

T-Cell Receptor/CD28-Mediated Activation of Human T Lymphocytes Induces Expression of Functional μ -Opioid Receptors

Christine Börner, Jürgen Kraus, Andrea Bedini, Burkhardt Schraven, and Volker Höllt

Department of Pharmacology and Toxicology (C.B., J.K., A.B., V.H.) and Department of Immunology (B.S.), University of Magdeburg, Magdeburg, Germany

Received February 4, 2008; accepted May 7, 2008

ABSTRACT

Opiates function as immunomodulators, partly by their effects on T cells. Opioids act via μ -, δ -, and κ -opioid receptors, among which the μ -type is of particular interest, because morphine-like opioids preferentially bind to it. Here we report that μ -opioid receptor mRNA was induced after CD3/28-mediated activation of primary human T lymphocytes and Jurkat T cells, neither of which expresses the gene constitutively. Moreover, a reporter gene construct containing 2624 base pairs of the μ -opioid receptor promoter was transactivated by CD3/28 stimulation. Transcriptional induction of the μ -opioid receptor gene was mediated by activator protein-1 (AP-1), nuclear factor- κ B, and nuclear factor of activated T cells (NFAT). NFAT was found to bind to three sequences of the μ -opioid receptor promoter, located at nucleotides -1064, -785, and -486. Although the -486 element is in close proximity to a putative AP-1 site, there was no evidence for a combined AP-1/NFAT

site. Furthermore, we demonstrated that the induction of interleukin-2 mRNA and protein in activated T cells was inhibited by morphine in cells, in which μ -opioid receptors had been induced by CD3/28 monoclonal antibodies (mAbs), and that this effect was blocked by the μ -opioid receptor-specific antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂. CD3/28 mAb-induced interleukin-2 transcription was also inhibited by the opioids fentanyl and looperamide. This indicates that the induced μ -opioid receptor mRNA is translated into functional receptor protein. Furthermore, a μ -opioid receptor-enhanced green fluorescent protein-fusion protein was localized in membranes of Jurkat cells and internalized in response to [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin but not morphine. In conclusion, these data emphasize the role of opioids in the modulation of T lymphocyte signaling.

Opioids are well known for their analgetic and euphoric effects. In addition, they potently modulate immune functions. For example, they increase the production of IL-12 in peritoneal macrophages (Peng et al., 2000), suppress splenic antibody responses (Rahim et al., 2001), and suppress the activity of natural killer cells (Roy et al., 1998). In activated T cells, in particular, morphine suppresses the expression of IFN- γ and IL-2, whereas it enhances the expression of IL-4 (Roy et al., 1997, 2001, 2005; Wang et al., 2003, 2007). The immunosuppressive effects of opioids may be disad-

vantageous for patients receiving morphine treatment. Effects of opioids ultimately depend on the expression of specific receptors, termed μ -, δ -, and κ -opioid receptors (Kieffer, 1995; Pol and Puig, 2004). Morphine and most of the clinically used opioids preferentially bind to μ -opioid receptors. In transgenic mice, in which these receptors are deleted, most of the immunomodulatory effects of morphine are missing as well, indicating that opioid effects in immune cells also are mediated via μ -opioid receptors (Gavériaux-Ruff et al., 1998; Roy et al., 1998). Although these receptors are not constitutively expressed in immune effector cells, they are induced in response to various stimuli. For example, the cytokines TNF and IL-4 induce the expression of μ -opioid receptors in T and B lymphocytes, monocytes, and granulocytes (Kraus et al., 2001, 2003a; Börner et al., 2004b). In addition, substances and stimuli

This work was supported by a research grant of the government of the state of Sachsen-Anhalt (N2/ND: Physiologie und Pathophysiologie signalübertragender Netzwerke im Immun- und Nervensystem) (to V.H. and B.S.) and a grant from the Deutsche Forschungsgemeinschaft (KR 1740/10-1) (to J.K.).

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.108.046029.

ABBREVIATIONS: IFN, interferon; AP-1, activator protein-1; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin; IL, interleukin; mAb, monoclonal antibody; NFAT, nuclear factor of activated T cells; NF- κ B, nuclear factor κ B; nt, nucleotide; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; tk, thymidine kinase; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunosorbent assay; CAT, chloramphenicol acetyltransferase; MOR, μ -opioid receptor; mu, mutated.

that cause induction of these cytokines also may cause induction of μ -opioid receptors. Thus, it was demonstrated that the expression and the release of IL-4 is induced by cannabinoids in T cells, which is followed by the induction of μ -opioid receptors (Börner et al., 2006). Likewise, it was reported recently that μ -opioid receptors are induced in HL-60 leukemia cells in response to the human immunodeficiency virus gp120, which involves TNF (Beltran et al., 2006). The regulation of μ -opioid receptor transcription by TNF and IL-4 is mediated by the transcription factors NF- κ B, and STAT6 and GATA3, respectively (Kraus et al., 2001, 2003a; Börner et al., 2004b, 2006). In addition, there is experimental evidence that the human μ -opioid receptor gene is transactivated by STAT1/3 (Börner et al., 2004a) and AP-1 (Börner et al., 2002). Because it is well established that T cell receptor-mediated activation of T cells leads to activation of transcription factors including AP-1, NF- κ B, and NFAT (Huang and Wange, 2004), we addressed the question of whether activation of T cells induces the expression of μ -opioid receptors and investigated which transcription factors contribute to this induction. Nothing is currently known about the transactivation of μ -opioid receptors by NFAT. Therefore, elucidating a possible involvement of this transcription factor in the transactivation of μ -opioid receptors in activated T cells was another aim of this report. Activation of NFAT in activated T cells is achieved by dephosphorylation and translocation into the nucleus (Li-Weber and Krammer, 2003). There, it binds to DNA elements with the consensus sequence motif 5'-WGGAAG-3', with W standing for A or T. In addition to such single NFAT sites, NFAT may bind together with AP-1 to composed NFAT/AP-1 sites, in which binding sites for both factors are in close proximity. It is noteworthy that the binding site for AP-1 itself in such composed elements may be very weak. As a consequence, AP-1 alone would only marginally bind to such sites, whereas the combination of AP-1 and NFAT shows remarkable binding (Sun et al., 1997). In addition, the question was addressed as to whether the μ -opioid receptor mRNA induced in activated T lymphocytes is translated into functional receptor protein. Therefore, the effects of opioids on the induction of IL-2 in activated T lymphocytes, which is established in a mouse model (Roy et al., 1997, 2004; Wang et al., 2007), was investigated. Finally, we investigated localization and internalization of a μ -opioid receptor-enhanced green fluorescent protein-fusion protein in Jurkat T cells to get a first impression of μ -opioid receptor trafficking in T lymphocytes.

