Mechanisms of Opioid-Mediated Inhibition of Human T Cell Receptor Signaling

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Opioids are widely used for the treatment of severe pain. However, it is also known that opioids, in particular morphine, cause immunosuppression. Therefore, their use may complicate treatment of persons with an already impaired immune system, e.g., patients suffering from cancer or AIDS. We investigated the mechanisms of opioid-induced immunosuppression in primary human T lymphocytes and the human T cell line Jurkat. We demonstrated that morphine and the endogenous opioid β-endorphin inhibited the transcription of IL-2 in activated human T lymphocytes as well as the activation of the transcription factors AP-1, NF-kB, and NF-αB, which transactivate IL-2. In addition, the TCR-induced calcium flux and MAPK activation were inhibited by the opioids, as well as proximal signaling events, such as the phosphorylation of the linker for activation of T cells and Zap70. A more detailed characterization of the mechanism revealed that incubation of T cells with the opioids caused a marked increase in cAMP. This in turn activated protein kinase A, which augmented the kinase activity of C-terminal Src kinase bound to phosphor-tyrosine. This inhibited the transcription of IL-2 in activated T cells, as well as the activation of the MAPK cascades. In this way, the signal is transduced and finally results in the activation of the transcription factors AP-1, NF-kB, and NFAT. Together, they transactivate the IL-2 transcription.

Opioids are the most potent analgesics and widely used for the treatment of severe pain such as cancer pain. However, a great number of studies have convincingly demonstrated that opioids, in particular morphine and derivatives, are immunosuppressive. This may cause unwanted side effects during pain treatment, especially in persons with an impaired immune system, e.g., patients suffering from cancer or AIDS. It is also reasonable to propose that the opioid-induced immunosuppression contributes to an increased prevalence of infections like pneumonia and HIV among opioid users and addicts (for reviews, see Refs. 1 and 2). Studies in mouse models have suggested that one of the mechanisms by which opioids cause immunosuppression is the inhibition of IL-2 transcription in activated T lymphocytes (3–6). Thus, it was demonstrated that heroin and methadone inhibit the IL-2 production of activated murine splenocytes (3). Furthermore, it was shown that opioid-mediated inhibition of c-fos mRNA (4) and opioid-induced modulation of the cAMP response element-binding transcription factors CREB, CREM, ICER (5, 6) are involved in the inhibition of IL-2. However, detailed molecular mechanisms, which link opioids to TCR signaling and lead to its inhibition, have not been clearly defined. Furthermore, little is known about opioid-induced immunosuppression in human T cells.

It is known that the TCR-mediated activation of T cells induces a defined series of signaling events which culminate in the transcription of the gene for the T cell growth factor IL-2 (7–10). In the nonactivated state, i.e., in resting T cells, the cascade is tonically repressed by constitutive phosphorylation of the negative regulatory site of Lck at Tyr505. This phosphorylation is mediated by the kinase C-terminal Src kinase (Csk), which is complexed in resting T cells with the transmembrane adapter protein Cbp/PAG (phosphoprotein associated with glycosphingolipid-enriched microdomain/Csk-binding protein). Upon engagement of the TCR, Cbp/PAG is dephosphorylated. This leads to a release of Csk from Cbp/PAG, resulting in a loss of the inhibitory effect of Csk on Lck. These events lead to activation of Lck and initiation of the TCR-induced signaling cascade (11–13). One of the earliest events in this cascade is the Lck-mediated phosphorylation and thereby activation of the protein tyrosine kinase Zap70. Activated Zap70 in turn phosphorylates the transmembrane adapter protein linker for activation of T cells (LAT) on at least four tyrosine residues, leading to the assembly of the calcium-initiation complex (14). These events lead to the induction of calcium flux and also to the activation of the MAPK cascades. In this way, the signal is transduced and finally results in the activation of the transcription factors AP-1, NF-kB, and NFAT. Together, they transactivate the IL-2 transcription.
gene, which is almost entirely regulated at the level of transcription. After being released, IL-2 induces a variety of well-defined subsequent immune responses.

