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Cannabinoid Inhibition of Adenylate Cyclase-mediated Signal Transduction and Interleukin 2 (IL-2) Expression in the Murine T-cell Line, EL4.IL-2*

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Cannabinoid receptors negatively regulate adenylate cyclase through a pertussis toxin-sensitive GTP-binding protein. In the present studies, signaling via the adenylate cyclase/cAMP pathway was investigated in the murine thymoma-derived T-cell line, EL4.IL-2. Northern analysis of EL4.IL-2 cells identified the presence of 4-kilobase CB2 but not CB1 receptor-subtype mRNA transcripts. Southern analysis of genomic DNA digests for the CB2 receptor demonstrated identical banding patterns for EL4.IL-2 cells and mouse-derived DNA, both of which were dissimilar to DNA isolated from rat. Treatment of EL4.IL-2 cells with either cannabinol or Δ^9-THC disrupted the adenylate cyclase signaling cascade by inhibiting forskolin-stimulated cAMP accumulation which consequently led to a decrease in protein kinase A activity and the binding of transcription factors to a CRE consensus sequence. Likewise, an inhibition of phorbol 12-myristate 13-acetate (PMA)/ionomycin-induced interleukin 2 (IL-2) protein secretion, which correlated to decreased IL-2 gene transcription, was induced by both cannabinol and Δ^9-THC. Further, cannabinoid treatment also decreased PMA/ionomycin-induced nuclear factor binding to the AP-1 proximal site of the IL-2 promoter. Conversely, forskolin enhanced PMA/ionomycin-induced AP-1 binding. These findings suggest that inhibition of signal transduction via the adenylate cyclase/cAMP pathway induces T-cell dysfunction which leads to a diminution in IL-2 gene transcription.

Cannabinoid compounds mediate many if not most of their actions on the immune and central nervous system through interactions with cannabinoid receptors. Their functional expression and involvement in mediating cannabinoid-associated effects has been demonstrated on a variety of cell types and is supported by various distinct lines of evidence. The most compelling include stereospecific activity (1, 2), inhibition of adenylate cyclase activity (3–5), high affinity specific binding which reaches saturation (2, 6–8), the cloning of cannabinoid receptor genes CB1[1] (7), CB1A (9), and CB2 (10), and the general lack of cannabinoid activity in CHO cells, a cell line lacking these receptors (7). However, transfection of either the CB1 or CB2 gene into CHO cells confers sensitivity of this cell line to adenylate cyclase inhibition which correlates with the binding affinity of the respective cannabinoid ligands (11, 12).

Two major types of cannabinoid receptors have thus far been identified. CB1 is expressed in greatest abundance in the brain (8, 13), but can also be found at low levels in peripheral tissues, notably in spleen (2). CB2, to date, has only been identified within the immune system (10).[2] Human CB2 shares approximately 44% identity with the human CB1 receptor which increases to 68% identity when comparing only the transmembrane domains that make up the receptor binding pocket (10). In addition, a variant of CB1 has recently been identified, CB1A, which is a truncated form of CB1, lacking 167 bp that code for the N terminus of the extracellular receptor domain (9).

It is well established that cannabinoids produce inhibitory effects on immunologic responses (14, 15). A strong correlation exists between the magnitude of inhibition by cannabinoids of adenylate cyclase activity, their binding affinity, and their respective potency in suppressing certain immune function responses (16).[2] One of the most sensitive immune responses to inhibition by cannabinoids that we have thus far identified is the IgM antibody-forming cell response to the T-cell dependent antigen, sheep erythrocytes (2, 17). This response is dependent on macrophages for antigen recognition, processing, and presentation, and on the activation of T-cells for the secretion of essential lymphokines required in B-cell growth and differentiation (18, 19). Interestingly, primary antibody-forming cell responses to T-cell independent antigens, either 2,4-dinitrophenol-Ficoll which requires macrophages as accessory cells (20, 21), or to lipopolysaccharide, a polyclonal B-cell activator with no requirements for accessory cell help (22–24), are refractory to inhibition by cannabinoids. The aforementioned profile of cannabinoid activity (*i.e.* sensitivity of T-cell-dependent, but not T-cell independent, humoral immune responses by cannabinoids) has been demonstrated with Δ^9-THC both *in vivo* and *in vitro* (17). Proliferative responses to T-cell specific mitogens and to allogeneic class II histocompatibility antigens are also inhibited by cannabinoids (17). Although these findings strongly suggest that the T-cell is a sensitive target for

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[1] The abbreviations used are: CB, cannabinoid receptor; Δ^9-THC, Δ-9-tetrahydrocannabinol; G protein, guanine nucleotide-binding protein; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin 2; bp, base pair(s); kb, kilobase(s); rc, recombinant; RT-PCR, reverse transcription-polymerase chain reaction; IS, internal standard; DTT, dithiothreitol; PKA, protein kinase A; CRE, cAMP response element; ELISA, enzyme-linked immunosorbent assay; CREB, cAMP response element-binding protein; ATF, activating transcription factor; PKI, protein kinase A inhibitor.

[2] A. R. Schatz, M. Lee, R. Condie, and N. E. Kaminski, submitted for publication.

inhibition by cannabinoids, it is notable that B-cells also exhibit adenylate cyclase inhibition following cannabinoid treatment which is indicative that this cell-type also expresses functional cannabinoid receptors.[2] The lack of an effect by cannabinoids on humoral immune responses to T-cell independent antigens suggests that the adenylate cyclase/cAMP signaling pathway is not critical for immunoglobulin production by B-cells. The involvement of adenylate cyclase inhibition in immune dysfunction is supported by the observation that membrane-permeable cAMP analogs (dibutyryl-cAMP and 8-bromo-cAMP) reverse Δ^9-THC-mediated suppression of both the sRBC antibody-forming cell response and lymphoproliferation following PMA plus ionomycin stimulation (16). Treatment of splenocytes with pertussis toxin also abrogates Δ^9-THC inhibition of the sRBC antibody-forming cell response and the inhibition of adenylate cyclase activity (16).

