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Analysis of Promoter Regions Regulating Basal and Interleukin-4-Inducible Expression of the Human CB1 Receptor Gene in T Lymphocytes

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ABSTRACT

The majority of effects of cannabinoids are mediated by the two receptors CB1 and CB2. In addition to neuronal cells, CB1 receptors are expressed in T lymphocytes, in which they are involved in cannabinoid-induced T helper cell biasing. Although basally expressed only weakly in T cells, CB1 receptors are up-regulated in these cells by stimuli such as cannabinoids themselves. This effect is mediated by interleukin-4. In this study, we investigated basal and interleukin-4-inducible expression of the CB1 gene in T lymphocytes. In a promoter analysis, two regions [nucleotides (nts) −3086 to −2490 and nts −1950 to −1653] were identified, which suppress basal transcription of the gene in Jurkat T cells, whereas the region between nts −648 and −559 enhanced basal CB1 transcription. Interleukin-4 markedly induced transcription of CB1 in Jurkat cells and primary human T cells. Experiments using transcription factor decoy oligonucleotides demonstrated that STAT6 mediates regulation of the gene by interleukin-4. Using reporter gene assays and the transcription factor decoy oligonucleotide approach, a binding site for STAT6 was identified at nt −2769 on the human CB1 gene promoter. Interleukin-4 also caused up-regulation of functional CB1 receptor proteins. In interleukin-4 pretreated, but not in naive Jurkat cells, the CB1 agonist *R*(+)-methanandamide caused a significant inhibition of forskolin-induced cAMP formation. This effect was blocked by the CB1-selective antagonists *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-mo rpholinyl-1*H*-pyrazole-3-carboxamide (AM281). Taken together, these data show that CB1 receptors are expressed and up-regulated by interleukin-4 in T lymphocytes, which enables CB1-mediated communication to cells of other systems, such as neuronal cells.

The effects of cannabinoids are mediated by a variety of receptors, which include the originally defined cannabinoid receptors CB1 and CB2, but also other receptors like TRP channels, peroxisome proliferator-activated receptors, and orphan G-protein-coupled receptors like GPR55 and GPR119 (Brown, 2007). The majority of effects is mediated by CB1 and CB2, which belong to the class of G-protein-coupled receptors. Earlier, CB1 was termed "central" cannabinoid receptor, because its expression is most abundant in neuronal cells. In contrast, the CB2 receptor was termed "peripheral" because of its expression in peripheral tissue, including immune tissue (Howlett et al., 2002). Today, this classification needs to be redefined, because CB1 receptors are also expressed in many peripheral tissues, particularly in immune effector cells (Klein et al., 2004; Börner et al., 2007). Moreover, recent reports also suggest the expression of CB2 receptors in neuronal cells (Ashton et al., 2006; Gong et al., 2006). According to the expression profile of their receptors, cannabinoids most prominently modulate neuronal and immune functions. Moreover, the cannabinoid system provides a biochemical basis for multiple neuroimmune interactions. In addition to neuronal functions, it was shown that CB1 receptors are involved in several anti-inflammatory effects of cannabinoids, such as the up-regulation of the interleukin (IL)-1 receptor antagonist and the inhibition of proinflammatory cytokines like IL-6 and IL-8 (Molina-Holgado et al., 2003; Nakajima et al., 2006). Furthermore, it was demonstrated that CB1 receptors are involved in cannabinoid-induced T-helper cell biasing, promoting the differentiation of

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ABBREVIATIONS: IL, interleukin; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl(4-methoxyphenyl)methanone; CAT, chloramphenicol acetyl transferase; tk, herpes simplex thymidine kinase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-mo rpholinyl-1*H*-pyrazole-3-carboxamide; nt, nucleotide(s); kb, kilobase(s); ELISA, enzyme-linked immunosorbent assay; bp, base pair.

T-helper cells type 2 (Klein et al., 2004). Despite considerable effects mediated by CB1 receptors in T lymphocytes, their expression level in resting T lymphocytes is very low (Daaka et al., 1995, 1996). Similar results were found by our group; however, we recently reported that expression of the CB1 gene in T lymphocytes may be markedly up-regulated by stimuli, including cannabinoids themselves (Börner et al., 2007). This feedback induction involves several steps and requires, as one of the first steps, cannabinoid-induced expression of IL-4. Once released from the cells, IL-4 then induces transcription of the CB1 gene. IL-4 is the typical cytokine released from T-helper cells type 2 and has anti-inflammatory properties (Curfs et al., 1997). Effects of IL-4 are normally mediated by the transcription factors GATA3 and STAT6. They bind to *cis*-active, regulatory elements, which are normally located on the target genes' promoters. A typical binding sequence for GATA3 is 5′-AGATAA-3′. All seven members of the STAT transcription factor family bind to similar sequences, the majority of which contain the six base pair palindrome 5′-TTCN$_{2-4}$GAA-3′. In an attempt to define sequences, which were specific for the individual members of this family, DNA elements with defined, extended eight base pair palindromes (e.g., 5′-TTCCN$_2$GGAA-3′) were reported to specifically bind STAT6 (Kraus et al., 2003b). Thus far, little is known about regulatory promoter elements of the human CB1 gene, because its genomic organization, intron/exon structure, and promoter sequences were determined only recently (Zhang et al., 2004). The CB1 gene is located on chromosome 6, where it covers more than 20 kb and consists of 4 exons. The region that codes for two protein isoforms of the gene is entirely located on exon 4. A first analysis revealed a marked promoter activity of sequences flanking the 5′ end of exon 1 in neuronal cell lines (Zhang et al., 2004). Basing on these studies, one aim was to characterize the molecular basis for the transcriptional induction of the gene in T cells in response to the cytokine IL-4, which includes the identification of IL-4-responsive promoter elements and the characterization of the transcription factors binding to these sequences. Another aim was to identify regions on the promoter of the human CB1 gene, which may be involved in controlling the basal transcription of the gene in Jurkat cells as a model for T lymphocytes.