The modulation of the IL-2 production of activated T cells by opioids is pharmacologically of great importance, since it contributes to the immunosuppressive side effects of drugs like morphine. In addition, there may be a physiological regulation of activated T cells by endogenous opioid peptides like β-endorphin. However, the question of if and how β-endorphin regulates the TCR-mediated signaling cascade and whether this is involved in the regulation of physiological T cell responses is less well documented. Earlier studies using the opioid receptor antagonist naloxone have suggested that β-endorphin exerts an inhibitory effect on T cell functions like IL-2 production (15) and mitogen-induced splenocytes proliferation (16). In line with this observation, it was demonstrated more recently that mice lacking β-endorphin showed enhanced splenocyte proliferation and enhanced levels of IL-2 mRNA compared with wild-type mice (17).

Effects of opioids on T lymphocytes require the expression of specific opioid receptors (for reviews, see Refs. 18 and 19). Drugs of the morphine type, and thus most of the clinically used opioids, bind preferentially to μ opioid receptors. The expression of functional μ opioid receptors on human T lymphocytes is induced by TNF and IL-4 (20–22). In addition, expression of μ opioid receptors is also induced indirectly by drugs and stimuli, which increase the expression of TNF and IL-4, like cannabinoids (23). As demonstrated recently by our group, also anti-CD3/anti-CD28-mediated activation of human T cells induces the expression of functional μ opioid receptors (24). Studies in mice have shown that morphine is only effective on mitogen-activated T cells, which may be explained by an induction of μ opioid receptors after T cell activation (4, 5, 25). Thus, there is reason to believe that the expression of μ opioid receptors on human T lymphocytes allows the modulation of T cell responses by ligands for μ opioid receptors, such as morphine. The endogenous opioid β-endorphin activates both μ and δ opioid receptors. It was reported that low levels of δ opioid receptors are expressed in Jurkat T lymphocytes and that their expression is induced in response to IL-4 (26), and anti-CD3/anti-CD28 (our unpublished observations). In murine T cells, it was shown that anti-CD3 increases the expression of δ opioid receptors (27). These observations suggest that also δ opioid receptor agonists can modulate T cell functions. In this report, we identified mechanisms by which opioids suppress the TCR-triggered signaling cascade in activated human T lymphocytes.

Materials and Methods

T cell culture, induction of opioid receptors, T cell activation, and reagents

PBMC were isolated from heparinized blood collected from healthy volunteers as previously described (28). Primary human T cells and the human T cell line Jurkat were cultivated in RPMI (1640 medium (Lonza Verviers) supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Lonza Verviers). For our experiments, no opioid receptors were added to the culture media. The medium was replaced by fresh medium without IL-4 and the cells were cultured for an additional 24 h. An aliquot of the cells was tested to assure the induction of μ and δ opioid receptor transcripts. For these cells, the term “opioid receptor-expressing” cells is used throughout this publication. For activation of T cells, 100 μl each of anti-CD3 and anti-CD28 was used per 10^6 cells. The sources for anti-CD3 (OKT3) and anti-CD28 (14C5.2) were the hybridoma supernatants produced in our laboratories (28). As opioids, morphine (Synophrm), β-endorphin (Sigma-Aldrich), and D-penicillamine2-D-penicillamine5-enkephalin (Sigma-Aldrich) were used at 1 μM. Antagonists were CTAP (μ opioid receptor specific) and naltrindole (δ opioid receptor specific), both used at 250 nM and purchased from Tocris. The protein kinase A (PKA) inhibitor (R)-adenosine cyclic 3’,5’-hydrogenphosphorothioate (cAMPS-Rp; Tocris) was used at 100 μM. The PKA activator 8-Br-cAMP (Tocris) was used at 5 μM. As phosphodiesterase inhibitors, IBMX (500 nM; Sigma-Aldrich), rolipram (10 μM; Tocris), BRL50481 (10 μM; Tocris), and dipyridamole (100 μM; Tocris) were used. FMA (Sigma-Aldrich) was used at 100 nM.

Chronic opioid treatment and IL-2 transcription

Experiments were performed similarly to a described protocol (29). Briefly, opioid receptor-expressing Jurkat cells (10^6 per sample) were incubated for 4 days on anti-CD3/anti-CD28-coated dishes with opioids and opioid antagonists. Then cells were restimulated with anti-CD3/anti-CD28 for 15 min to induce IL-2 mRNA and lysed after 4 h for quantitative IL-2 RT-PCR. The restimulation and final 4-h incubation of the cells was performed in the presence of opioids to exclude “withdrawal”-like effects.

