of the ERK1/ERK2 inhibitor PD98059 had no effect on chronic morphine-induced CD4+ T cell IL-4 promoter activity (Fig. 7B). However, pretreatment with the p38 MAPK inhibitor SB203580 significantly inhibited chronic morphine-induced CD4+ T cell IL-4 promoter activity in a concentration-dependent manner (Fig. 7B). These results suggest that morphine-induced activation of the IL-4 promoter is mediated through a p38 MAPK signaling pathway, but is independent of the PKA or ERK1/ERK2 signaling mechanism.

**Chronic morphine treatment in vitro induces anti-CD3/anti-CD28 Ab-stimulated IL-4 protein production through a p38 MAPK-dependent mechanism**

To determine the roles of these three intracellular signal transduction mechanisms in chronic morphine-induced IL-4 protein synthesis, splenocytes were pretreated with vehicle, the PKA inhibitors H89 (0.5 and 1 μM) and myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or the p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). Purified T lymphocytes were transfected with pIL-4 firefly luciferase reporter plasmid and cotransfected with pRL-TK plasmid driving Renilla luciferase as described in Materials and Methods. Lysates were harvested after 6 h and assayed for firefly and Renilla luciferase activities. Data are presented as relative light units after correction for transfection efficiency by normalization with pRL-TK driving Renilla luciferase. Each group was run in quadruplicate, and the experiments were repeated twice. Each bar represents the mean of eight determinations ± SE. * in B, Pretreatment with SB203580 significantly inhibits IL-4 promoter activity compared with either vehicle or PD98059 pretreatment (p < 0.001).

**FIGURE 7.** The effects of PKA and MAPK inhibitors in vitro on chronic morphine treatment-induced IL-4 promoter activity. Splenocytes were pretreated with vehicle, the PKA inhibitor H89 (0.5 and 1 μM) and myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or the p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). Purified T lymphocytes were transfected with pIL-4 firefly luciferase reporter plasmid and cotransfected with pRL-TK plasmid driving Renilla luciferase as described in Materials and Methods. Lysates were harvested after 6 h and assayed for firefly and Renilla luciferase activities. Data are presented as relative light units after correction for transfection efficiency by normalization with pRL-TK driving Renilla luciferase. Each group was run in quadruplicate, and the experiments were repeated twice. Each bar represents the mean of eight determinations ± SE. * in B, Pretreatment with SB203580 significantly inhibits IL-4 promoter activity compared with either vehicle or PD98059 pretreatment (p < 0.001).

**FIGURE 8.** A and B, The effects of PKA and MAPK inhibitors in vitro on chronic morphine treatment-induced IL-4 protein synthesis. C, Positive control showing the efficacy of doses of signaling inhibitors on anti-CD3/CD28 Ab-induced IFN-γ synthesis. Splenocytes were pretreated with vehicle, the PKA inhibitor H89 (0.5 and 1 μM) and myristoylated PKI (14–22) amide (0.5 and 1 μM), the ERK1/ERK2 inhibitor PD98059 (10 and 50 μM), or the p38 MAPK inhibitor SB203580 (0.1 and 1 μM) for 2 h, then treated with medium (RPMI 1640 and 10% NCS) containing either vehicle or morphine (100 ng/ml). CD4+ T lymphocytes were harvested and treated as described in Materials and Methods, then assayed for IL-4 and IFN-γ immunoprotein. Each group was run in quadruplicate, and the experiments were repeated twice. Each bar represents the mean of eight determinations ± SE. * in C, Significant difference of all treatment groups compared with the activated/vehicle group (p < 0.001).
pretreatment with the p38 MAPK inhibitor SB203580 resulted in a significant and dose-dependent inhibition of chronic morphine-induced CD4+ T cell IL-4 protein synthesis (Fig. 8B). To verify that the concentrations of PKA and MAPK inhibitors used were capable of inhibiting the respective signaling pathway, the effects of these inhibitors on IFN-γ synthesis were determined. Although the PKA inhibitor H89 and the ERK1/2 inhibitor PD98059 had no effect on the morphine-induced increase in IL-4 protein synthesis, they significantly inhibited IFN-γ protein synthesis.

Discussion

Three major opioid receptors types have been identified by molecular cloning techniques (μ-, κ-, and δ-opioid receptors) that differ in their affinity for various opioid ligands. These opioid receptors are members of the G (inhibitory) protein-coupled seven-transmembrane receptor superfamily (18). In this investigation we studied morphine as an opioid agonist and demonstrated that morphine treatment in vivo induces a cytokine profile consistent with transmembrane receptor superfamily (18). In this investigation we inhibited IL-2 and IFN-γ synthesis. Although increased cytoplasmic cAMP levels in Th1 and Th2 cell functions are reflected in the induction of cytokine-cAMP cascade exerts an effect on T lymphocytes as a functional mechanism.