Materials and Methods

Cell Culture, Reagents, and Activation of T Cells. Primary human peripheral blood T cells and Jurkat (Kab 14) cells were cultivated at 37°C and 5% CO₂ in RPMI 1640 medium (Lonza Verviers SPRL, Verviers, Belgium) supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Lonza Verviers SPRL). The source for CD3 and CD28 mAbs was the supernatant from hybridoma cell lines, which are established in our laboratories (Smida et al., 2007). Activation of T cells for RNA isolation and RT-PCR: 10⁶ cells were pelleted and resuspended in 100 μ l of CD3 and 100 μ l of CD28 mAbs. Cells were activated at 37°C for 30 min. The activation was stopped with 1 ml of ice-cold phosphate-buffered saline, cells were pelleted again, resuspended in

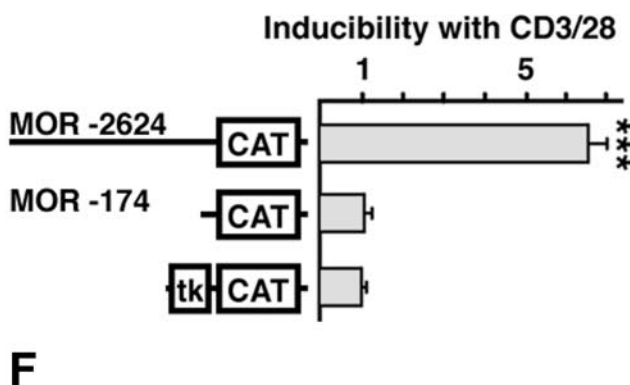
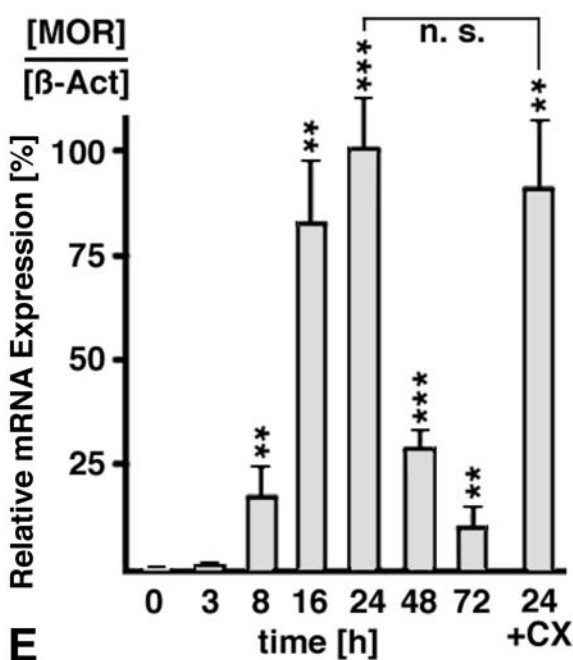
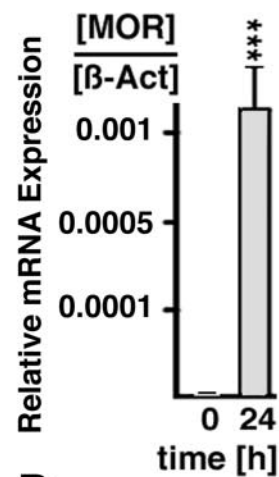
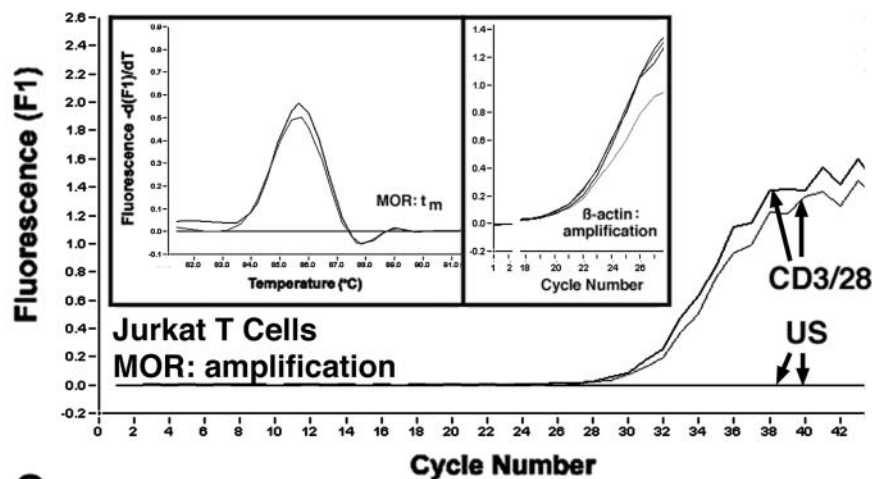
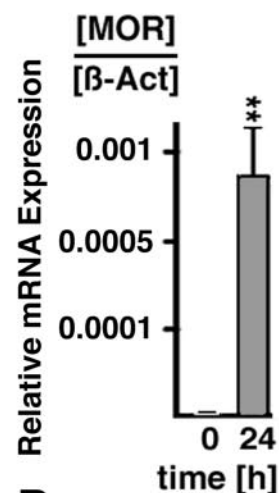
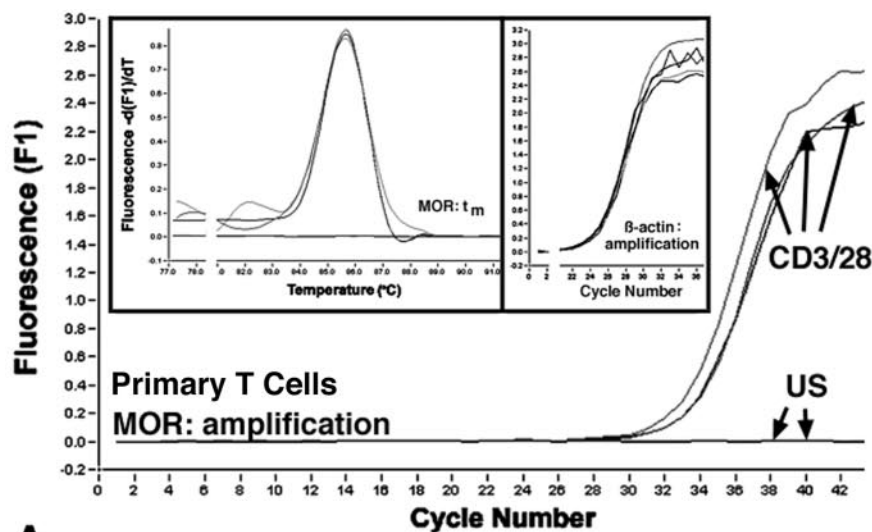
growth medium, and incubated for a desired time. For activation of T cells on CD3/28 mAb-coated culture dishes (10 cm²), the dishes were incubated overnight at 4°C with 12.5 μ g of anti-mouse immunoglobulins (Dako Cytomation, Glostrup, Denmark). After that, they were coated over night at 4°C with 120 μ l of CD3 and CD28, each. Then, cells were incubated on the coated dishes. As opioids, morphine (1 μ M; Synopharm, Barsbüttel, Germany), fentanyl (15 nM; Grünenthal, Aachen, Germany), loperamide (1.5 μ M; Sigma, Taufkirchen, Germany), and DAMGO (1 μ M; Sigma) were used. CTAP (Tocris, Bristol, UK) was used at 250 nM. Cycloheximide (Sigma) was used at a concentration of 10 μ g/ml.

Isolation of RNA and RT-PCR. Isolation of RNA and quantitative real-time RT-PCR specific for μ -opioid receptor and β -actin transcripts has been described in detail in a recent publication from our group (Börner et al., 2007). Quantitative real-time RT-PCR was done in a total volume of 20 μ l on a LightCycler instrument using the LightCycler-Fast Start DNA Master SYBR Green I kit (both from Roche, Mannheim, Germany) according to the manufacturer's suggestions. IL-2-specific RT-PCR was performed with 5'-GAAGGCCA-CAGAATGAAACATCT-3' and 5'-CTGTTTCAGAAATCTACAATG-GTTG-3' primers as follows: preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 65°C, and 10 s at 72°C (specific T_m = 81.75°C).