Materials and Methods

Cell Culture, Transfection, and Reagents. Primary human peripheral blood T cells and Jurkat E6.1 cells were cultivated in RPMI-1640 medium (Cambrex Bio Science S.p.r.l., Verviers, Belgium) supplemented with 10% fetal calf serum (PAN-BIOTECH GmbH, Aidenbach, Germany) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Cambrex Bio Science). Plasmid DNA for transfections was purified by QIAGEN Plasmid Kit columns (QIAGEN, Hilden, Germany). Transfection of Jurkat cells has been performed by electroporation with 15 μg of DNA per 5×10^6 cells according to a preset protocol on a Gene Pulser Xcell (Bio-Rad Laboratories, Munich, Germany). Equimolar amounts of different constructs were transfected using pBluescript DNA to fill up to 15 μg. The day after transfection, cells received fresh medium. After 72 h of transient expression, the reporter gene was assayed via a chloramphemicol acetyl transferase (CAT)-ELISA purchased from Roche (Mannheim, Germany). For stimulation experiments, IL-4 (5 ng/ml; R&D Systems, Wiesbaden, Germany) or vehicle (PBS buffer) was used. The CB1 receptor agonist *R*(+)-methanandamide was purchased from Sigma (Taufkirchen, Germany). The CB1 antagonists AM251 and AM281 and the CB2 antagonist AM630 were purchased from Tocris (Bristol, UK).

Reporter Gene Constructs. All reporter gene constructs are based on the pBLCAT2/pBLCAT3 system and contain the CAT reporter gene. Plasmid pBLCAT2 additionally contains the tk minimal promoter (Luckow and Schütz, 1987). The series of phCB1-CAT plasmids contain sequences of the human CB1 promoter instead of the tk promoter in front of CAT. Their construction was as follows: the CB1 gene is contained on human genomic clone RZPDB737B0120D (RZPD, Berlin, Germany). A SacI fragment of this clone spanning from nt −3086 to +142 (referring to the first nucleotide of exon 1; Zhang et al., 2004) of the CB1 gene was excised and cloned into the polylinker of the promoterless pBLCAT3 vector yielding phCB1-CAT-3086. Five prime deletion constructs of this plasmid were constructed either by site specific restriction enzyme deletion (−2490, AfeI; −1950, SpeI; −1653, EagI; and −648, SphI) or by a deletion strategy using the sequence-unspecific enzyme Bal31 (−1099, −955, −875, −559, and −223). Plasmid pS1-tk-CAT contains the sequence 5′-GATCTTAGGTTTCTGCTTGGAATTTCATAGAATTCAGTGAAGAAACCCAACGTCAATCAGAAG-3′ cloned into the multiple cloning site 5′ to the tk promoter. Plasmid pS2-tk-CAT has the insertion 5′-GATCTGGCTGAGTTGAGATACAAGATTTCCATGGAATACCTTTCACAGGTTGATGCTGACG-3′ and pmuS2-tk-CAT has the insertion 5′-GATCTGGCTGAGTTGAGATACAAGATGTCGATGGAGTACCTTTCACAGGTTGATGCTGACG-3′ in front of the tk promoter. The inserted DNAs were synthesized by Metabion (Martinsried, Germany). The correct insertion of sequences and the deletions of all plasmids were verified by sequencing.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA from T cells was extracted using the Nucleospin RNA II kit from Macherey-Nagel (Düren, Germany). One microgram of total RNA was used for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, RNase H minus (Promega, Mannheim, Germany), and diluted to 50 μl. Two microliters of cDNA was used for RT-PCR reactions. Quantitative real-time RT-PCR was performed in a total volume of 20 μl on a LightCycler instrument using the LightCycler-Fast Start DNA Master SYBR Green I kit (both from Roche). Conditions were as follows: β-actin, 5′-GGTCCACACCCGCCACCAG-3′ and 5′-CAGGTCCAGACGCAGGATGG-3′ primers; preincubation, 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 60°C, and 22 s at 72°C. CB1, 5′-CACCTTCCGCACCATCACCAC-3′ and 5′-GTCTCCCGCAGTCATCTTCTCTTG-3′ primers; preincubation: 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 68°C, and 10 s at 72°C. The primers were synthesized by Metabion.