Analysis of transcription factor activity

Oligonucleotides containing binding sequences (core-binding sequences are underlined) for the transcription factors AP-1 (5’-AACATATGATTCACCAGGCCA-3’), NF-κB (5’-AAAGTTGGAGGACCTCCAGGCCT-3’), and NFAT (5’-GCCCAAAGAGGAATAATGTTCATA-3’) were cloned 5’ to the herpes simplex thymidine kinase promoter of the reporter gene plasmid pBLCAT2 (30). These constructs were transfected into opioid receptor-expressing Jurkat cells by electroporation (210 V, 950 μF) using a Gene Pulser II (Bio-Rad). The day after transfection, cells were transferred to anti-CD3/anti-CD28- or anti-mouse Ig-coated dishes, with or without morphine. After 72 h of transient expression, the reporter gene was assayed via a chloroamphenol acetyl transferase-ELISA purchased from Roche.

Quantitative real-time RT-PCR

Isolation of RNA and quantitative real-time RT-PCR for β-actin, μ, and δ opioid receptor transcripts have been described earlier (20, 22, 26). IL-2-specific RT-PCR was performed with 5’-GAAGGCCACAGAAGCTAAACATCT-3’ and 5’-CTGTTCAGAAATTCACACTGAAATG-3’ primers, with a preincubation for 8 min at 95°C and 50 cycles with 5 s at 95°C, 5 s at 65°C, and 10 s at 72°C (specific Tm = 81, 75°C). Quantitative real-time RT-PCR was done in a total volume of 20 μl on a LightCycler instrument using a LightCycler Fast Start DNA Master SYBR Green I kit (both from Roche) according to the manufacturer’s suggestions.

Surface expression of TCR

After washing in PBS, cells were incubated with murine anti-CD3 for 30 min at 4°C. Secondary goat anti-mouse-FIT! Abs (Dianova) were incubated for 30 min at 4°C and the surface expression of the TCR were measured using a FACScalibur flow cytometer (BD Biosciences).

Western blots

Western blots were performed as previously described (22, 23, 31). For activation, 2.5 × 10^6 opioid receptor-expressing cells per sample were pelleted, resuspended in anti-CD3/anti-CD28, and incubated at 37°C for 2 min. The activation was stopped with 1 ml of ice-cold PBS, cells were pelleted again, and lysed. For protein detection, the following Abs were used: primary Abs were actin C-11 (Santa Cruz Biotechnology); phospho-p44/42 MAPK Tyr202/Tyr204 (Cell Signaling Technology/New England Biolabs). All Abs were from rabbits; the secondary Ab was anti-rabbit IgG (GE Healthcare). Anti-PAG (MEM255) hybridoma was made in the laboratory of Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic) and grown and supernatant was collected in our institute. The secondary Ab anti-mouse-HRP was obtained from Dianova.

cAMP measurement

Opioid receptor-expressing cells were incubated with morphine and β-endorphin for 0 h (controls) up to 24 h. Then cells were lysed with 50 mM HCl for 30 min on ice. The competitive cAMP-ELISA was performed according to a described procedure (32).

Down-regulation of Cbp/PAG with small interfering RNA (siRNA)

For the construction of silencing plasmids, the sequences targeting human Cbp/PAG (5’-AAGCGTACAGACTCTCCAACA-3’), mouse Cbp/PAG

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Opioids inhibit TCR-induced IL-2 mRNA formation and transcription factor activities. A, Effects on IL-2 mRNA. Opioid receptor-expressing Jurkat cells were simultaneously incubated for 4 days on anti-CD3/anti-CD28-coated culture dishes along with opioid receptor agonists (morphine: 1 μM, μ opioid receptor specific; β-endorphin (β-End): 1 μM, μ and δ specific; DPDPE: 1 μM, δ specific) and antagonists (CTAP: 250 nM, μ specific; naltrindole (Naltrin): 250 nM, δ specific), as indicated below. Then cells were restimulated in the presence of opioids with anti-CD3/anti-CD28 for 15 min to induce IL-2 mRNA. Before harvesting, cells were incubated for another 4 h in the presence of opioids. Quantitative IL-2 RT-PCR is shown relative to that of β-actin (β-act). The anti-CD3/anti-CD28-induced IL-2 mRNA amount of samples that contained no opioids (controls) was set to 100%. The means of at least three independent experiments performed in triplicate ± SEM are shown. Secondary comparisons are indicated by brackets (p < 0.05; **, p < 0.01; and ***, p < 0.001). B, Effects on transcription factor activities. Opioid receptor-expressing Jurkat cells were transfected with thymidine kinase (tk)-chloramphenicol acetyl transferase (CAT) reporter genes with binding sites for the transcription factors AP-1, NF-κB, and NFAT. Cells were incubated either on anti-CD3/anti-CD28- or Ig-coated dishes in the presence and absence of morphine (Mo; 1 μM) for 72 h as indicated. The means of at least three independent experiments performed in triplicate ± SEM are displayed. Secondary comparisons are indicated by brackets (p < 0.05; **, p < 0.01; and ***, p < 0.001).