Another immunological response restricted to T-cells that cannabinoids modulate is the production of interleukin-2 (IL-2). In both spleen and lymph node cells of adult mice, Δ^9-THC has been shown to inhibit IL-2 production (25). IL-2 is an essential cytokine in stimulating both humoral and cell-mediated immune responses whose transcription is strictly regulated at two levels, cell-type specificity and activating stimuli. Helper T-cells produce IL-2 in response to antigen recognition by signaling through the T-cell receptor. The EL4.IL-2 thymoma, an IL-2-producing T-cell line which can be readily induced to express IL-2 synchronously in 70–80% of the cells in a given culture (26), is a widely used model for studying IL-2 regulation. When stimulated with phorbol ester and calcium ionophore, EL4.IL-2 cells show many of the same gene induction events exhibited by antigen-stimulated T-helper cells (26, 27). Consequently, the focus of the present studies was to investigate the effects of cannabinoids on signal transduction through the adenylate cyclase/cAMP pathway in T-cells and on IL-2 expression using EL4.IL-2 cells.

MATERIALS AND METHODS

Chemicals—Δ^9-THC and cannabinol were provided by the National Institute on Drug Abuse.

Animals and Cell Line—Virus-free female B6C3F1 mice, 4 or 6 weeks, were purchased and cared for as described previously (2). The C57BL/6 mouse lymphoma, EL4.IL-2, was obtained from ATCC (TIB 181) and cultured in RPMI 1640 medium supplemented with 100 units of penicillin/ml, 100 units of streptomyocin/ml, 2 mM L-glutamine (Life Technologies, Inc.), 50 μM 2-mercaptoethanol, and 10% bovine calf serum (Hyclone, Logan, UT).

Probes—Both the 1.1-kb human CB2 (GenBank™ accession number X74328) and 1.4-kb human CB1 (X54937) probes were cut out of pBluescriptII SK+ vectors (generous gifts from Merck Frosst Canada, Inc.), using *Eco*RI and *Hin*dIII. Only the translated portion of both cDNA genes was used in probing either Southern or Northern blots. The CB2 probe consists of base pairs 126 to 1209, and the CB1 probe consists of base pairs 148 to 1564. The 1.1-kb mouse IL-2 (*Xho*I) probe, cloned by Yokota *et al.* (28), contains the complete IL-2 coding sequence and was obtained from ATCC (39892). All probes were randomly primed using [^{32}P]dATP (29).

Northern Blot Analysis—Total RNA was isolated using either Tri Reagent (Molecular Research Center, Cincinnati, OH) or the guanidium isothiocyanate method of Chomczynski and Sacchi (30). Poly(A) RNA was purified by oligo(dT)-cellulose chromatography (Life Technologies, Inc.) (31). Total RNA or mRNA was fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membrane (Amersham), and cross-linked to the membrane using the UV Stratalinker 1800 (Stratagene). Blots were hybridized overnight at 42 °C in 50% formamide, 5 × SSPE, 10 × Denhardt's, 2% SDS, 7% dextran sulfate, yeast tRNA at 130 μg/ml, then washed twice for 5 min in 2 × SSPE, 0.5% SDS, twice for 15 min in 1 × SSPE, 1% SDS, and twice for 15 min in 0.1 × SSPE, 0.1% SDS if needed, and exposed to DuPont film at −80 °C in the presence of intensifying screens.

Southern Blot Analysis—Genomic DNA was isolated from mouse tail, rat tail, or EL4.IL-2 cells by incubating overnight at 55 °C in 0.5% SDS, 400 mM NaCl, 20 mM Tris, pH 7.6, and 2 mM EDTA, pH 8.0, with 0.5 mg/ml Proteinase K. DNA was extracted twice with phenol:chloroform and then ethanol-precipitated. Approximately 10 μg of genomic DNA was incubated at 37 °C overnight with the appropriate restriction enzyme, separated on a 0.8% agarose gel in 1 × TAE, blotted onto nylon membrane (Amersham), and UV-cross-linked. Prior to blotting, gels were denatured for 40 min in 1.5 M NaCl, 0.5 M NaOH and then neutralized for 40 min in 1.5 M NaCl, 0.5 M Tris, pH 7.4. Southern blots were prehybridized at 68 °C in 6.25 × SSPE, 7% SDS, 20 × Denhardt's, and 0.2 mg/ml yeast tRNA. Overnight hybridization included the addition of 6 ml of 50% dextran sulfate as well as the probe. Blots were washed and exposed to film as stated above.

Preparation of Internal Standard for RT-PCR—An artificial or recombinant mRNA (rcRNA) was used for an internal standard (IS) containing specific PCR primer sequences for IL-2 that were added to RNA samples in a dilution series. A rat β-globin sequence was used as the spacer gene for the IL-2 IS. This method, developed by Vanden Heuvel (32, 33), avoids sample-to-sample variation of reference gene expression (*e.g.* β-actin) as well as gene-to-gene differences in amplification efficiency. The primer sequences from 5′ to 3′ for IL-2 are: forward primer = TGCTCCTTGTCAACAGCG, and reverse primer = TCATCATCGAATTGGCACTC. The IS primer design from 5′ to 3′ is as follows: IS forward primer = T7 promoter (TAATACGACTCACTATAGG), IL-2 forward primer (as stated above), and rat β-globin forward primer (GGTGCTTGGAGACAGAGGTC); and IS reverse primer = $(dT)_{18}$, IL-2 reverse primer (as stated above), and rat β-globin reverse primer (TCCTGTCAACAATCCACAGG). PCR reaction conditions for making the internal standard were performed as stated previously using 100 ng of rat genomic DNA (32). PCR-amplified products were purified using the Wizard PCR Prep DNA purification system (Promega) and transcribed into RNA using Promega's Gemini II *In Vitro* Transcription System. The rcRNA was subsequently treated with RNase-free DNase to remove the DNA template. After quantitating, the following calculations were performed in order to calculate the molecules/μl of internal standard (330 × bp is an approximation for the molecular weight of the internal standard): (((μg/μl RNA (from 260 nm reading))/(330 μg/μmol/bp × bp IS)) × 6.02 × 10^{17} molecules/μmol.