Reporter Gene Constructs and Transfection of Jurkat Cells. All reporter gene constructs are based on the pBLCAT2/3 system (Luckow and Schütz, 1987). Cloning of the reporter gene constructs MOR-2624 and MOR-174 is described in detail elsewhere (Kraus et al., 2001, 2003a). Construction of the NFAT-tk-CAT plasmids was performed according to a method described previously (Kang and Inouye, 1993). The oligonucleotides with the sequences 5'-GATCCGATTGTGTTTAGGAAATTTTGTTCATTGGTAAACC-3' (for -2253-tk-CAT), 5'-GATCAGTCTCTAGGAAATCTCTGTACA-3' (for -1542-tk-CAT), 5'-GATCTATTATGTGGCTTTTCCTA-GAATT-3' (for -1064-tk-CAT), 5'-GATCAATTGGGGAAATGAG-GAACG-3' (for -785-tk-CAT), and 5'-GATCCAAAAGGCGCTG-GAAAATTGAGTGATGTTAGCCCC-3' (for -486-tk-CAT) were inserted into the BamHI site 5' to the tk promoter of pBLCAT2. Only the top strand is given. All plasmids were sequenced to verify the correct insertions of the oligonucleotides. Transfection of Jurkat cells has been performed by electroporation with 15 μ g of DNA per 5 \times 10⁶ cells according to a preset protocol on a Gene Pulser Xcell (Bio-Rad, München, Germany). The day after transfection, cells received fresh medium and were activated by incubation on CD3/28-coated dishes with normal culture medium at 37°C and 5% CO₂. Controls were incubated on dishes coated with anti-mouse immunoglobulins only. After 72 h of transient expression, the reporter gene was assayed via a CAT-ELISA (Roche).

Decoy Oligonucleotide Approach. The decoy oligonucleotide approach is described in detail in previous publications from our group (Kraus et al., 2003a, 2003b; Börner et al., 2004a). The sequences for the decoy oligonucleotides were as described previously or as follows (only the top strand is given; putative binding sequences are underlined): AP-1 -2388, 5'-AAACATATGATTTCACCAGGCA-3'; AP-1 -1434, 5'-TTACCTATGAGTTATCTGTTT-3'; mu-AP-1, 5'-CCTAAGGAGAGTCAAGAGAAC-3', all NF- κ B decoys, see Kraus et al. (2003a), consensus/classic NFAT, 5'-GCCCAAAGAGGAAAATTTGTT-TCATA-3', mu-NFAT, 5'-GCCCAAAGAGGTCCATTTGTTTCATA-3'; NFAT -2253, 5'-TGTGTTTAGGAAATTTGTTCATT-3'; NFAT -1542, 5'-GTCTCTAGGAAATCTCTGTAAAC-3'; NFAT -1064, 5'-TTATGTG-GCTTTTCCTAGAATT-3'; NFAT -785, 5'-AATTGGGGAAATGAG-GAA-3'; and NFAT -486, 5'-AAGGCGCTGGAAAATTGAGTGATGT-TAGCC-3'.

EMSA and Nuclear Extracts. As probes and competitor DNA, the same double-stranded oligonucleotides as described under Decoy Oligonucleotide Approach were used. Nuclear extracts were isolated from Jurkat cells 30 min after CD3/28 activation. The extraction procedure and the EMSA were performed as described previously (Kraus et al., 2001).



Inhibition of IL-2 Expression. Experiments investigating the inhibition of IL-2 mRNA were performed similarly to a protocol described previously (Roy et al., 2004). In brief, primary human T cells and Jurkat cells (10^6 per sample) were incubated for 4 days on CD3/28-coated dishes to induce μ -opioid receptors, whereas controls were incubated on dishes coated with anti-mouse immunoglobulins only. At the same time, cells were coincubated with opioids, CTAP, or vehicle. Then cells were stimulated again with CD3/28 mAbs for 30 min to induce IL-2 and were lysed after 4 h for quantitative IL-2 RT-PCR (see above). For IL-2 protein determination, the supernatants of cells were assayed by ELISA (R&D Systems, Wiesbaden, Germany) 6 and 24 h after CD3/28 mAbs restimulation.

Localization and Internalization Studies. A μ -opioid receptor (N-terminal)-enhanced green fluorescent protein (C-terminal)-fusion plasmid, which contains cDNA of the rat μ -opioid receptor gene, was transfected into Jurkat cells by electroporation as described above and analyzed 24 h after transfection by confocal microscopy (Leica TCS-NT laser-scanning confocal microscope; Leica, Jena, Germany). Before analysis (pinhole <60 nM), cells were exposed to 1 μ M DAMGO or 1 μ M morphine for 1 to 4 h or left untreated.

Results

CD3/28-Mediated Activation of Human T Lymphocytes Induces μ -Opioid Receptor Transcription. First, we addressed the question of whether the μ -opioid receptor gene was induced in response to activation of T lymphocytes. Primary human peripheral blood T cells contained no detectable amounts of μ -opioid receptor-specific transcripts. However, there was a de novo induction of μ -opioid receptor transcription when cells were stimulated with a combination of CD3 and CD28 mAbs (Fig. 1A). Quantification analysis showed that the amount of μ -opioid receptor-specific transcripts in the primary cells was 0.00085 times that of transcripts for the housekeeping gene β -actin (Fig. 1B). Stimulation of primary T cells with activators targeting more downstream signaling components such as 12-*O*-tetradecanoylphorbol-13-acetate, and 12-*O*-tetradecanoylphorbol-13-acetate plus ionomycin similarly induced transcription of the μ -opioid receptor gene (data not shown). Similar to the experiments in primary T cells, activation of cells of the human T cell line Jurkat with CD3/28 mAbs induced μ -opioid receptor-specific transcripts, whereas resting Jurkat cells had no detectable amounts of μ -opioid receptor transcripts (Fig. 1C). The amount of μ -opioid receptor-specific transcripts in the Jurkat cells was 0.00115 times that of β -actin (Fig. 1D). The Jurkat cell line was then used as a model to study molecular mechanisms underlying μ -opioid receptor gene induction in

response to T-cell activation. In these cells, the mRNA was detectable after 3 h and was maximal at the 24 h time point. At later time points, mRNA amounts decreased again (Fig. 1E). Induction of μ -opioid receptor transcription in response to T-cell activation did not require novel protein synthesis, as shown in experiments using cycloheximide (Fig. 1E). The mRNA amount measured after CD3/28-mediated activation of the cells was similar to that after CD3 stimulation alone, whereas triggering of CD28 alone did not induce detectable μ -opioid receptor transcripts (data not shown). Next, the inducibility of reporter gene constructs was tested (Fig. 1F). A construct containing the μ -opioid receptor promoter up to nt -2624, which was transiently transfected into Jurkat cells, was responsive to CD3/28 stimulation, whereas construct MOR-174, which contains only core promoter sequences of the μ -opioid receptor, and the reporter plasmid pBLCAT2, which contains the herpes simplex thymidine kinase promoter instead of the μ -opioid receptor promoter, were not. This indicates that the sequences between -2624 and -174 are responsible for mediating the CD3/28 response.