Calcium measurement

Cells (three times 10^5 cells/ml) in RPMI 1640 medium (phenol red free; Invitrogen) containing 10% FCS were loaded with 5 μg/ml Indo-1-AM (MobiTec) at 37°C for 45 min. After washing, cells were incubated in RPMI 1640 medium (phenol red free) supplemented with 10% FCS at 37°C for an additional 45 min. Cells were measured using an LSR I flow cytometer (BD Biosciences) equipped with a 325-nm laser. Cells were stimulated with anti-CD3 and the ratio of the two emission wavelengths (512/20 nm and 400/40 nm) was recorded over time. To confirm proper Indo-1 loading and the viability of cells, ionomycin (10 μg/ml; Sigma-Aldrich) was used. The kinetics of the data were analyzed using FlowJo software (Tree Star).

Statistical analysis

For statistical evaluations between two groups of samples, Student’s t tests were performed. Multiple comparisons were performed with ANOVA, followed by Tukey’s multiple comparison posttest. Asterisks indicate significantly different values (p < 0.05; **, p < 0.01; and ***, p < 0.001).

Results

Effects of morphine and β-endorphin on the transcription of IL-2

Activation of the TCR leads to the induction of IL-2. A number of reports demonstrated that chronic morphine treatment of activated T cells inhibits this induction in mice (3, 4, 6). Fig. 1A shows the effects of chronic opioid treatment on IL-2 transcription in opioid receptor-expressing Jurkat cells (i.e., cells in which opioid receptors were induced; see Materials and Methods) after stimulation with a combination of anti-CD3 and anti-CD28. Similar to murine cells, morphine caused a significant inhibition of anti-CD3/anti-CD28-induced IL-2 mRNA production in the human cells. This effect was mediated by μ opioid receptors, since it was blocked by CTAP, a specific μ opioid receptor antagonist. Similarly, β-endorphin, which is a ligand for μ and δ opioid receptors, caused inhibition of anti-CD3/anti-CD28-induced IL-2 transcription. This effect was also mediated by μ opioid receptors, since it was blocked by CTAP, but not by naltrindole, a specific δ opioid receptor antagonist. To test possible effects on the anti-CD3/anti-CD28-induced IL-2 mRNA production mediated by δ opioid receptors more specifically, we additionally tested the specific δ opioid receptor agonist d-penicillamine2-d-penicillamine5-enkephalin (DPDPE). However, treatment of the cells with DPDPE had no significant effect on IL-2 transcription. Additionally, the effect of morphine on the activities of the transcription factors AP-1, NF-κB, and NFAT were investigated. As shown in Fig. 1B, morphine significantly inhibited the anti-CD3/anti-CD28-induced IL-2 transcription.
FIGURE 3. Opioids inhibit the TCR-induced phosphorylation of Zap70, LAT, and MAPK. Primary human T cells received a single dose of opioids (morphine or β-endorphin; both 1 μM) and were incubated with the drugs for the indicated time periods. Then the cells received fresh medium and were either left untreated or activated with anti-CD3/anti-CD28 for 2 min and lysed. Blots were probed for phospho-specific proteins (P-) and actin as a control. Examples of representative experiments are depicted, which were performed at least twice in duplicate. A, Effects of morphine in opioid receptor-expressing cells. B, The effect of morphine in opioid receptor-expressing cells is blocked by simultaneous addition of the μ opioid receptor antagonist CTAP (250 nM). C, Effects of β-endorphin (β-End) or opioid receptor-expressing cells. D, The effect of β-endorphin in opioid receptor-expressing cells is blocked by simultaneous addition of the δ opioid receptor-specific antagonist naltrindole (Naltrin; 250 nM). E and F, Temporal dissection of the effects of morphine (E) and β-endorphin (F) in opioid receptor-expressing cells. A quantification of two individual experiments performed in duplicate is depicted on top (asterisks indicate samples significantly different from opioid-untreated, anti-CD3/anti-CD28-activated samples; secondary comparisons are indicated by brackets; ***, p < 0.01 and ****, p < 0.001). G, The effect of the opioids on the TCR-mediated phosphorylation of Zap70, LAT, and MAPK is absent in naive T cells, which do not express μ opioid receptors.