Quantitative Competitive RT-PCR—RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) as described by Chomczynski (34) and Chomczynski and Mackey (35). In order to avoid any DNA contamination, RNA samples were incubated with RNase-free DNase for 15 min at 37 °C in 10 mM $MgCl_2$, 1 mM DTT, 25 units of RNasin, 10 mM Tris, 1 mM EDTA, phenol:chloroform-extracted, and precipitated in isopropyl alcohol. Competitive RT-PCR was performed as outlined in Gilliland *et al.* (36, 37), except that rcRNA was used as an internal standard (IS) instead of genomic DNA with 8 aliquots of rcRNA IS from 10^2 to 10^9 molecules made for each RNA treatment group. Briefly, total RNA and IS rcRNA of known amounts were reverse-transcribed into cDNA using oligo$(dT)_{15}$ as primers. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol each of IL-2 forward and reverse primers, and 2.5 units of *Taq* DNA polymerase was added to the cDNA samples. Samples were then heated to 94 °C for 4 min and cycled 30 times at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s after which an additional extension step at 72 °C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. The IL-2 primers produce a 391-bp amplified product from the cellular RNA and a 474-bp product from the IS rcRNA. Quantitation was performed using the Gel Doc 1000 (Bio-Rad) where the amount of IL-2 mRNA present is determined as described by Gilliland *et al.* (36) Briefly, the ratio of the volume of the IS rcRNA to IL-2 RNA bands is plotted against the amount of IS rcRNA (in molecules) added to each reaction. The point at which the ratio of IS (rcRNA) to IL-2 mRNA is equal to 1 signifies the "cross-over" point which represents the amount of IL-2 molecules present in the initial RNA sample. After performing the 10^2 to 10^9 range-finding experiment, a second set of much narrower internal standard dilutions (*i.e.* 10^5 to 10^8 molecules/tube) were examined in order to quantitate RNA message levels more accurately.

cAMP Determinations—EL4.IL-2 cells were washed with RPMI 1640, resuspended in RPMI 1640 with 1 mg/ml fatty acid-poor bovine serum albumin (Calbiochem), and adjusted to 5 × 10^5 cells/ml. 500-μl aliquots of cells were treated with vehicle (0.1% ethanol), Δ^9-THC (3.2–32 μM), or cannabinol (3.2–32 μM) and incubated for 10 min at room temperature. The appropriate cell preparations were then stimulated with 50 μM forskolin (Sigma) for 15 min at 37 °C in 5% CO_2 or were treated in combination with PMA (80 nM)/ionomycin (1 μM) plus 5 μM forskolin for 5 min. The extraction, release, and quantitation of cAMP from cells were performed as described previously (16).

Analysis of PKA Activity—EL4.IL-2 cells were washed in Earle's

balanced salt solution and lysed in ice-cold lysis buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT, 0.1% Triton X-100, and 10 μg/ml each of leupeptin and aprotinin) at 4 °C by gentle sonication in order to maintain the functional integrity of the cannabinoid receptor as it is anchored in the membrane (*i.e.* twice at 60 Hz for 5 s). Lysates were centrifuged at 270 × *g* for 2 min, and aliquots of the cell extract (supernatant) were incubated with appropriate concentrations of cannabinol or Δ^9-THC for 5 min in triplicate for use in the PKA assay (Life Technologies, Inc.). The reaction mixture of 40 μl contained 10 μl of cell extract, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP (20 μCi/ml γ-[^{32}P] ATP), 0.25 mg/ml bovine serum albumin, 50 μM Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide). The background level of each group was measured in the presence of 1 μM PKI-(6–22)-amide and total activity with 10 μM cAMP. Samples were incubated at 37 °C for 10 min. Phosphocellulose discs were then spotted with 20 μl of sample followed by two acid washes (1% (v/v) phosphoric acid) and two water washes. The amount of ^{32}P was quantified by scintillation counting.

Electromobility Shift Assay—A previously described method was used to extract nuclei for electrophoretic mobility shift assays (38). Briefly, treated and untreated EL4.IL-2 cells were lysed with HB buffer (10 mM HEPES, 1.5 mM $MgCl_2$), and nuclei were pelleted by centrifugation at 6700 × *g* for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) which contained 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and leupeptin after which the samples were centrifuged at 17,500 × *g* for 15 min, and the supernatant was retained. Nuclear extracts (3 μg) were incubated with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled CRE or AP-1 probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin and leupeptin) for 10 min on ice. DNA binding activity was separated from free probe using a 4% acrylamide gel in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried for 1.5 h and autoradiographed for analysis.

IL-2 ELISA Assay—EL4.IL-2 cells were washed in RPMI 1640 and resuspended in RPMI 1640 complete with 5% bovine calf serum at 5 × 10^5 cells/ml. Vehicle (0.1% ethanol), Δ^9-THC (3.2–32 μM), or cannabinol (3.2–32 μM) was added to appropriate flasks and incubated for 10 min at room temperature. Stimulation included the addition of PMA (80 nM) and ionomycin (1 μM) for 24 h at 37 °C in 5% CO_2 after which supernatants were collected and assayed in triplicate using a mouse interleukin-2 ELISA kit (Becton Dickinson Labware, Bedford, MA). The IL-2 sample levels were determined by comparison to a standard curve of recombinant mouse IL-2.

Statistical Analysis of Data—The mean ± S.E. was determined for each treatment group of a given experiment. When significant differences occurred, treatment groups were compared to the vehicle controls using a Dunnett's two-tailed *t* test (39).

RESULTS

Northern and Southern Blot Analysis of EL4.IL-2—In trying to find a suitable *in vitro* modeling system for CB2, the native expression of cannabinoid receptors and the similarity of EL4.IL-2 cells to primary lymphoid tissue was examined. First, when probed with CB2, Northern analysis of EL4.IL-2 poly(A) RNA expression revealed one band at ~4.0 kb which is similar to results seen in mouse splenic tissue (Fig. 1*A*). Interestingly, the mouse splenic and EL4.IL-2 mRNA band size also corresponds nicely to the cloning of a 3.7-kb full-length mouse CB2 cDNA sequence recently submitted to GenBank™ (accession number X86405) by Shire *et al.* In contrast to the peripheral cannabinoid receptor, CB1 expression was not detectable in EL4.IL-2 cells at the level of poly(A)-purified RNA (Fig. 1*B*) or by RT-PCR (data not shown). Positive controls of both neuronal and lymphoid poly(A) RNAs were included for CB1 and CB2 expression, and no cross-hybridization was seen between the probes. Second, Southern blots were probed with CB2 in order to confirm similarity between the EL4.IL-2 and the mouse (B6C3F1) genome. For all restriction enzymes used, both mouse and EL4.IL-2 genomic DNA demonstrated the same banding patterns. According to Shire's mouse CB2 cDNA sequence, one set of restriction enzymes cuts only once within the CB2 gene, namely *Bam*HI, *Eco*RI, and *Sty*I (Fig. 2*A*),