Decoy Oligonucleotide Approach. The transcription factor decoy oligonucleotide approach, its efficiency, and specificity were described in detail in previous publications from our group (Kraus et al., 2003a,b; Börner et al., 2004a,b). In general, in the decoy oligonucleotide approach, short double-stranded oligonucleotides with specific binding sequences for transcription factors are introduced into living cells. In the cells, transcription factors interact with the excess of decoy oligonucleotides rather than bind to the natural regulatory motifs of genes. Thus, the decoys selectively disrupt the function of a desired transcription factor. Because the decoys act within living cells, they are highly specific. The sequences of the decoy oligonucleotides used for the experiments described in Fig. 3 were as follows: STAT6, 5′-CTAGTTCTTCTCAGAAGCATATGT-3′; nSTAT6, 5′-CTAGTTGATCTCAGATCCATATGT-3′; GATA3, 5′-CTAGAGGAAGTCTTCAGATAAAAAAGATAACAA-3′; and nGATA3, 5′-CTAGAGGAAGTCTTCACTTAAAAAACTTAACAA-3′. The sequences of the decoy oligonucleotides used for the experiments described in Fig. 5 (S1, S2, muS2) are given in the figure (only the top strand is given). As demonstrated earlier, Jurkat cells take up the decoy oligonucleotides passively (Kraus et al., 2003a). Cells were incubated with decoy oligonucleotides (160 nM) for 16 h before stimulation. Decoy oligonucleotides were synthesized by Metabion (Martinsried, Germany) as complementary single strands.

cAMP Measurement. Cells were incubated with IL-4 or vehicle for up to 4 days to up-regulate expression of CB1. The induction of

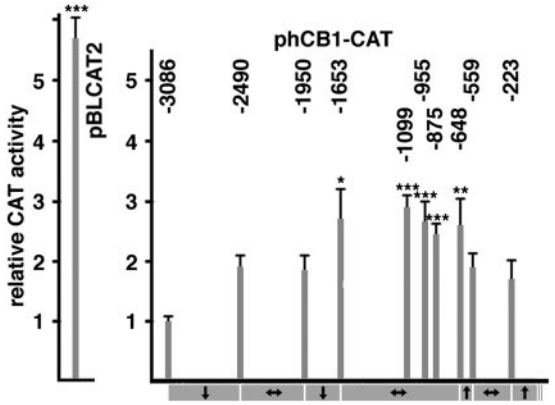


Fig. 1. Basal activity of the CB1 promoter in Jurkat cells. CAT reporter gene activities plus S.E.M. of at least three independent experiments performed in triplicate obtained after transient transfection of Jurkat cells are displayed relative to the activity of construct phCB1-CAT-3086. Left, activity of the pBLCAT2 reference and vector plasmid containing the herpes simplex thymidine kinase minimal promoter instead of the CB1 promoter. Right, activities of a series of 5′ promoter deletion constructs of phCB1-CAT-3086. Activities of all samples were compared with those of construct phCB1-CAT-3086 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Below, putative regulatory input of promoter regions is indicated by arrows.

CB1 mRNA in the IL-4 prestimulated cells compared with the vehicle-treated cells was verified by quantitative RT-PCR in an aliquot of the cells. Specific antagonists were added 1 h before forskolin addition. Cells were then incubated with forskolin (25 μM) alone or forskolin plus different concentrations of $R(+)$-methanandamide for 15 min at 37°C. Cells were lysed with 50 mM HCl for 30 min on ice. Acetylation of the samples and cAMP ELISA were performed according to a described procedure (Horton et al., 1992).

Statistical Analysis. For statistical evaluations between two groups of samples, Student's t tests were performed. Multiple comparisons were performed with analysis of variance, followed by Tukey's multiple comparison post test. Asterisks indicate significantly different values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Results

To characterize promoter regions that may be involved in the regulation of the basal transcription of the CB1 gene in T cells, transient transfections were performed in Jurkat cells with plasmids containing various parts of the CB1 promoter fused to the CAT reporter gene (Fig. 1). Compared with the activity of the tk promoter contained in plasmid pBLCAT2, there was a weaker but substantial reporter gene activity of the longest construct comprising 3086 bp of the CB1 promoter, which was set to 1.0. The effects of sequential 5′