Activities of all three transcription factors in opioid receptor-expressing Jurkat cells.

Effects of morphine and β-endorphin on the anti-CD3/anti-CD28-induced signaling cascade of T cells

One of the early events in T cell activation is mobilization of intracellular calcium. As shown in Fig. 2A, incubation of opioid receptor-expressing Jurkat cells with morphine for up to 2 h gradually inhibited the TCR-induced calcium flux by >60% (Fig. 2B). In contrast, morphine had no effect on the calcium flux in naive T cells, which do not express μ opioid receptors (Fig. 2C).

To obtain further insight into the mechanism(s), by which opioids inhibit anti-CD3/anti-CD28-induced IL-2 transcription, we next investigated their effects on the signaling cascade downstream of the TCR. The activation states of MAPK, LAT, and Zap70 were determined in Western blot experiments using phospho-specific Abs (Fig. 3). Activation of opioid receptor-expressing primary human T cells with anti-CD3/anti-CD28 for 2 min induced a robust phosphorylation of MAPK, LAT, and Zap70. However, this phosphorylation was markedly reduced when the cells were incubated with morphine for 2–8 h before their activation (Fig. 3A). Simultaneous incubation of the T cells with morphine and the μ opioid receptor-specific antagonist CTAP abolished the effect, indicating that it is mediated via μ opioid receptors (Fig. 3B). Similar effects were observed when opioid receptor-expressing primary T cells were incubated with β-endorphin (Fig. 3C). The inhibitory effect of β-endorphin on anti-CD3/anti-CD28-induced MAPK, LAT, and Zap70 phosphorylation was abolished after coincubation of the cells with β-endorphin and CTAP, but not after coincubation with β-endorphin and naltrindole, a specific antagonist for the δ opioid receptor (Fig. 3D). This indicates that the inhibitory effect of β-endorphin is also mediated via μ opioid receptors. As shown in Fig. 3, E and F, the inhibitory effects of morphine and β-endorphin on the TCR-triggered activation of MAPK are clearly visible at time points later than 80 min of incubation of the cells with the drugs. The effects of the opioids were not observed in naive T cells, which did not express μ opioid receptors (Fig. 3G).

To more precisely identify the level at which μ opioid receptors and the TCR communicate, we examined Lck, the kinase responsible for initiating TCR signaling and the activation of Zap70. Lck is phosphorylated in unstimulated T cells at Tyr505, by which signaling downstream of the TCR is prevented. We observed that in opioid receptor-expressing primary T cells (Fig. 4) and Jurkat cells (data not shown) the dephosphorylation of Lck at Tyr505 is also inhibited by opioids. In contrast, the opioids did not inhibit the TCR-induced dephosphorylation of Lck at Tyr505 in naive cells, which do not express μ opioid receptors (Fig. 4). As a control, and to rule out the possibility that the effect of the opioids might be due to down-regulation or internalization of the TCR, the presence of TCR on the cell surface was determined by flow cytometry. It was shown that 98% of the opioid receptor-expressing Jurkat cells were positive for the TCR. After 2 h of morphine treatment, the number of TCR-positive cells did not change significantly, indicating that morphine does not influence the surface expression of the TCR (data not shown).
Agonists for the μ opioid receptor inhibit TCR signaling via the PKA-Cbp/PAG/Csk-Lck pathway