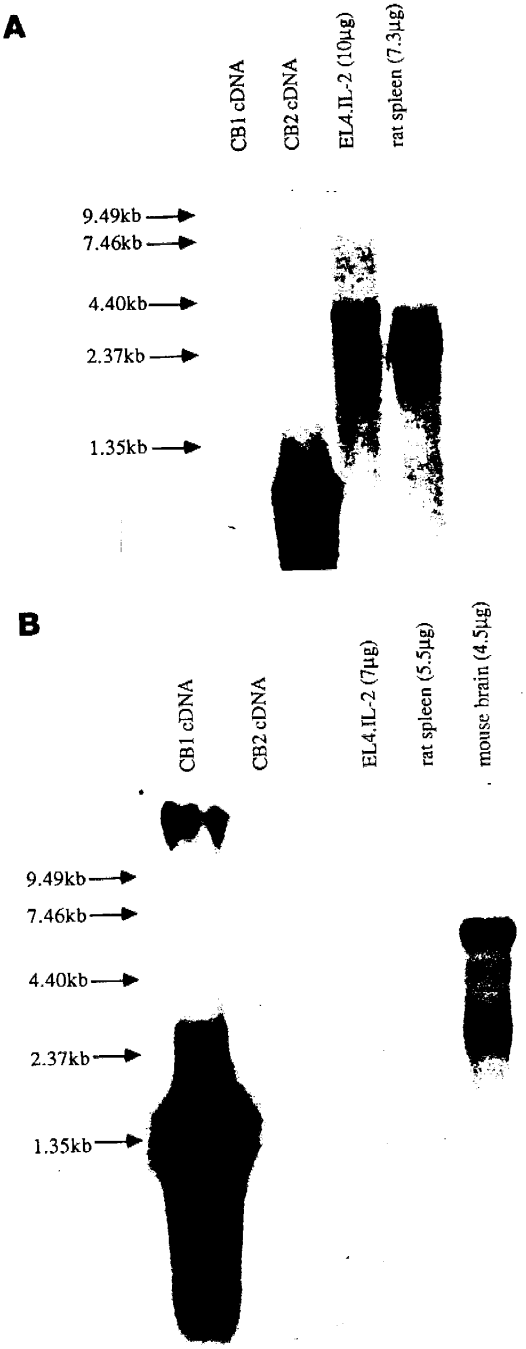

FIG. 1. **Northern blot analysis of CB2 and CB1 mRNA expression in EL4.IL-2 cells.** Poly(A) RNA was isolated from B6C3F1 mice, Sprague-Dawley rats, and EL4.IL-2 cells by the guanidium isothiocyanate method followed by oligo(dT)-cellulose chromatography. RNA samples were electrophoresed on a 1.2% formaldehyde-agarose gel, blotted onto nylon membrane, and hybridized to either a 1.1-kb human ^{32}P-labeled CB2 probe or a 1.4-kb human ^{32}P-labeled CB1 probe. For all blots, both CB1 and CB2 cDNA lanes were included to ensure that no cross-hybridization exists between the two probes. *A*, Northern blot analysis of EL4.IL-2 cells and rat spleen probed with CB2. *B*, Northern blot analysis of EL4.IL-2 cells, rat spleen, and mouse brain when probed with CB1. These results are representative of at least three individual blots.

and the other set of restriction enzymes cuts at least twice within the CB2 gene, namely *Ban*I, *Bst*XI, and *Sty*I (Fig. 2*B*). The expected sizes for mouse genomic DNA digested with *Ban*I, *Bst*XI, or *Sty*I restriction enzymes that cut twice within the CB2 gene are 824 bp, 775 bp, and 666 bp, respectively, which can be seen in the Southern blot. In addition, the Southern analysis banding patterns for the rat (Sprague-Dawley)