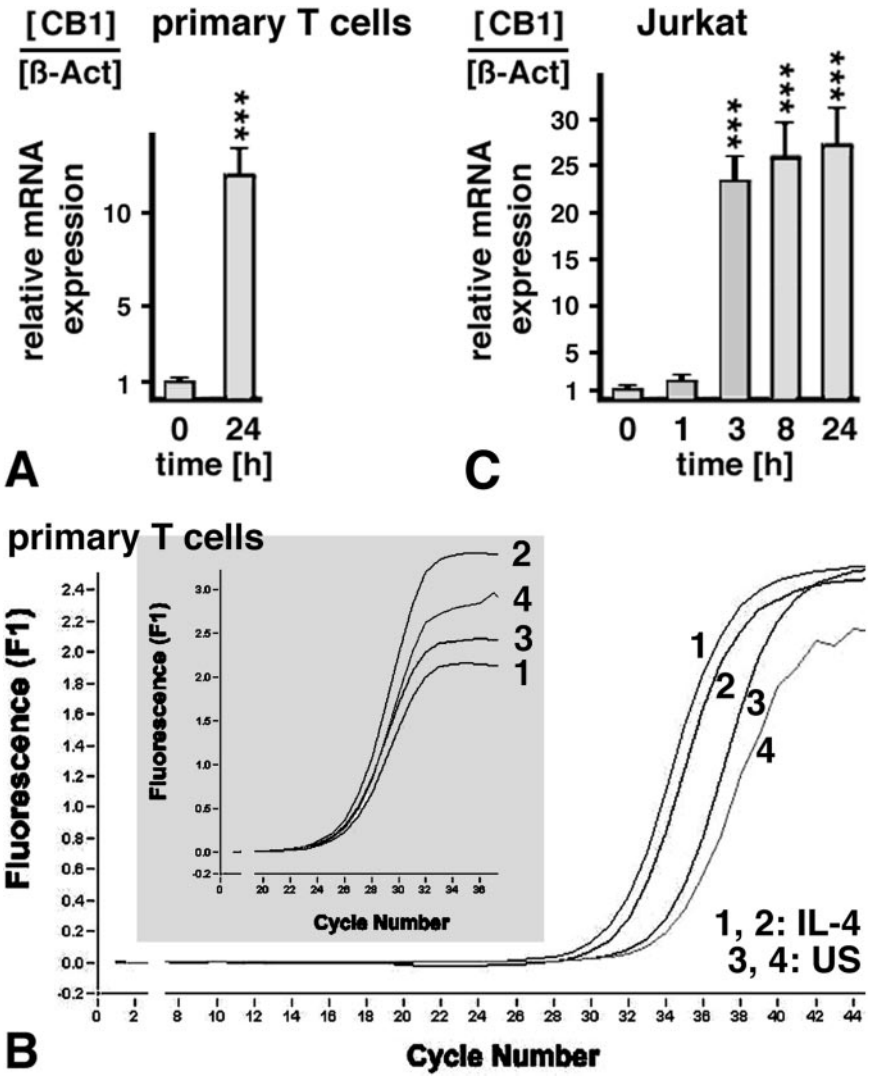


Fig. 2. Induction of CB1 transcription in T lymphocytes in response to IL-4. A and B, CB1 induction in primary human T cells. Cells were stimulated with IL-4 (5 ng/ml) for 24 h, and CB1-specific transcripts were determined relative to those of the housekeeping gene β-actin by quantitative real-time PCR. B, example of a representative experiment. The higher amount of transcripts in IL-4-treated cells is seen by the left-shifted curves for these samples (crossing points for sample number 1, 31.02; 2, 31.61) compared with the controls (crossing points 3, 33.82; 4, 34.30). The PCR for β-actin for the same samples is shown in the shaded box (crossing points 1, 26.14; 2, 25.81; 3, 26.01; 4, 26.02). US, unstimulated samples. C, time course for the induction of CB1 in Jurkat cells. Cells were stimulated with IL-4 for the indicated times and CB1-specific transcripts were determined by quantitative real-time PCR. A and C, 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 (***, $p < 0.001$).

promoter deletions were tested next. An increase in the reporter gene activity was observed after the deletion of promoter sequences from −3086 to −2490, indicating that these sequences contain *cis*-active, negative regulatory elements. The deletion of sequences between nts −1950 and −1653 further increased reporter gene activity, indicating additional negative elements located in these sequences. Little effect on promoter activity was observed after sequential deletion of sequences up to nt −648. The sequences between nts −648 and −559 are likely to contain elements, which act as enhancers in Jurkat cells, because deletion of them resulted in decreased reporter gene activity. Further deletion (construct −223) had little effect on reporter gene activity.

Induction of CB1 Transcription in T Lymphocytes in Response to IL-4. An earlier investigation from our group demonstrated up-regulation of CB1 in T cells in response to cannabinoids, which is mediated by IL-4 (Börner et al., 2007). Here, we investigated the direct effect of IL-4 on the transcription of the human CB1 gene. IL-4 induced a significant increase in the rate of transcription of the gene in primary T cells from peripheral human blood (Fig. 2, A and B) and in Jurkat T cells (Fig. 2C). Experiments in Jurkat cells additionally revealed that the transcriptional induction is almost complete after 3 h of IL-4 stimulation.