AP-1, NF- κ B, and NFAT Transactivate μ -Opioid Receptor Transcription in Response to T Cell Activation. In general, T cell receptor-mediated activation results in the activation of AP-1, NF- κ B, and NFAT. In earlier studies, we already demonstrated transactivation of the μ -opioid receptor gene by AP-1 and NF- κ B in neuronal SH SY5Y and Raji B cells and localized binding sites for these factors (Börner et al., 2002; Kraus et al., 2003a) (Fig. 2A). To study which transcription factors transactivate μ -opioid receptor gene expression in response to T cell receptor activation, oligonucleotides containing the AP-1 and NF- κ B binding sequences of the μ -opioid receptor promoter were used as transcription factor decoys to disrupt transactivation by these two factors. Induction of μ -opioid receptor transcription in response to CD3/28 (Fig. 2B, lanes a and b) was dramatically blocked by all of the AP-1 (Fig. 2B, lanes c and d) and NF- κ B (Fig. 2B, lanes f to h) decoy oligonucleotides, indicating that both transcription factors are involved in this induction. As controls, decoy oligonucleotides with point mutations were used, which had been shown previously to no longer bind AP-1 and NF- κ B (Fig. 2B, lanes e and i) (Börner et al., 2002; Kraus et al., 2003a). Next, a possible involvement of NFAT in the CD3/28-induced transcription of the μ -opioid receptor gene was investigated. Induction of μ -opioid receptor transcription after T-cell activation (Fig. 3A, lanes a and b) was significantly inhibited by decoy oligonucleotides containing a

Fig. 1. Activation of T lymphocytes with a combination of CD3 and CD28 mAbs induces μ -opioid receptor transcription. A and C, representative experiments showing the detection of MOR transcripts in primary human T lymphocytes (A) and Jurkat T cells (C) by quantitative real-time RT-PCR. Cells were stimulated with CD3/28 mAbs for 30 min or were treated identically with vehicle (growth medium, US). Twenty-four hours later, cells were lysed, and RT-PCR was performed. Amplification is only observed in the stimulated cells. The boxes show the melting curves, which are typical for μ -opioid receptor transcripts ($T_m = 85.7^\circ\text{C}$), and the amplification of the housekeeping gene β -actin of the same samples, for which the crossing points of amplification are all within the range 24 ± 0.5 (primary T cells) and 21.3 ± 0.2 (Jurkat cells). B and D, quantification analysis of MOR-specific transcripts in primary human T lymphocytes (B) and Jurkat T cells (D) relative to β -actin transcripts. At least three independent experiments were performed in duplicate and are shown plus S.E.M. (**, $p < 0.01$; ***, $p < 0.001$; n.s., not significant). E, determination of the time course for the μ -opioid receptor mRNA induction in Jurkat cells. Cells were stimulated with CD3/28 mAbs for 30 min and then incubated for the indicated times in growth medium and μ -opioid receptor-specific transcripts were determined by quantitative real-time RT-PCR relative to those of β -actin. The amount of transcripts at the 24 h time points was set to 100%. The 24-h time point was additionally performed in the presence of cycloheximide (24 + CX). Values of at least three independent experiments performed in duplicate plus S.E.M. are shown. All samples were compared with the 0 h time point. A secondary comparison is indicated by a bracket (**, $p < 0.01$; ***, $p < 0.001$; n.s., not significant). F, inducibility of the human μ -opioid receptor gene promoter in response to CD3/28 stimulation. Jurkat cells were transiently transfected with chloramphenicol acetyl transferase (CAT) reporter gene constructs containing parts of the μ -opioid receptor promoter (MOR-2624 and MOR-174) and the construction vector pBLCAT2 with the herpes simplex thymidine kinase minimal promoter (tk) instead of μ -opioid receptor promoter sequences. The next day, medium was replaced and cells were incubated either on CD3/28 mAb-coated dishes (stimulated samples) or immunoglobulin-coated dishes (respective controls) for 72 h. Results of at least three independent experiments performed in triplicate plus S.E.M. are displayed (***, $p < 0.001$).

classic NFAT site (Fig. 3A, lane c), indicating that this transcription factor is additionally involved in transactivation of the μ -opioid receptor gene.

Characterization of NFAT Sites on the μ -Opioid Receptor Gene Promoter. The promoter of the human μ -opioid receptor gene contains several sequences, which are similar to NFAT sites (Fig. 3B). Transcription factor decoy oligonucleotides comprising the putative NFAT elements of the μ -opioid receptor promoter at nts -1064, -785, and -486 significantly inhibited up-regulation of μ -opioid receptor in response to activation of Jurkat cells (Fig. 3A, lanes f to h), whereas the oligonucleotides comprising the putative elements at nts -2253 and -1542 had no effect (Fig. 3A, lanes d and e). This shows that at least three sequence elements (located at nts -1064, -785, and -486 on the μ -opioid receptor gene promoter) bind to NFAT in intact Jurkat cells. To confirm these data, *in vitro* binding studies using EMSAs were performed (Fig. 4A). Incubation of a classic NFAT probe (lane 1) together with CD3/28-stimulated Jurkat cell nuclear extract, yielded three shifted bands (lane 2). The addition of homologous competitor DNA, which lets the specific band(s) disappear, demonstrated that the specific band was the uppermost band (lane 3, large arrow). Appearance of the specific band was not affected using competitor DNA comprising the -2253 and -1542 elements, indicating that these sequences do not bind NFAT (lanes 4 and 5). In contrast, formation of the specific band was inhibited using competitor DNA comprising the -1064, -785, and -486 elements, showing binding of NFAT to these sequences (lanes 6–8). In line with these results, incubation of nuclear extract with probes comprising the -1064, -785, and -486

elements yielded a similar band shift pattern as observed with the classic probe, which was abolished by competition with a classic NFAT binding sequence (lanes 9–14). To test the transactivating potency of the μ -opioid receptor NFAT sites transfection studies were performed (Fig. 4B). In accordance with the above results, reporter gene constructs with the herpes simplex thymidine kinase promoter under the control of the -1064, -785, and -486 elements were responsive to CD3/28 in transfected Jurkat cells, whereas the constructs with the other putative elements were not.

The -486 NFAT Element Is Not a Composed NFAT/AP-1 Binding Site. It is known that NFAT and AP-1 may bind to composed DNA elements. In this case, the AP-1 site may have only weak homology to the classic AP-1 consensus binding sequence 5'-TGASTCA-3' in which S stands for C or G. Therefore, we asked whether the -486 NFAT site, which is followed by a putative AP-1 site, may be such a composed element (Fig. 5A). We used the decoy oligonucleotide strategy, assuming that removal of either NFAT or AP-1 would cause a reduction in the CD3/28-mediated inducibility of a reporter construct containing this element, if it was indeed a composed element. Thus, inducibility of construct -486-tk-CAT in response to CD3/28 in transfected Jurkat cells was measured in the presence of NFAT and AP-1 decoy oligonucleotides and oligonucleotides with mutations, which do not bind these transcription factors (Fig. 5B). However, NFAT but not AP-1 decoy oligonucleotides inhibited reporter gene activity, indicating that the element does not bind the AP-1 complex in intact cells and therefore most likely is not a composed NFAT/AP-1 element.