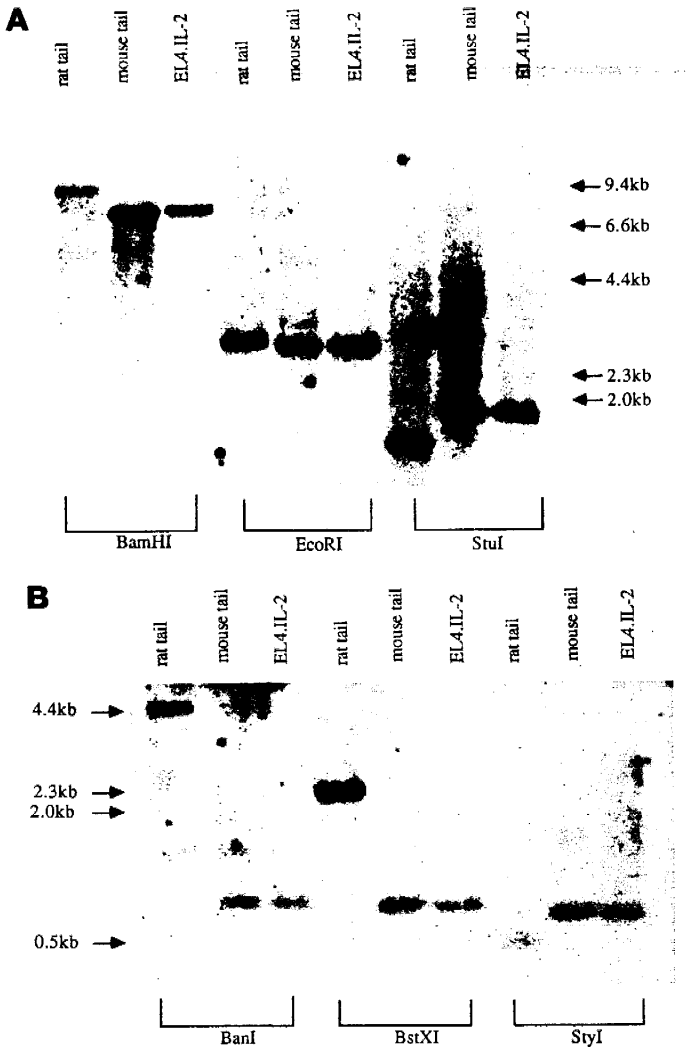

FIG. 2. **Southern blot analysis of genomic DNA from rat, mouse, and EL4.IL-2 cells.** Genomic DNA was isolated by phenol extraction/ethanol precipitation, digested with various restriction enzymes, electrophoresed on an 0.8% agarose gel, blotted onto a nylon membrane, and probed with a 1.1-kb human CB2 sequence. *A*, Southern analysis with restriction enzymes (*Bam*HI, *Eco*RI, *Stu*I) that cut only once within the CB2 gene. *B*, Southern analysis with restriction enzymes that cut at least twice within the CB2 gene. *Ban*I yields an expected 824-bp digest, *Bst*XI yields an expected 775-bp digest, and *Sty*I yields an expected 666-bp product. These Southern blots are representative of at least two separate experiments.

genomic DNA were different from mouse and EL4.IL-2 for almost every restriction enzyme used.

Effects of Δ^9-THC and Cannabinol on the Regulation of the cAMP Cascade—The effect of Δ^9-THC and cannabinol on forskolin-stimulated adenylate cyclase activity was measured in EL4.IL-2 cells (Fig. 3). Forskolin alone markedly activated adenylate cyclase and elevated cAMP levels at least 25-fold over naive levels in this murine lymphoma. Pretreatment with Δ^9-THC before forskolin stimulation significantly decreased cAMP levels by 20–35% over a dose range of 12–32 μM. Cannabinol also markedly inhibited adenylate cyclase activity by 17–52% over a dose range of 3.2–32 μM. Viability of EL4.IL-2 cells before and after forskolin stimulation was greater than 92% for all drug concentrations tested. Further characterization of the regulation of the cAMP cascade in EL4.IL-2 cells demonstrated that PMA/ionomycin treatment modestly enhances adenylate cyclase activity (Fig. 4) as previously shown in primary leukocyte preparations (16, 40). Interestingly, an increase in cAMP accumulation was measurable when cells

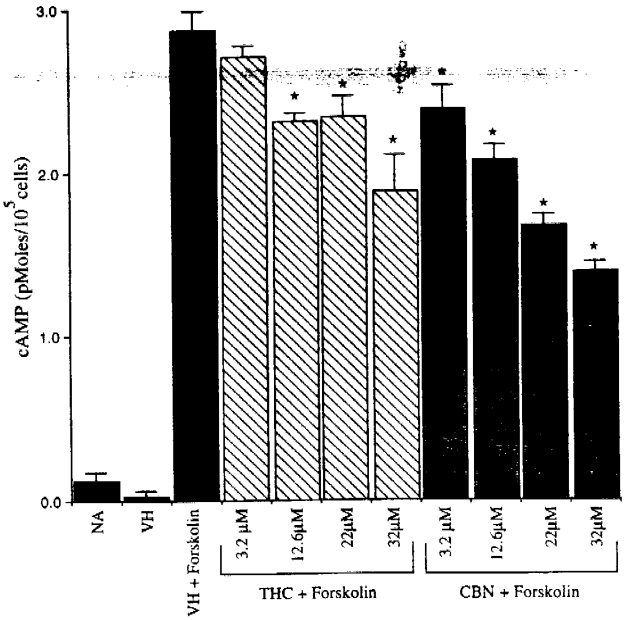

FIG. 3. **Effect of cannabinoids on forskolin-stimulated adenylate cyclase activity in EL4.IL-2 cells.** EL4.IL-2 cells at 5×10^5 cells/ml were incubated with either vehicle (1.0% ethanol), Δ^9-THC, or cannabinol for 10 min followed by a 15-min stimulation with forskolin (50 μM). Intracellular cAMP concentrations (picomoles) are expressed as the mean ± S.E. of triplicate determinations from one of at least three representative experiments. *, $p < 0.05$ as determined by Dunnett's t test as compared with the forskolin control group.

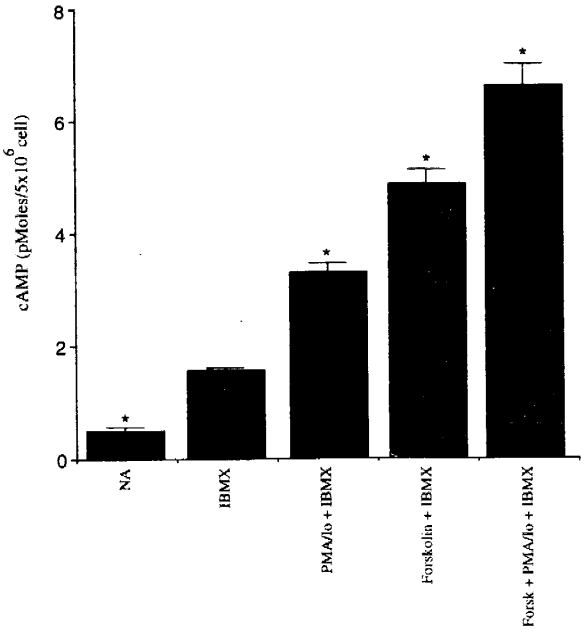

FIG. 4. **Enhancement of intracellular cAMP by PMA/ionomycin stimulation.** EL4.IL-2 cells were treated with isobutylmethylxanthine (100 μM), PMA (80 nM), ionomycin (1 μM), and/or forskolin (5 μM) for 5 min. Quadruplicate cell suspensions were used for each group. The *bars* represent the mean ± S.E. as determined for each group for one of three representative experiments. *, $p < 0.05$ as determined by Dunnett's t test as compared to the isobutylmethylxanthine control group.

were stimulated with either PMA/ionomycin alone or in combination with forskolin suggesting an additivity with the combined treatment (*i.e.* forskolin plus PMA/ionomycin *versus* forskolin alone). It is also notable that PMA alone but not ionomycin produced a modest enhancement in adenylate cyclase activity in EL4.IL-2 cells (data not shown). In following the cAMP cascade, cannabinoid treatment was also inhibitory