Transcriptional Induction of CB1 in Response to IL-4 Is Mediated by the Transcription Factor STAT6 but Not GATA3. The next question to address was which transcription factor mediated the induction of the gene by IL-4. In T cells, most transcriptional effects of IL-4 are mediated by the transcription factors STAT6 and GATA3. Therefore, transcription factor decoy oligonucleotides against these factors were introduced into Jurkat cells before the stimulation with IL-4. In the cells, the stimulus-induced transcription factors then bind to the excess of decoy oligonucleotides rather than to the gene's regulatory elements, which results in attenuation of the induction of the gene. The specificity of the decoy oligonucleotides for the desired transcription factors and that of the respective mutated negative control oligonucleotides had been shown in previous reports from our group (Börner et al., 2006, 2007). Decoy oligonucleotides directed against STAT6 completely abolished IL-4-mediated induction of CB1 (Fig. 3), indicating that this factor plays a pivotal role in the induction of CB1 by this cytokine. Control decoy oligonucleotides, in which the STAT6 binding site was destroyed (nSTAT6), had no effect on the induction. In contrast to STAT6, decoy oligonucleotides directed against GATA3 had no effect on the induction of the gene, indicating that GATA3 is not involved in up-regulation of the gene by IL-4.

Characterization of a STAT6 Element on the CB1 Gene Promoter. To characterize IL-4-responsive sequences on the promoter of the human CB1 gene, reporter gene assays in transiently transfected Jurkat cells were performed. A significant inducibility with IL-4 was revealed for construct phCB1-CAT-3086, which comprises 3086 bp of sequences flanking 5′ to exon 1 of the CB1 gene (Fig. 4). The loss of inducibility after deletion of 5′ sequences up to nt −2490 demonstrated that the IL-4-responsive sequences are located between nts −3086 and −2490 and indicated that these sequences contain one or more STAT6 binding sites. Sequence comparisons within this region suggested two possible STAT6 elements. First, a tandem repeat of the 5′-TTC . . . GAA-3′ motif, which is common to the binding sequences for all members of the STAT family (S1 sequence; Fig. 5A); second, an eight base pair motif, similar to a STAT6 binding sequence defined previously (S2 sequence; Fig. 5A) (Kraus et al., 2003b). Therefore, we next tested whether sequences comprising these motifs were sufficient to confer inducibility with IL-4 on the heterologous tk promoter of pBLCAT2. As shown in Fig. 4, only S2 conferred inducibility with IL-4. A mutated form of this element (muS2 sequence; Fig. 5A) used as a control, in which the binding site for STAT6 was destroyed, and S1 were not responsive to IL-4. Next, it was tested whether S2, when used as a decoy oligonucleotide, would inhibit the IL-4-mediated up-regulation of the gene in Jurkat cells (Fig. 5A). Indeed, S2 decoy oligonucleotides completely abolished the IL-4-inducible transcription of the gene, whereas S1 and muS2 did not, confirming that S2 is the STAT6 site of the CB1 gene. In addition, we tested wether S2 decoy oligonucleotides had any effect on

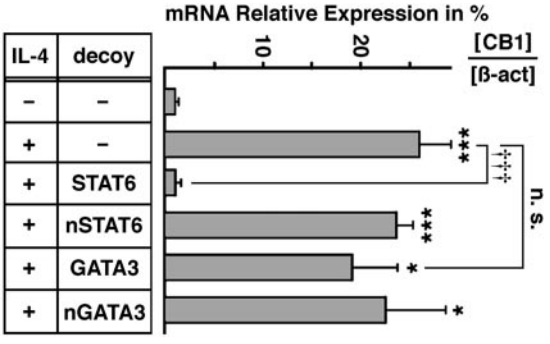


Fig. 3. Transcriptional induction of CB1 in Jurkat cells in response to IL-4 is mediated by the transcription factor STAT6 but not GATA3. The effects of decoy oligonucleotides with classic binding sites for STAT6 and GATA3 or mutations, which do not bind the factors (nSTAT6, nGATA3), on the induction of the gene in response to IL-4 were tested. Cells were incubated without or with decoy oligonucleotides (160 nM), as indicated for 16 h. After this time, IL-4 was added at 5 ng/ml as indicated. After another 8 h, cells were lysed and then subjected to quantitative real-time PCR. At least three independent experiments were performed in triplicate. Asterisks indicate significantly different values compared with non–IL-4-stimulated cells. Secondary comparisons are indicated by brackets (*, $p < 0.05$; ***, †††, $p < 0.001$; n.s., not significant).