It is known that the phosphorylation of the inhibitory Tyr^505 of Lck is mediated by Csk (33). Csk is recruited to the membrane by binding to Cbp/PAG (11), which, in turn, enhances its activity (34). The activity of Csk can be further enhanced by PKA-activated phosphorylation of Csk (35). Thus, activation of PKA via elevated levels of cAMP exhibits an inhibitory effect upon TCR signaling by stabilizing the inhibited state of Lck (36). Therefore, we monitored the intracellular cAMP concentration in Jurkat cells after morphine and β-endorphin treatment (Fig. 5). In opioid receptor-expressing cells, both substances caused a significant decrease in the cAMP formation at the 15-min time point. However, longer incubation of the cells with the opioids caused a significant elevation of cAMP, which reached a maximum of about 886% after morphine- and β-endorphin treatment (Fig. 5B). In contrast, the opioids did not affect the cAMP concentration in naive T cells, which do not express μ opioid receptors (Fig. 5B). In the opioid receptor-expressing cells, the μ opioid receptor-specific antagonist CTAP blocked both the morphine- and β-endorphin-induced increase in cAMP levels, whereas the δ opioid receptor-specific antagonist naltrindole had no effect (Fig. 5C). Furthermore, pertussis toxin inhibited the morphine- and β-endorphin-induced increase in cAMP levels, indicating that the effect is mediated by G_12 proteins (Fig. 5C). Next, we aimed to find a possible explanation for the terminal decrease in cAMP that occurs between the 4- and 24-h time points and that brings cAMP levels back to control levels. Speculating that phosphodiesterases might be involved, we found that coinucubation of cells with morphine and the unspecific phosphodiesterase inhibitor IBMX inhibited this decrease. Thus, cAMP levels remained at maximal levels at the 8- and 16-h time points (data not shown) and the 24-h time point (Fig. 5D). To more specifically determine the types of phosphodiesterases, which are involved in this cAMP decrease, we next performed transient transfection of siRNA directed against human Cbp/PAG, the inhibitory effect of which on the anti-CD3/anti-CD28-induced phosphorylation of Lck at Tyr^505 is mediated by Csk (33). Csk is recruited to the membrane by binding to Cbp/PAG (11), which, in turn, enhances its activity (34). The activity of Csk can be further enhanced by PKA-activated phosphorylation of Csk (35). Thus, activation of PKA via elevated levels of cAMP exhibits an inhibitory effect upon TCR signaling by stabilizing the inhibited state of Lck (36). Therefore, we monitored the intracellular cAMP concentration in Jurkat cells after morphine and β-endorphin treatment (Fig. 5). 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To more specifically determine the types of phosphodiesterases, which are involved in this cAMP decrease, we next tested rolipram (inhibitor of phosphodiesterase 4), BRL50481 (inhibitor of phosphodiesterase 7), and dipyridamole (inhibitor of phosphodiesterases 5, 6, 8, 10, and 11) (Fig. 5D). Our results indicated that the phosphodiesterases 4 and 7 are involved in the terminal decrease in cAMP, since rolipram and BRL50481 significantly reduced the cAMP decrease. Applied together, both substances inhibited the cAMP decrease to a similar extent as IBMX did alone. To test whether the ability of opioids to inhibit TCR signaling is dependent on cAMP-activated PKA, we applied the PKA antagonist cAMPS-Rp. Indeed, preincubation of opioid receptor-expressing primary T cells (Fig. 6) and Jurkat cells (data not shown) with cAMPS-Rp for 1 h before opioid incubation completely abolished the inhibitory effects of morphine and β-endorphin on the anti-CD3/anti-CD28-induced phosphorylation of MAPK and Zap70 and dephosphorylation of Lck. To investigate whether Cbp/PAG/Csk is involved in the inhibition of TCR signaling by μ opioid receptor agonists, we down-regulated the expression of Cbp/PAG using siRNA (Fig. 7). As shown in Fig. 7A, transient transfection of siRNA directed against human Cbp/PAG resulted in an ~90% down-regulation of the protein compared with a control (scrambled) siRNA. Transfection of siRNA specific for the mouse protein, which has two nucleotide exchanges compared with the human gene, resulted in an inhibition of about one-third of Cbp/PAG expression compared with the control siRNA. Transfection of control siRNA had no detectable effect on Cbp/ PAG expression compared with untransfected, naive cells (data not shown). In opioid receptor-expressing Jurkat cells transfected with siRNA directed against human Cbp/PAG, the inhibitory effect of
were performed at least two times in duplicate. (P-) and actin as shown. Examples of typical experiments are depicted, which were performed at least two times in duplicate.