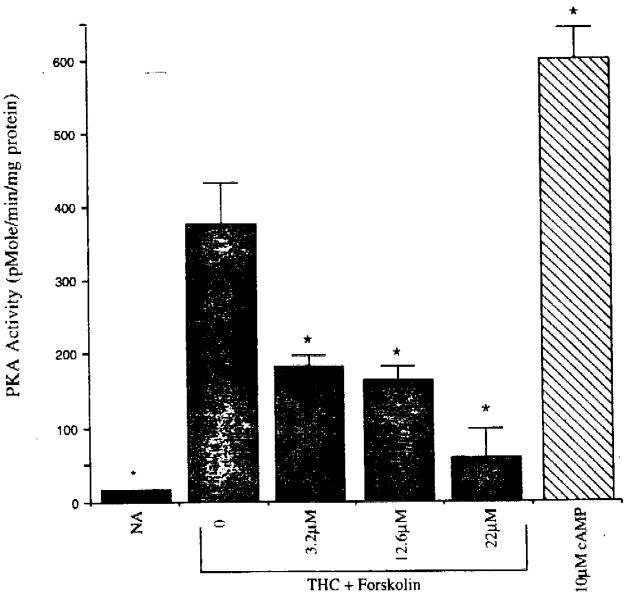

FIG. 5. **Effects of Δ^9-THC on PKA activation by forskolin in EL4.IL-2 cells.** Cell extracts preincubated with vehicle (0.1% ethanol), Δ^9-THC, or cannabinol for 5 min were incubated in reaction mixture at 37 °C for 10 min in the presence or absence of forskolin (50 μM). Triplicate samples were used for each group. The *bars* represent the mean ± S.E. as determined for each group for the representative of two independent experiments. *, $p < 0.05$ as determined by Dunnett's *t* test as compared to the forskolin control group.

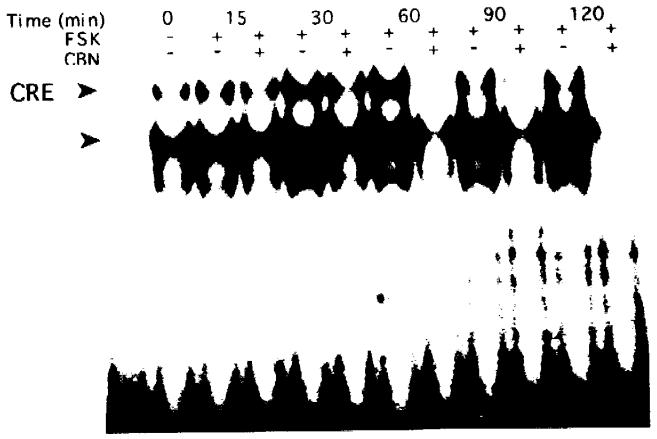

FIG. 6. **Inhibition of CREB binding with cannabinol treatment in forskolin-stimulated EL4.IL-2 cells.** Nuclear extract (3 μg) from treated and untreated EL4.IL-2 cells were incubated with 0.5 μg of poly(dI-dC) and ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. *Lane 1* represents free probe, and *lane 2* indicates unstimulated EL4.IL-2 cells. The *arrows* identify a major and minor band signifying two distinct CRE nuclear factor binding complexes. Results are representative of two separate experiments.

on PKA activity as evidenced by a dose-related decrease shown in Fig. 5. Δ^9-THC abrogated forskolin-stimulated PKA levels by 52, 57, and 84% at 3.2, 12, and 22 μM, respectively, and cannabinol decreased PKA levels by 57, 70, and 76% over the same dose range (data not shown). The terminal step of this signaling pathway, binding of activated transcription factors to CRE regulatory sites, is well established to peak around 30 min following activation of the cAMP cascade (41). In the present studies, nuclear extracts from untreated cells incubated in the presence of a ^{32}P-CRE oligomer resulted in the identification of two distinct protein/DNA binding complexes which recognized the CRE motif as evidenced by the presence of a major and a very faint minor band (Fig. 6, *lane 2*). CRE binding was increased by forskolin treatment within 30 min and persisted through 120 min poststimulation indicating the involvement of the cAMP cascade. However, stimulation of cells with forskolin in the presence of cannabinol resulted in a noticeably diminished magnitude of CRE binding at all time points assayed after 30 min.

Effects of Δ^9-THC and Cannabinol on IL-2 mRNA Expression and Protein Secretion—EL4.IL-2 cells were treated with either Δ^9-THC or cannabinol before stimulation with PMA/ionomycin in order to assess the effect of cannabinoids on IL-2 production in a T-cell line. This subline of EL4.IL-2 cells produces an average of 2500 units/ml of murine IL-2 when stimulated with PMA for 24 h which is indistinguishable from normal, concanavalin A-stimulated spleen cells, having the same molecular weight and isoelectric point heterogeneity (27). First, Northern analysis of PMA/ionomycin-stimulated (6 h) EL4.IL-2 cells demonstrated a decrease in IL-2 RNA expression when incubated with either Δ^9-THC or cannabinol (22 μM) as seen in Fig. 7*A*. Unstimulated EL4.IL-2 cells showed no IL-2 RNA message. Second, quantitative RT-PCR analysis, using a rcRNA internal standard (IS), of IL-2 expression from PMA/ionomycin-stimulated EL4.IL-2 cells (6 h) demonstrated a dose-dependent decrease in IL-2 mRNA expression in stimulated cells (Fig. 7*B*). The magnitude of inhibition at the transcriptional level ranges from approximately 20–60% over a cannabinoid concentration range of 3.2 to 22 μM. Similar results were obtained with splenocytes that were stimulated with PMA/ionomycin in the presence and absence of either Δ^9-THC or cannabinol. No PCR product bands were seen in PCR-processed RNA when reverse transcriptase was omitted, confirming that no DNA contamination was present. An example of a quantitative RT-PCR gel for the EL4.IL-2 and splenocyte PMA/ionomycin-stimulated RNA can be seen in Fig. 7*C* with the IS resolving at 474 bp and the IL-2 RNA resolving at 391 bp. Confirming the cannabinoid-mediated inhibition of IL-2 transcription, IL-2 protein secretion was dose-dependently inhibited in splenocytes and EL4.IL-2 cells following PMA/ionomycin stimulation for 24 h as measured by ELISA (Fig. 8). Over a dose range of 3.2–32 μM, cannabinol significantly decreased IL-2 secretion in EL4.IL-2 cells up to 60% of the maximal PMA/ionomycin stimulation and decreased splenocyte IL-2 activity up to 40%. Like cannabinol, Δ^9-THC also abrogated IL-2 secretion at 12–32 μM from 2–48% in EL4.IL-2 cells and 8–55% in splenocytes as compared to maximal, PMA/ionomycin-stimulated IL-2 production. Interestingly, cannabinol caused a greater inhibition of IL-2 secretion in EL4.IL-2 cells, and Δ^9-THC caused the greatest IL-2 inhibition in splenocytes. No difference in cell viability (>90%) was observed before or after stimulations or between vehicle or drug-treated groups.