Previous investigators have constructed models in various cell lines to investigate the nature of μ-opioid receptor signal transduction mechanisms. Avidor-Reiss et al. (10) investigated the role of adenylyl cyclase superactivation in μ-opioid receptor-transformed Chinese hamster ovary cells after chronic opioid treatment. These investigators demonstrated that although acute morphine treatment (10 min) led to inhibition of forskolin-stimulated cytoplasmic cAMP accumulation, chronic morphine treatment (>4 h) resulted in adenylyl cyclase superactivation and increased forskolin-stimulated cAMP levels. It is unclear in these investigations whether μ-opioid receptor-transfected Chinese hamster ovary cells have general applicability to signal transduction in other cell systems, specifically CD4+ T cells. We demonstrate that chronic morphine treatment 1) increases CD4+ T cell cytoplasmic cAMP level in response to anti-CD3/anti-CD28 activation in a concentration-dependent manner, and 2) increases forskolin-induced cytoplasmic cAMP through a PTX-sensitive receptor. Study of forskolin-stimulated cAMP generation in intact cells measures the activity of the G protein-coupled receptor adenylyl cyclase axis (19). These data are consistent with the concept that chronic morphine treatment of CD4+ T cells evokes adenylyl cyclase supersensitization in a concentration-dependent manner. Of interest is the observation that PTX pretreatment in vitro almost completely inhibits opioid-mediated IL-4 protein synthesis, consistent with CD4+ T cell signal transduction occurring through a classical G protein-coupled mechanism.

Chronic morphine treatment-induced cytoplasmic cAMP has potent immunomodulatory properties. Studies in T cells have led to the concept that signal transduction through the adenylyl cyclase-cAMP cascade exerts an effect on T lymphocytes as a function of differentiation state. The contrasting effects of cAMP on Th1 and Th2 cell functions are reflected in the induction of cytokine synthesis. Although increased cytoplasmic cAMP levels inhibit IL-2 and IFN-γ production, they induce the synthesis of IL-4, IL-5, and other Th2-type cytokines (20–23).

cAMP-mediated signal transduction in CD4+ T cells may proceed through a number of possible pathways, including the classical PKA-mediated pathway and MAK-mediated routes. PKA plays a significant role in cAMP-mediated signal transduction in many cells, with PKA type 1 being the major isozyme in T cells (24). PKA is composed of catalytic and regulatory subunits. cAMP activates PKA by binding to the regulatory subunit, leading to dissociation of the complex. Teschendorf et al. (25) investigated the effect of PKA activation on protein synthesis and secretion in the Th2 cell line D10.G4.1 and demonstrated that TCR-induced production of IL-4 was enhanced by low concentrations of PKA activators. Using the Th2 clone D10.G4.1, Ariai et al. (26) reported that overexpression of the constitutively active form of PKA (SRRPKA, catalytic subunit) significantly increased the expression of IL-4. In Th1 cells, which express only minor amounts of GATA-3, transfection of Th1 cells with retroviruses expressing the catalytic subunits of PKA or GATA-3 demonstrate that 1) dibutyryl-cAMP further increased the effect of ectopic overexpression of GATA-3, leading to an increase in IL-4 protein synthesis; and 2) overexpression of catalytic subunits of PKA was able to replace the effect of dibutyryl-cAMP in the induction of IL-4 protein synthesis (27). In contrast to these data, our study shows that although chronic morphine treatment increases p-CREB, a downstream substrate of PKA, treatment of splenocytes with a PKA inhibitor, H89 or PK1 (14–22) amide, did not antagonize the morphine-induced increase in anti-CD3/anti-CD4 Ab-stimulated IL-4 promoter activity and IL-4 protein synthesis. These results suggest that the morphine-induced increases in IL-4 promoter activity and IL-4 protein levels are mediated through a PKA-independent pathway.

Opioid signal transduction has also been demonstrated to operate through MAPK-mediated pathways. There are a number of MAPK systems, including ERKs and the p38 MAPKs. We and other investigators have shown that μ-opioid G protein-coupled opioid receptors have been demonstrated to stimulate ERK1 and ERK2, and it is assumed that this mechanism is operable, in part, in CD4+ T cells (28, 29). Investigations into the role of p38 MAPK in opioid receptor signaling are few. Chen et al. (15) has investigated the role of the p38 MAPK pathway in CD4+ T cell differentiation. Using the D10 cell line, these investigators report that cAMP-induced effects in Th2 cells occur independently of the PKA pathway. Instead, they demonstrate that cAMP stimulates activation of p38 MAPK in Th2 cells. Consistent with these observations, we demonstrate that chronic morphine treatment resulted in an increase in the anti-CD3/CD28 Ab-induced increase in both ERK1/2 and P38 MAPK activities. However, pretreatment with only the p38 MAPK inhibitor significantly antagonized morphine-induced IL-4 promoter activity and IL-4 protein levels.

In summary, our data demonstrate that chronic morphine treatment 1) increases CD4+ T cell IL-4 and GATA-3 mRNA accumulation and IL-4 protein synthesis through a PTX-sensitive receptor, 2) results in a dose-dependent increase in CD4+ T cell cytoplasmic cAMP concentration, and 3) increases the forskolin-stimulated cytoplasmic cAMP level through a PTX-sensitive receptor. We also demonstrate that the morphine-induced increase in IL-4 promoter activity or IL-4 immunoprotein expression is independent of the PKA or ERK1/ERK2 pathway; rather, it is mediated through a p38 MAPK-dependent pathway.

Disclosures

The authors have no financial conflict of interest.

References