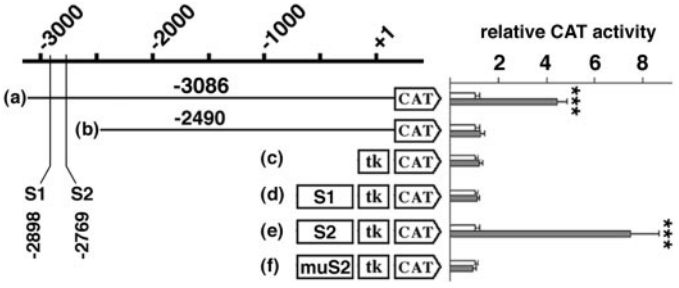


Fig. 4. Characterization of a STAT6 element on the CB1 gene promoter. According to sequence analysis, the promoter of the human gene contains two possible STAT6 sites, termed S1 and S2. Jurkat cells were transiently transfected with CAT reporter gene constructs. Their inducibility in response to IL-4 (5 ng/ml) is depicted on the right side. a and b, two constructs containing 3086 and 2490 bp of the CB1 promoter. c, the construction vector pBLCAT2 with the herpes simplex thymidine kinase minimal promoter (tk) instead of CB1 promoter sequences. d to f, constructs with CB1 sequences encompassing S1, S2, and a mutated sequence of S2, muS2. For the sequences of S1, S2, and muS2, see Fig. 5. At least three independent experiments, each in triplicate, were performed. For significant differences, IL-4-stimulated samples (dark bars) were compared with unstimulated controls (light bars; ***, $p < 0.001$).

basal transcriptional activity (Fig. 5B). Therefore, construct −3086 was transiently expressed in Jurkat cells in the presence of decoy oligonucleotides. However, neither S2 nor muS2 decoy oligonucleotides had any effect on basal activity of this construct, indicating that STAT6 is not involved in the regulation of basal CB1 transcription.

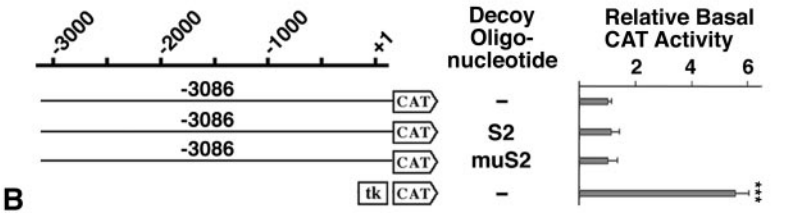
Inhibition of cAMP Accumulation. The above data show the induction of CB1 at the level of RNA. Next, we addressed the question of whether there was also an increase in the amount of functional CB1 receptor protein after stimulation of Jurkat cells with IL-4. It is known that short-term binding of cannabinoids to their receptors causes inhibition of forskolin-induced cAMP accumulation via coupling to G_i proteins (Felder et al., 1995). First, the kinetics of induction of functional receptors was studied (Fig. 6A). In Jurkat cells prestimulated with IL-4 for 1 and 2 days, *R*(+)-methanandamide, which preferentially binds to CB1 receptors, caused no inhibition of forskolin-induced cAMP accumulation. In contrast, prestimulation of cells with IL-4 for 3 and 4 days caused a significant inhibition of forskolin-induced cAMP accumulation by *R*(+)-methanandamide. This effect was blocked by the CB1-selective antagonists AM251 and AM281, indicating that it is mediated by CB1. Furthermore, the effect in cells stimulated for 4 days was dose-dependent (Fig. 6B). In contrast, in naive, non–IL-4-prestimulated cells, *R*(+)-methanandamide only caused an inhibition of forskolin-induced cAMP formation at the highest concentration tested (Fig. 6B). Although *R*(+)-methanandamide preferentially binds to CB1 receptors at 500 nM, a possible activation of endogenous CB2 receptors by *R*(+)-methanandamide was excluded by the addition of the CB2-specific antagonist AM630 (500 nM) to all samples of the experiments shown in Fig. 6. Taken together, these data indicate that the IL-4-prestimulated cells contain significantly more functionally expressed CB1 receptor protein than unstimulated cells.