Effects of Cannabinol on PMA/Ionomycin-induced Binding of trans-Acting Factors to an AP-1 Motif—Although the above studies show that cannabinoids inhibit signaling events mediated through the cAMP cascade leading to the activation and binding of *trans*-acting factors to the CRE, to date no CRE motifs have been identified in the IL-2 promoter. However, members of the CREB/ATF family can dimerize with Fos and Jun to bind AP-1 motifs (42, 43). Further, studies utilizing anti-CREB and anti-Fos/Jun antibodies established binding of all three proteins to the AP-1 proximal site (−151 to −145) of the IL-2 promoter following activation of a variety of T-cell preparations (44). Based on this, we performed experiments to determine whether activation of EL4.IL-2 cells by PMA/ionomycin in the presence of cannabinol would inhibit the binding of *trans*-acting factors to the AP-1 proximal site in the IL-2 promoter. Time course studies revealed that PMA/ionomycin-induced increase in binding to this

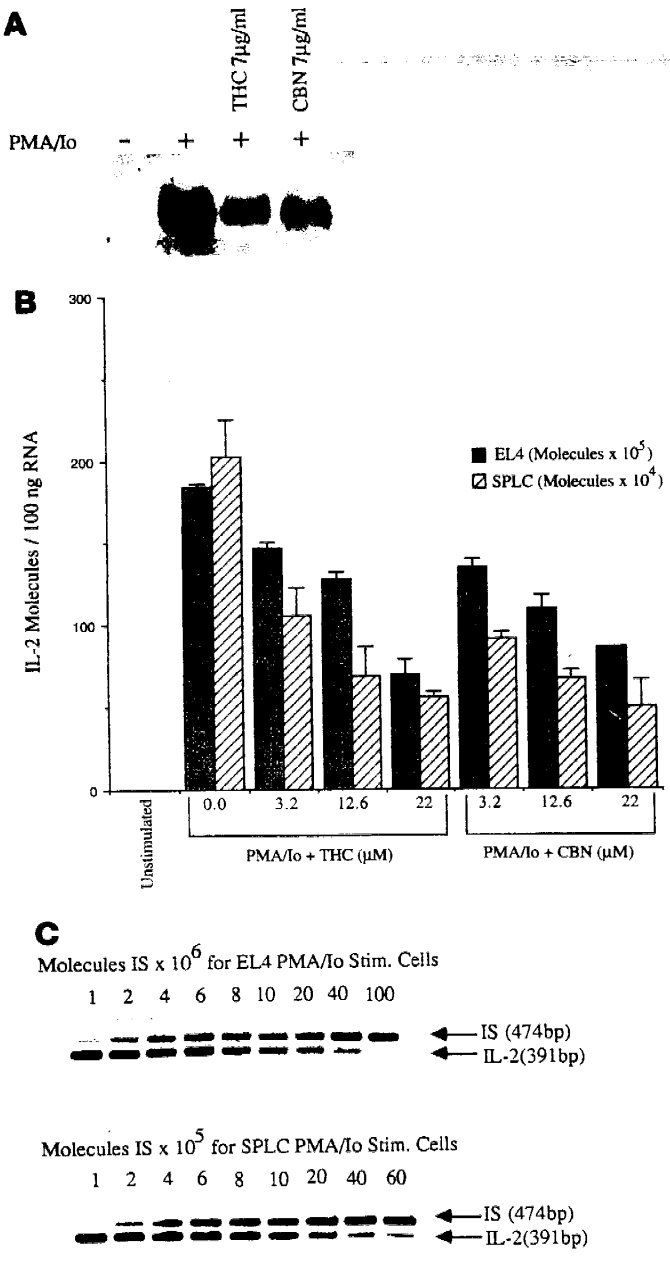

FIG. 7. **Effect of cannabinoids on IL-2 gene expression in EL4.IL-2 cells.** EL4.IL-2 cells (5×10^5 cells/ml) and splenocytes (*SPLC*) (5×10^6 cells/ml) were treated with vehicle (0.1% ethanol), Δ^9-THC, or cannabinol for 10 min, then stimulated with PMA (80 nM) and ionomycin (*Io*) (1 μM), and incubated for 6 h at 37 °C in 5% CO_2 after which cells were harvested and homogenized in Tri reagent for total RNA isolation. Viability was greater than 90% for all groups at the end of stimulation. *A*, Northern blot analysis of total RNA (5 μg/lane) from EL4.IL-2 PMA/ionomycin-stimulated cells when probed with a 1.1-kb mouse IL-2 sequence. Drug treatments were at 22 μM for both Δ^9-THC and cannabinol (*CBN*). *B*, summary graph of IL-2 gene expression for all PMA/ionomycin-stimulated cannabinoid treatment groups as determined by quantitative RT-PCR. After electrophoresis using a 3% NuSieve 3:1 gel, DNA bands were quantitated, and the ratio of internal standard (IS) to IL-2 RNA sample was calculated in order to generate a standard curve for each treatment group. All standard curve R^2 values were greater than 0.92 for all of the treatment groups. Each *bar* represents the molecules of IL-2 per 100 ng of RNA for both EL4.IL-2 and splenocytes calculated from the standard curve and represents the average from two experiments. *C*, representative quantitative RT-PCR gels of IL-2 mRNA in EL4.IL-2 and spleen PMA/ionomycin-stimulated cells. Nine internal standard rcRNA dilutions from 1×10^6 to 1×10^8 molecules/sample for EL4.IL-2 cells and 1×10^5 to 6×10^6 molecules/sample for splenocytes were added to each different treatment RNA sample.