In contrast, applying control siRNA had no influence on the inhibition of Zap70 (Fig. 7B). Applying siRNA specific for the mouse opioid receptor agonists inhibited TCR signaling via the PKA-Cbp/PAG pathway. If this was indeed the case, then the PMA/ionomycin-induced activation of MAPK, in which Lck-regulated TCR activation is bypassed, should not be influenced by opioids. Indeed, although the incubation of opioid receptor-expressing Jurkat cells with morphine and β-endorphin for 4 h produced elevated cAMP levels, which are required for the activation of PKA, the PMA/ionomycin-induced activation of MAPK was not influenced (Fig. 8).

Discussion

In this report, we addressed the question how opioids inhibit the TCR-mediated production of IL-2 in human T lymphocytes. We investigated the effects of morphine, as the pharmacologically most important opioid, and β-endorphin, as an endogenous opioid. The mechanisms are summarized in a schematic diagram presented in Fig. 9.

One of the first steps in these opioid-T cell interactions was an increase in cAMP, which evoked by both opioids and mediated by μ opioid receptors. Thus far, detailed mechanisms for the regulation of cAMP by μ opioid receptor ligands are poorly understood. It has been suggested from studies in other cells that the activation of μ opioid receptors leads to an initial decrease in cAMP levels, which is due to their coupling to the inhibitory G<sub>ia/o</sub> proteins. Under certain experimental conditions, e.g., removal of the ligand, a compensatory, sometimes overshooting increase in cAMP levels at a single, fixed time point only may be insufficient to establish a role for this molecule in the opioid-mediated modulation of TCR signaling, because of the biphasic, time-dependent modulation of the cAMP levels caused by opioids. For the first time, we demonstrated that simple incubation of human T cells with opioids, without any further manipulation like removal of the opioid effect of the opioids. Together, these data suggest that μ opioid receptor agonists inhibit TCR signaling via the PKA-Cbp/PAG/Csk-Lck pathway. If this was indeed the case, then the PMA/ionomycin-induced activation of MAPK, in which Lck-regulated TCR activation is bypassed, should not be influenced by opioids.
opiod, induced a short, initial decrease in cAMP levels, which was followed by a marked increase in cAMP. This increase was sensitive to pertussis toxin and thus mediated by $G_{i/o}$ proteins. The elevated cAMP levels were most pronounced ~2–4 h after opioid addition. Thereafter, they decreased again, reaching control levels at the 24-h time point. This final decrease in cAMP levels was due to phosphodiesterase activity, catalyzed by the phosphodiesterases 4 and 7.

It is known that agents, which lead to increased cAMP levels, may inhibit the TCR-activated signaling cascade of T lymphocytes via the PKA-Cbp/PAG/Csk-Lck pathway (8). In line with this model, our experiments using a PKA inhibitor strongly suggest that the effect is mediated via this enzyme. The data using siRNA demonstrated the involvement of Cbp/PAG. Since interactions between Cbp/PAG and Csks are crucial for the function of the “master regulator” Lck, these data suggest that $\mu$ opioid receptor ligands inhibit TCR signaling by superactivating Csks and thereby inhibiting Lck. The stable phosphorylation of the inhibitory Tyr505 within the C terminus of Lck produced by the opioids is likely to lead to intramolecular binding between the Src homology 2 domain and the inhibitory phospho-tyrosine of Lck resulting in the formation of a closed conformation. This prevents interaction of Lck with its substrates, e.g., the TCR complex, resulting in a blockade of TCR-mediated signaling and IL-2 induction.

References

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Disclosures
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18. Kieffer, B. L. 1995. Recent advances in molecular recognition and signal trans-
4. Roy, S., R. B. Chapin, K. J. Cain, R. G. Charboneau, S. Ramakrishnan, and
24. Borner, C., J. Kraus, A. Bedini, B. Schraven, and V. Holf, 2008. T cell receptor/ CD28-mediated activation of human T lymphocytes induces expression of func-
17. Kieffer, B. L. 1995. Recent advances in molecular recognition and signal trans-
15. Luckow, B., and G. Schutz. 1987. CAT constructions with multiple unique re-
4. Roy, S., R. B. Chapin, K. J. Cain, R. G. Charboneau, S. Ramakrishnan, and