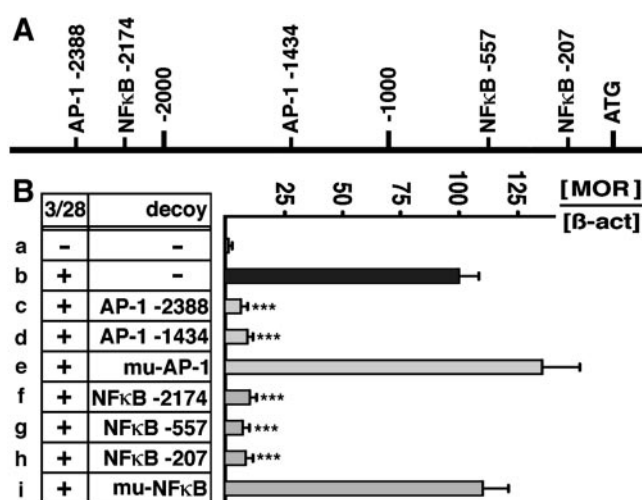


Fig. 2. AP-1 and NF- κ B transactivate μ -opioid receptor transcription in response to activation of Jurkat cells. A, schematic drawing of the μ -opioid receptor gene promoter showing previously localized binding sites for AP-1 and NF- κ B. B, effects of transcription factor decoy oligonucleotides comprising the two binding sites for AP-1 (lanes c and d) and the three sites for NF- κ B (lanes f to h) of the μ -opioid receptor gene promoter, or mutations, which do not bind the factors (mu-AP-1, mu-NF- κ B, lanes e and i) on the induction of the gene in response to CD3/28-stimulation (lanes a and b). Cells were incubated without or with decoy oligonucleotides (160 nM) as indicated for 16 h. Then cells were activated with CD3/28 mAbs for 30 min as indicated. After another 24 h, cells were lysed and subjected to quantitative real-time RT-PCR. At least three independent experiments were performed in triplicate. Asterisks indicate significantly reduced mRNA amounts compared with the stimulated cells without decoy oligonucleotides shown in lane b (***, $p < 0.001$).

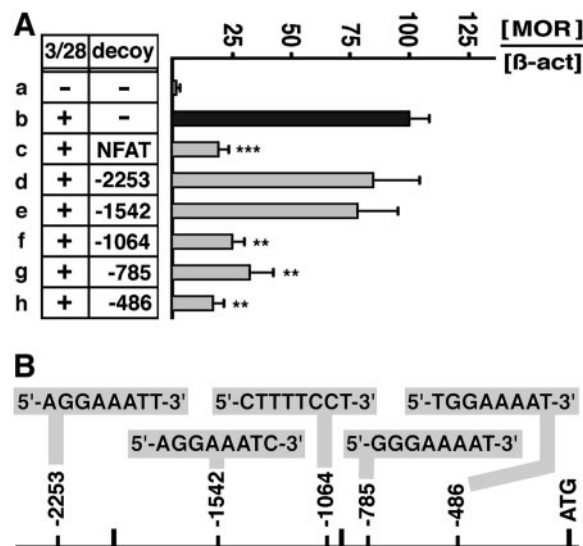


Fig. 3. NFAT transactivates μ -opioid receptor transcription in response to activation of Jurkat cells. A, effects of transcription factor decoy oligonucleotides comprising a consensus NFAT binding site (lane c) and five putative NFAT binding sites of the μ -opioid receptor gene (lanes d to h) on the induction of the gene in response to CD3/28-stimulation (lanes a and b). Cells were incubated without or with decoy oligonucleotides (160 nM) as indicated for 16 h. Then, cells were activated with CD3/28 mAbs for 30 min as indicated. After 24 h, cells were lysed and subjected to quantitative real-time RT-PCR. At least three independent experiments were performed in triplicate. Asterisks indicate significantly reduced mRNA amounts compared with the stimulated cells without decoy oligonucleotides shown in lane b (**, $p < 0.01$; ***, $p < 0.001$). B, schematic drawing of the μ -opioid receptor gene promoter showing the putative NFAT binding sequences.

CD3/28 mAbs Induce Functional μ -Opioid Receptors in Human T Lymphocytes. It is commonly known that activation of T cells leads to induction of IL-2 within a few hours. Data from a mouse model suggest that morphine inhibits this IL-2 induction. Therefore, the effect of the drug on the IL-2 production of human T lymphocytes was investigated next. In activated primary human T cells (Fig. 6A) morphine caused a significant inhibition of the IL-2 mRNA production, which, however, was observed in cells only in which μ -opioid receptors were induced previously by CD3/28. Morphine had no effect on naive T cells. In addition, the effect of morphine was completely blocked by the μ -opioid receptor-specific antagonist CTAP, indicating that it is mediated by this type of receptor exclusively. In addition, morphine caused an inhibition in the amount of CD3/28 mAb-induced IL-2 protein (Fig. 6B). These experiments indicate that the CD3/28 mAb-mediated induction of μ -opioid receptor transcription is followed by the induction of functional receptor

protein. A similar inhibition of CD3/28 mAb-induced IL-2 mRNA by morphine was found in Jurkat cells (Fig. 6C). The inhibitory effect of morphine was blocked by CTAP (data not shown). Furthermore, it was found that fentanyl, an opioid that is important in anesthesiology, and looperamide, a widely used peripheral μ -opioid receptor agonist, significantly inhibited the CD3/28 mAb-induced IL-2 mRNA in Jurkat cells (Fig. 6C). This indicates that the inhibitory effect of opioids on IL-2 is not restricted to morphine.

μ -Opioid Receptors Are Located in the Membranes of T Lymphocytes and Internalize after Ligand Treatment. Next we asked whether μ -opioid receptors are localized in the membrane of T cells and whether they internalize in response to ligands (Fig. 7). Transient expression of a μ -opioid receptor-enhanced green fluorescent protein-fusion protein in Jurkat cells and confocal microscopy suggested that μ -opioid receptors are located in the cell membranes. Similar to neuronal cells, treatment with the peptidic μ -opioid receptor agonist DAMGO caused internalization of the receptors in the Jurkat cells. The effect was maximal at the 2 h time point. Thereafter, receptors were increasingly found in the membranes again. In contrast to DAMGO, but again similar to neuronal cells, morphine only tended to result in receptor internalization.