Discussion

In this study, we characterized the transcriptional induction of the human CB1 gene in T lymphocytes in response to IL-4 and defined a STAT6 binding element on the gene's promoter as the structural requirement for this type of regulation. In addition, an IL-4-mediated increase in functional CB1 receptors was demonstrated. Furthermore, basal transcription of the gene was investigated in Jurkat cells, which serve as a model for T lymphocytes. In the Jurkat cells, two CB1 promoter regions had marked inhibitory input on the gene's basal transcription (spanning from nts −3086 to −2490 and nts −1950 to −1653), whereas the region spanning from nts −648 to −559 rather enhanced transcription (see Fig. 1). It is interesting that a different pattern of basal CB1 promoter activity has been observed in NG108-15 and N1E-115 cells, serving as neuronal model cells expressing the CB1 gene. In the neuronal cells, regions with negative effects on basal transcription were located roughly between −2 and −1 kb, whereas regions upstream of −2 kb and regions downstream of −1 kb enhanced basal transcription (Zhang et al., 2004). Thus, at least the distal promoter sequences located upstream of approximately −2 kb had contrary effects on basal transcription of the CB1 gene in the immune and the neuronal cells, indicating different control mechanisms in cell types derived from different tissue. In general, the basal expression of CB1 is much weaker in immune cells compared with neuronal cells. This situation is well reflected by the CB1 promoter activity. Thus, basal transcriptional activity of the CB1 promoter in T lymphocytes was lower than that of plasmid pBLCAT2, which lacks additional enhancer elements, whereas the activities in the neuronal cells were reported to be similar to a highly active, SV40 enhancer-driven reporter gene (Zhang et al., 2004). Although the two different sets of experiments may not directly be comparable because of the use of different control-reporter genes, they reflect the much weaker expression rate of the gene in T cells compared with distinct neuronal cells. The silencing effects of the distal promoter regions defined in the experiments presented here may additionally contribute to the weak expression of the gene in T cells. Several transcriptional start sites for the CB1 gene have been reported, and other promoter regions upstream of other exons have been discussed (Zhang et al., 2004). Nevertheless, the marked promoter activity of the sequences flanking exon 1, in both neuronal and T cells, supports the hypothesis that these sequences constitute the main promoter of the gene. The hypothesis is further sup-

Decoy Oligonucleotide		Relative Inducibility of human CB1 mRNA	
		−	↗ + IL-4
–	–	1.0 ± .1	25.8 ± 3.3***
5'-AATTTCATAGAATTCAGTGAAGAAAC-3'	S1	1.0 ± .2	32.5 ± 5.8**
5'-AAGAT TTCCATGGAA TACCT-3'	S2	1.0 ± .1	.8 ± .1
5'-AAGAT GTCGATGGAG TACCT-3'	muS2	1.0 ± .1	21.7 ± 3.2***

A



B

Fig. 5. Decoy oligonucleotides containing the putative CB1 STAT6 sequence S2 attenuate induction of the CB1 gene in response to IL-4 but do not influence basal transcription. A, effect of decoy oligonucleotides on IL-4-inducible transcription. Jurkat cells were preincubated with the indicated decoy oligonucleotides for 16 h and then stimulated with IL-4 (5 ng/ml, 8 h) in the presence of the decoy oligonucleotides. Their effects on IL-4 inducibility of the CB1 mRNA relative to β-actin was revealed by quantitative real-time PCR. In S1, the tandem repeat of the 5′-TTC. . . GAA-3′ motif is underlined. In S2, the STAT6 motif is boxed. MuS2 contains three mutations, which destroy the STAT6 motif. At least three independent experiments, each in duplicate, were performed. IL-4-stimulated samples were compared with unstimulated controls (**, $p < 0.01$; ***, $p < 0.001$). B, effect of decoy oligonucleotides on basal transcription. CAT reporter gene activities plus S.E.M. of at least two independent experiments performed in triplicate obtained after transient transfection of Jurkat cells are displayed. As indicated, some samples were performed in the presence of decoy oligonucleotides added 1 day before transfection until lysis of cells. Samples were compared with the activity of the construct −3086 without decoys (***, $p < 0.001$).