Discussion

The induction of IL-2 is a hallmark of T-cell activation, which is followed by various immune responses (Koretzky and Myung, 2001; Simeoni et al., 2005). Here, we demonstrated for the first time that functional μ -opioid receptors are induced in response to T cell receptor activation as well. We showed that opioids decrease the production of IL-2 from activated T cells via the induced μ -opioid receptors, which may contribute to their immunosuppressive actions. Such feedback mechanisms possibly have not only pharmacological but also physiological impact. Thus, in addition to commonly used opioid drugs like morphine, it is reasonable to propose that endogenous μ -opioid receptor agonists like β -endorphin modulate the T-cell response. It will be interesting to

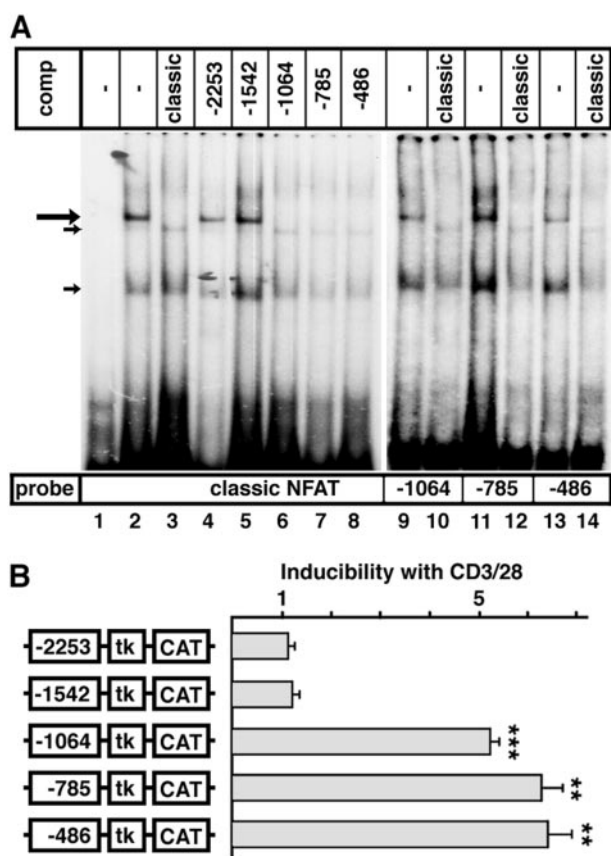


Fig. 4. Characterization of NFAT elements on the μ -opioid receptor gene promoter. **A**, EMSAs identifying NFAT sites. Representative examples showing the formation of NFAT complexes (large arrow) with various labeled probes (indicated on the bottom of the gels). The addition of competitor DNAs (10 pmol, equaling approximately a 50-fold molar excess) is indicated above the gels. A classic or consensus NFAT binding site and the five putative sequences of the μ -opioid receptor promoter (see Fig. 3B) were tested. **B**, transfection experiments demonstrating inducibility of the μ -opioid receptor NFAT elements by CD3/28. Jurkat cells were transiently transfected with CAT reporter gene constructs containing the herpes simplex thymidine kinase promoter (tk) under the control of the five putative NFAT sites of the μ -opioid receptor promoter. The next day, medium was replaced, and cells were incubated either on CD3/28 mAb-coated dishes (stimulated samples) or immunoglobulin-coated dishes (respective controls) for 72 h. Results of at least three independent experiments performed in triplicate plus S.E.M. are shown (**, $p < 0.01$; ***, $p < 0.001$).

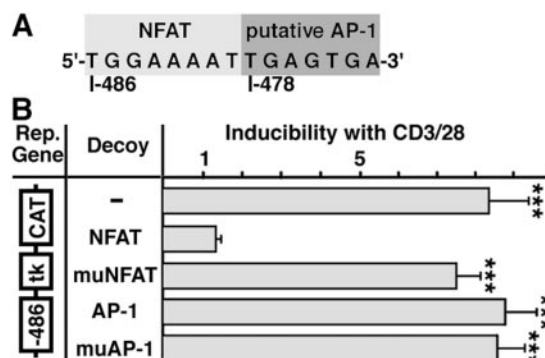


Fig. 5. The -486 NFAT element is not a combined NFAT/AP-1 element. **A**, sequence of the NFAT site at nt -486 and the putative AP-1 site at nt -478. **B**, Jurkat cells were transiently transfected with the -486-tk-CAT reporter gene construct (see Fig. 4B) and treated with decoy oligonucleotides directed against NFAT and AP-1 and mutated oligonucleotides, which do not bind these factors (muNFAT, muAP-1). The effect of the decoy oligonucleotides on the CD3/28-mediated inducibility of the reporter construct was measured. Results of at least three independent experiments performed in triplicate plus S.E.M. are shown (***, $p < 0.001$).

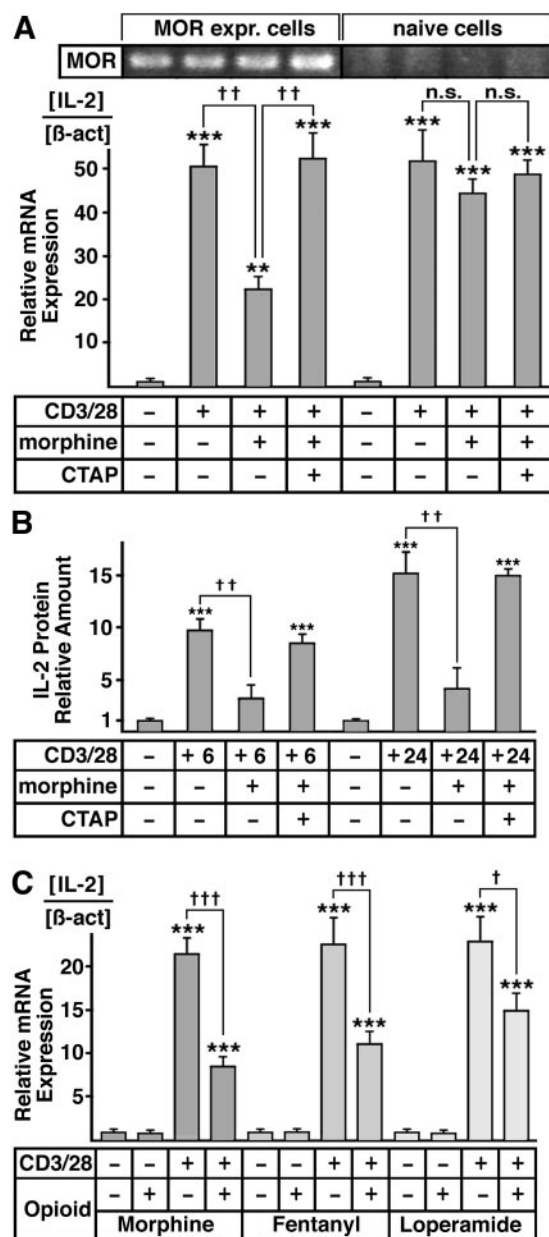


Fig. 6. CD3/28 mAbs-induced μ -opioid receptors mediate the inhibition of IL-2 transcription of activated T cells by opioids. **A**, IL-2 mRNA inhibition in primary human T cells. Cells were incubated for 4 days either on CD3/28 mAb-coated dishes to induce μ -opioid receptors (left) or on dishes coated with anti-mouse immunoglobulins only (right). Aliquots of these cells were taken, and induction of μ -opioid receptor transcripts was confirmed by RT-PCR (shown in the gel). At the same time, cells were coincubated with morphine, CTAP, or vehicle as depicted below. Then cells were restimulated with CD3/28, as depicted below, for 30 min to induce IL-2 mRNA and lysed after 4 h for quantitative IL-2 RT-PCR, which is shown relative to that of β -actin. Results of at least three independent experiments performed in triplicate plus S.E.M. are shown. Samples were compared with CD3/28-unstimulated controls (**, $p < 0.01$; ***, $p < 0.001$). Secondary comparisons are indicated by brackets ($\dagger\dagger$, $p < 0.01$; n.s., not significant). **B**, inhibition of IL-2 protein expression in primary human T cells. Six and 24 h after restimulation with CD3/28 (as indicated below), supernatants were assayed for IL-2 protein by ELISA. Results of two independent experiments plus S.E.M. are shown. (Samples were compared with CD3/28-unstimulated controls; ***, $p < 0.001$; secondary comparisons are indicated by brackets, $\dagger\dagger$, $p < 0.01$). **C**, IL-2 mRNA inhibition in Jurkat T cells. Cells were incubated for 4 days on CD3/28 mAb-coated dishes with or without opioids as indicated. Then, cells were restimulated with CD3/28, as depicted below, for 30 min to induce IL-2 mRNA. After 4 h, cells were lysed for quantitative IL-2 RT-PCR, which is shown relative to that of β -actin. Results of at least

determine how opioids communicate with the T-cell machinery in detail.

In an earlier publication, it was demonstrated by others that activation of T cells leads to the induction of a morphine binding site (Madden et al., 2001). Because the authors did not detect μ -opioid receptor transcripts, it was speculated that this binding site is different from μ -opioid receptors. To date, however, such nonclassic opioid binding sites or receptors in lymphocytes are not established and characterized. In contrast, there is increasing evidence for an inducible expression of μ -opioid receptors in immune effector cells (Kraus et al., 2001, 2003a; Börner et al., 2004b; Beltran et al., 2006). In general, the amounts of induced μ -opioid receptor transcripts are very low and require highly sensitive RT-PCR. In earlier publications from our group, in which we described the induction of μ -opioid receptor transcription in immune effector cells, we either used the highly sensitive nested RT-PCR or real-time RT-PCR. Using a quantitative approach, we reported recently that the amount of specific μ -opioid receptor transcripts in T cells after induction of the gene by cytokines is up to 200 times lower compared with certain neuronal cells (Börner et al., 2007). Nevertheless, we also showed that this amount is sufficient to induce functional receptor protein by demonstrating that the receptors mediate typical effects of opioids in T cells, like the induction of IL-4, which was reported previously by others (Roy et al., 2005).

In general, opioids and their receptors are regarded as a "neuronal" system. However, there is increasing knowledge about the expression and function not only of opioid receptors but also of endogenous opioids in cells of the immune system (Peterson et al., 1998; Stein et al., 2003; Pol and Puig, 2004; Roy et al., 2006; Sacerdote, 2006). In addition to a regulatory function for the μ -opioid system in activated T cells, which is suggested here, the μ -opioid system is involved in the regulation of T helper cells. Thus, opioids induce the expression of IL-4, which is the prototypical cytokine expressed in T helper type 2 cells and maintains their differentiation (Roy et al., 2005). In turn, IL-4 enhances the expression of μ -opioid receptors, which may support the effect of opioids to increase IL-4 (Kraus et al., 2001). On the other hand, morphine inhibits the expression of IFN- γ , which is the prototypical

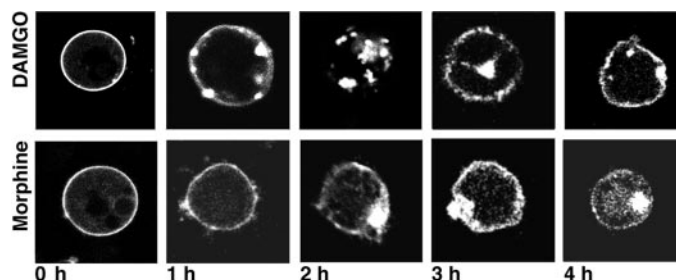


Fig. 7. Localization and internalization of μ -opioid receptors in Jurkat cells. A μ -opioid receptor-enhanced green fluorescent protein-fusion plasmid was transiently expressed in Jurkat cells and analyzed by confocal microscopy. Before analysis, cells were exposed to 1 μ M DAMGO or 1 μ M morphine for 1 to 4 h or left untreated as indicated. Examples of a representative experiment (of four individual transfections) are shown.

three independent experiments performed in triplicate plus S.E.M. are shown. Samples were compared with CD3/28-unstimulated controls for each opioid (***, $p < 0.001$). Secondary comparisons are indicated by brackets (\dagger , $p < 0.05$; $\dagger\dagger$, $p < 0.001$).

cytokine expressed in T helper type 1 cells (Wang et al., 2003). IFN- γ itself inhibits the expression of μ -opioid receptors (Kraus et al., 2006). As a consequence of these regulatory circuits, μ -opioid receptors are preferentially expressed in type 2 but not type 1 T helper cells (Kraus et al., 2006). Moreover, exogenous and endogenous μ -opioid ligands skew the T helper cell balance into the direction of type 2 T helper cells (Sacerdote et al., 1998, 2000; Roy et al., 2001, 2004). It is interesting that the transcription of μ -opioid receptors in response to IL-4 is mediated by the transcription factors STAT6 and GATA3, which is typical for several genes expressed in type 2 T helper cells (Kraus et al., 2001; Börner et al., 2004b, 2006). In general, this may emphasize the role of the μ -opioid system in lymphocytes. Activation of T cells leads to the activation of AP-1, NF- κ B, and NFAT. In this publication, we demonstrated that the μ -opioid receptor gene is transactivated by all of these factors in response to T-cell activation and determined binding sequences for NFAT. Often, NFAT binds together with AP-1 to composed NFAT/AP-1 sites, in which binding sites for both factors are in close proximity, and the homology to the classic AP-1 site may be rather low (Sun et al., 1997). Therefore, we considered the NFAT element at nt -486 to be such a composed element, because the sequence is followed by a sequence with some similarity to an AP-1 site. However, this sequence binds neither to AP-1 alone, as demonstrated in an earlier publication from our group (Börner et al., 2002), nor to an NFAT/AP-1 complex, as demonstrated here. Finally, trafficking of μ -opioid receptors in T cells was investigated. Similar to neuronal cells (Trafton et al., 2000; Bailey et al., 2003) we found localization of the receptors in the membranes of Jurkat cells. It is interesting that the receptors in Jurkat cells behave similarly to those in neuronal cells with regard to internalization upon agonist treatment. It was reported that the peptidic μ -opioid receptor agonist DAMGO caused rapid and marked internalization of the receptors in neuronal cells, whereas morphine caused very weak internalization only (Trafton et al., 2000; Bailey et al., 2003). In contrast to neuronal cells, our results suggest that the internalization process in response to DAMGO is slower in T cells than in neuronal cells. In general, μ -opioid receptor internalization in neuronal cells is far from being understood, and this report only can provide a first idea of this phenomenon in T cells.

In conclusion, activation of T cells by antigens is a fundamental and complex process in immunity. Its complexity is further increased by the expression of μ -opioid receptors, because they allow pharmacologically used ligands like morphine and physiological ligands like β -endorphin to alter the T-cell response.

Acknowledgments

We thank Helga Tischmeyer and Camilla Merten for excellent technical assistance. The μ -opioid receptor-enhanced green fluorescent protein-fusion plasmid was a kind gift of Hermann Ammer (Institute of Veterinary Pharmacology, University of Munich, Ludwig-Maximilians-Universität).

References

- Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, and Henderson G (2003) Mu-opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. *J Neurosci* **23**:10515–10520.
- Beltran JA, Pallur A, and Chang SL (2006) HIV-1 gp120 up-regulation of the mu opioid receptor in TPA-differentiated HL-60 cells. *Int Immunopharmacol* **6**:1459–1467.