ported by our findings that these sequences contain a typical promoter element, which is the binding site for STAT6, conferring responsiveness of the gene to IL-4. It is interesting that the IL-4-responsive STAT6 site of the gene is located within a region that silences basal transcription of the gene. However, as indicated in the experiments, in which basal activity of this region was tested in the presence of STAT6 decoy oligonucleotides (see Fig. 5B), STAT6 seems not to influence basal transcription. In general, promoters seem to consist of a marked number of *cis*-active modules, to which specialized *trans*-acting factors bind. Some factors mediate stimulus-induced transcription, whereas others regulate basal transcription. Unless a cell is not subjected to a constant level of a certain stimulus, inducible factors contribute little to basal transcription of a gene. STAT factors are specialized factors that respond to cytokines and growth factors. Most transcriptional effects of IL-4 are mediated by the transcription factors STAT6 and GATA3 (Hou et al., 1994; Kraus et al., 2003b; Börner et al., 2006). Experiments using decoy oligonucleotides excluded the involvement of GATA3 in the IL-4-mediated up-regulation of CB1 but clearly established the role of STAT6 (Fig. 3). Once activated by Jak kinases, STAT6 dimers bind to defined regulatory sequences and enhance transcription of the target gene (Hou et al., 1994). Because of the close similarity of the binding sequences for all STAT members, thus far, the decoy oligonucleotide approach, with the oligonucleotides acting in living, intact cells, has proven to be the most selective method for studying DNA-STAT interactions. In contrast, classic in vitro binding assays such as electrophoretic mobility shift assays often gave unspecific results (Kraus et al., 2003b). The relevant regulatory sequence of the CB1 gene promoter, termed S2 in this study, is located at nt −2769. It consists of an extended, eight base pair palindrome and thus sticks to the rule defined previously for STAT6 binding sequences (Fig. 5A; Kraus et al., 2003b). In addition to transcriptional induction of the CB1 gene by IL-4, an increase in functional receptors in response to IL-4 stimulation of Jurkat cells was demonstrated. It was astonishing to find that although IL-4 caused an increase in CB1 mRNA after 3 h, an increase in functional CB1 protein was only observed after 3 days. This is probably due to an overall small amount of CB1 receptor protein in the T cells or to post-translational effects needed for full receptor activity. From a physiological standpoint, CB1 receptors in T cells may participate in the regulation of the T-helper cell balance. It is known that cannabinoids alter this balance in favor of the type 2 phenotype. Among other mechanisms, such as the inhibition of the expression of the T helper cell type 1 cytokine interferon-γ, cannabinoids lead to the induction of IL-4, which is a key factor for the T-helper cell type 2 differentiation and its maintenance (Curfs et al., 1997; O'Garra and Arai, 2000). This cannabinoid-induced induction of IL-4 is mediated by CB2 receptors, which are abundantly expressed in resting T lymphocytes (Börner et al., 2006). IL-4 then in turn induces genes, which further promote T-helper cell type 2 development and, as reported now, the CB1 receptor gene. CB1 additionally participates in T-helper cell biasing. It was shown that cannabinoids, via CB1 receptors, inhibit the expression of IL-12Rβ2, which is the binding and signal-transducing component of the IL-12 receptor complex, which is necessary for T-helper cell type 1 development (Klein et al., 2000, 2004). There is an increasing awareness that cannabinoids, via CB1 receptors, not only regulate neuronal functions but also potently modulate immune functions. Furthermore, being expressed on cells of the nervous and the immune system, CB1 receptors enable communication between the two systems and allow neuroimmune interactions. The extent of the CB1-mediated cross-talk may be regulated at the level of gene expression (e.g., by IL-4).

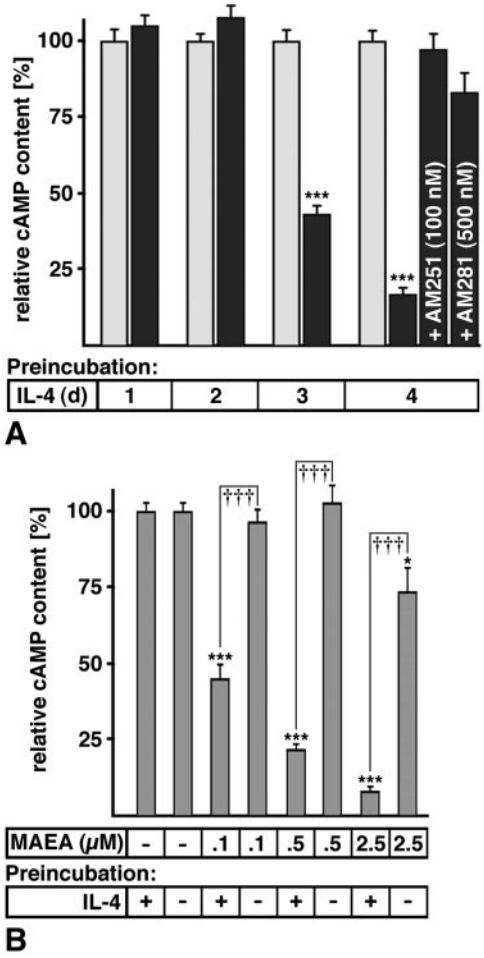


Fig. 6. Inhibition of forskolin-induced cAMP accumulation demonstrates functional CB1 receptors. A, time kinetics of receptor induction. As indicated, Jurkat cells were preincubated with IL-4 (5 ng/ml) for 1 to 4 days to up-regulate CB1 protein expression. The effect of the CB1-selective agonist *R*(+)-methanandamide (MAEA, 500 nM, black bars) on the forskolin-induced cAMP formation is shown compared with controls treated with forskolin alone (light gray bars). On day 4, the effect was additionally assayed in the presence of the CB1 antagonists AM251 and AM281. A possible activation of endogenous CB2 receptors by MAEA was excluded by the addition of the CB2-specific antagonist AM630 (500 nM) to all samples. The mean of at least two experiments performed in quadruplicate ± S.E.M. is depicted. For each time-point of prestimulation, MAEA-treated samples were compared with the respective controls (***, $p < 0.001$). B, effect of different concentrations of MAEA on the forskolin-induced cAMP formation in unstimulated Jurkat cells and cells prestimulated with IL-4 for 4 days. A possible activation of endogenous CB2 receptors by MAEA was excluded by the addition of the CB2-specific antagonist AM630 (500 nM) to all samples. The mean of at least two experiments performed in quadruplicate ± S.E.M. is depicted. Samples are compared with MAEA-unstimulated, IL-4-pretreated samples. Secondary comparisons are indicated by brackets (*, $p < 0.05$; ***, †††, $p < 0.001$).

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