- Börner C, Höllt V, and Kraus J (2002) Involvement of activator protein-1 in transcriptional regulation of the human mu-opioid receptor gene. *Mol Pharmacol* **61**:800–805.
- Börner C, Höllt V, and Kraus J (2006) Cannabinoid receptor type 2 agonists induce transcription of the μ -opioid receptor gene in Jurkat T cells. *Mol Pharmacol* **69**:1486–1491.
- Börner C, Kraus J, Schröder H, Ammer H, and Höllt V (2004a) Transcriptional regulation of the human mu-opioid receptor gene by interleukin-6. *Mol Pharmacol* **66**:1719–1726.
- Börner C, Stumm R, Höllt V, and Kraus J (2007) Comparative analysis of mu-opioid receptor expression in immune and neuronal cells. *J Neuroimmunol* **188**:56–63.
- Börner C, Wöltje M, Höllt V, and Kraus J (2004b) STAT6 transcription factor binding sites with mismatches within the canonical 5'-TTC.GAA-3' motif involved in regulation of delta- and mu-opioid receptors. *J Neurochem* **91**:1493–1500.
- Gavériaux-Ruff C, Matthes HW, Peluso J, and Kieffer BL (1998) Abolition of morphine-immunosuppression in mice lacking the μ -opioid receptor gene. *Proc Natl Acad Sci U S A* **95**:6326–6330.
- Huang Y and Wange RL (2004) T cell receptor signaling: beyond complex complexes. *J Biol Chem* **279**:28827–28830.
- Kang C and Inouye M (1993) One-step insertion of oligonucleotide linkers or adaptors to DNA using unphosphorylated oligonucleotides. *Biotechniques* **15**:659–668.
- Kieffer BL (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol Neurobiol* **15**:615–635.
- Koretzky GA and Myung PS (2001) Positive and negative regulation of T-cell activation by adaptor proteins. *Nat Rev Immunol* **1**:95–107.
- Kraus J, Börner C, Giannini E, Hickfang K, Braun H, Mayer P, Hoehe MR, Ambrosch A, König W, and Höllt V (2001) Regulation of μ -opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. *J Biol Chem* **276**:43901–43908.
- Kraus J, Börner C, Giannini E, and Höllt V (2003a) The role of nuclear factor κ B in tumor necrosis factor-regulated transcription of the human μ -opioid receptor gene. *Mol Pharmacol* **64**:876–884.
- Kraus J, Börner C, and Höllt V (2003b) Distinct palindromic extensions of the 5'-TTC.GAA-3' motif allow STAT6 binding in vivo. *FASEB J* **17**:304–306.
- Kraus J, Börner C, Lendeckel U, and Höllt V (2006) Interferon-gamma down-regulates transcription of the mu-opioid receptor gene in neuronal and immune cells. *J Neuroimmunol* **181**:13–18.
- Li-Weber M and Krammer PH (2003) Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat Rev Immunol* **3**:534–543.
- Luckow B and Schütz G (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res* **15**:5490.
- Madden JJ, Whaley WL, Ketelsen D, and Donahoe RM (2001) The morphine-binding site on human activated T-cells is not related to the mu opioid receptor. *Drug Alcohol Depend* **62**:131–139.
- Peng X, Mosser DM, Adler MW, Rogers TJ, Meissler JJ Jr, and Eisenstein TK (2000) Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages. *J Leukoc Biol* **68**:723–728.
- Peterson PK, Molitor TW, and Chao CC (1998) The opioid-cytokine connection. *J Neuroimmunol* **83**:63–69.
- Pol O and Puig MM (2004) Expression of opioid receptors during peripheral inflammation. *Curr Top Med Chem* **4**:51–61.
- Rahim RT, Meissler JJ Jr, Cowan A, Rogers TJ, Geller EB, Gaughan J, Adler MW, and Eisenstein TK (2001) Administration of mu-, kappa- or delta2-receptor agonists via osmotic minipumps suppresses murine splenic antibody responses. *Int Immunopharmacol* **1**:2001–2009.
- Roy S, Balasubramanian S, Sumandeep S, Charboneau R, Wang J, Melnyk D, Beilman GJ, Vatasery R, and Barke RA (2001) Morphine directs T cells toward TH2 differentiation. *Surgery* **130**:304–309.
- Roy S, Barke RA, and Loh HH (1998) MU-opioid receptor-knockout mice: role of mu-opioid receptor in morphine mediated immune functions. *Brain Res Mol Brain Res* **61**:190–194.
- Roy S, Chapin RB, Cain KJ, Charboneau RG, Ramakrishnan S, and Barke RA (1997) Morphine inhibits transcriptional activation of IL-2 in mouse thymocytes. *Cell Immunol* **179**:1–9.
- Roy S, Wang J, Charboneau R, Loh HH, and Barke RA (2005) Morphine induces CD4+ T cell IL-4 expression through an adenyl cyclase mechanism independent of the protein kinase A pathway. *J Immunol* **175**:6361–6367.
- Roy S, Wang J, Gupta S, Charboneau R, Loh HH, and Barke RA (2004) Chronic morphine treatment differentiates T helper cells to Th2 effector cells by modulating transcription factors GATA 3 and T-bet. *J Neuroimmunol* **147**:78–81.
- Roy S, Wang J, Kelschenbach J, Koodie L, and Martin J (2006) Modulation of immune function by morphine: implications for susceptibility to infection. *J Neuroimmune Pharmacol* **1**:77–89.
- Sacerdote P (2006) Opioids and the immune system. *Palliat Med* **20** (Suppl 1):S9–S15.
- Sacerdote P, di San Secondo VE, Sirchia G, Manfredi B, and Panerai AE (1998) Endogenous opioids modulate allograft rejection time in mice: possible relation with Th1/Th2 cytokines. *Clin Exp Immunol* **113**:465–469.
- Sacerdote P, Manfredi B, Gaspari L, and Panerai AE (2000) The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in BALB/c mice. *Blood* **95**:2031–2036.
- Simeoni L, Smida M, Posevitz V, Schraven B, and Lindquist JA (2005) Right time, right place: the organization of membrane proximal signaling. *Semin Immunol* **17**:35–49.
- Smida M, Posevitz-Fejfar A, Horejsi V, Schraven B, and Lindquist JA (2007) A novel negative regulatory function of the phosphoprotein associated with glycosphingolipid-enriched microdomains: blocking Ras activation. *Blood* **110**:596–615.
- Stein C, Schäfer M, and Machelska H (2003) Attacking pain at its source: new perspectives on opioids. *Nat Med* **9**:1003–1008.

Sun LJ, Peterson BR, and Verdine GL (1997) Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1. *Proc Natl Acad Sci U S A* **94**:4919–4924.

Trafton JA, Abbadie C, Marek K, and Basbaum AI (2000) Postsynaptic signaling via the [mu]-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J Neurosci* **20**:8578–8584.

Wang J, Barke RA, Charboneau R, Loh HH, and Roy S (2003) Morphine negatively regulates interferon-gamma promoter activity in activated murine T cells through two distinct cyclic AMP-dependent pathways. *J Biol Chem* **278**:37622–37631.

Wang J, Barke RA, and Roy S (2007) Transcriptional and epigenetic regulation of interleukin-2 gene in activated T cells by morphine. *J Biol Chem* **282**:7164–7171.

Address correspondence to: Dr. Jürgen Kraus, Dept. of Pharmacology and Toxicology, Magdeburg University, 44 Leipzigerstrasse, 39120 Magdeburg, Germany. E-mail: juergen.kraus@med.ovgu.de