AP-1 motif starting at 60 min with strong binding still quite evident at 4 h following activation (Fig. 9). Cells activated in the presence of cannabinol exhibited an attenuation of AP-1 binding at all of the time points measured. Further implicating a role for cAMP in the regulation of AP-1 binding was the finding that binding was enhanced when EL4.IL-2 cells were activated with PMA/ionomycin in the presence of forskolin as compared to PMA/ionomycin alone (Fig. 10). A modest forskolin-associated enhancement in AP-1 binding was observed beginning at 2 h which became more noticeable by 4 h as evidenced by the appearance of one major band and several minor bands. Similar enhancing effects by forskolin on AP-1 binding in phorbol ester/calcium ionophore-stimulated EL4.IL-2 cells have been shown at 3.5 h post-treatment (45).

DISCUSSION

In the present studies, we demonstrate in the mouse thymoma line EL4.IL-2 that cannabinoid treatment significantly inhibits modulation of the cAMP signal transduction pathway leading to a disruption in IL-2 transcription and production. The focus of our present studies is the T-cell which we and others have consistently found to be a sensitive immunologic target for alterations by cannabinoids both at a biochemical and functional level (17, 46).[2] The putative mechanism of action by cannabinoids is through cannabinoid receptors which are expressed on lymphoid and myeloid cells. Although the distribution of this class of receptors on specific cell types within the immune system has not been widely studied, the recent characterization of mouse splenic T-cells revealed an approximate density of 100–300 cannabinoid receptors per cell.[2] Radioligand binding, Northern analysis, and quantitative RT-PCR indicate that the subtype, CB2, is the primary form of the cannabinoid receptor expressed within the immune system; however, it is notable that low amounts of CB1 transcripts have also been identified in a variety of lymphoid preparations suggesting the possibility of modest CB1 protein expression on lymphoid and myeloid cells (2, 47).[2] Both receptor subtypes negatively regulate adenylate cyclase via a pertussis toxin-sensitive GTP-binding protein causing a decrease in cellular cAMP (4, 12, 16). This modulation of the cAMP cascade by cannabinoids is believed to be functionally relevant since either pertussis toxin pretreatment of cells or addition of exogenous cAMP analogs restored immune function in the presence of cannabinoids under *in vitro* conditions (16).

Northern analysis of EL4.IL-2 poly(A) RNA identified the presence of CB2 as a single band ~4 kb. Thus far, this transcript size for CB2 has only been identified in the mouse where it was originally described in spleen and thymus.[2] Although considerably larger than the 2.5-kb CB2 transcripts observed in rat and human (10), the recent cloning of a 3.7-kb mouse CB2 putative full-length cDNA sequence confirms the aforementioned species differences previously observed by Northern analysis of CB2.[2] No significant species differences have been observed by this receptor subtype in ligand binding characteristics (10)[2] or in the relative size of the CB2 protein as expressed on T-cell membranes.[3] Conversely, CB1 transcripts were not detectable either by Northern analysis or RT-PCR in EL4.IL-2 cells. Comparison of mouse and EL4.IL-2 genomic DNA digests with restriction enzymes known to cut within the CB2 gene indicated no differences by Southern analysis in CB2 banding patterns, suggesting a high degree of similarity in the CB2 gene between primary mouse immunocytes and the EL4.IL-2 cell line. As expected, the rat genome demonstrated different Southern analysis banding patterns for CB2 in com-

[3] R. B. Crawford, S. G. Hwang, and N. E. Kaminski, unpublished observation.

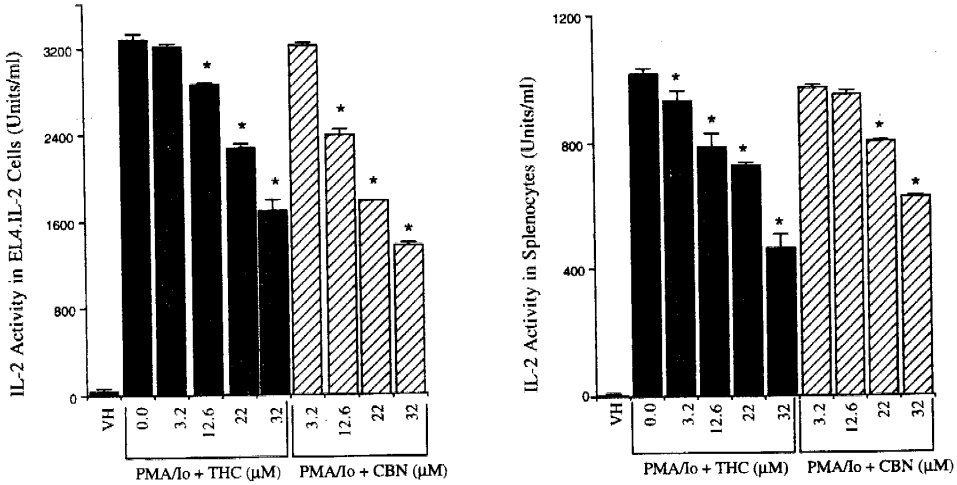

FIG. 8. **Inhibition of IL-2 protein secretion by cannabinoid treatment in PMA/ionomycin-stimulated EL4.IL-2 cells.** EL4.IL-2 cells (5×10^5 cells/ml) in RPMI complete with 5% bovine calf serum were treated with vehicle (0.1% ethanol), Δ^9-THC, or cannabinol for 10 min before stimulating cells with PMA (80 nM) and ionomycin (1 μM) for 24 h at 37 °C in 5% CO_2. After stimulation, supernatants were collected and assayed in triplicate using an ELISA kit. Viability was greater than 90% for all groups at the end of the stimulation. The IL-2 sample levels were determined by comparison to a standard curve of recombinant mouse IL-2 and are expressed as the mean ± S.E. as determined for each group from two independent experiments. *, $p < 0.05$ as determined by Dunnett's *t*-test as compared to the PMA/ionomycin control group.

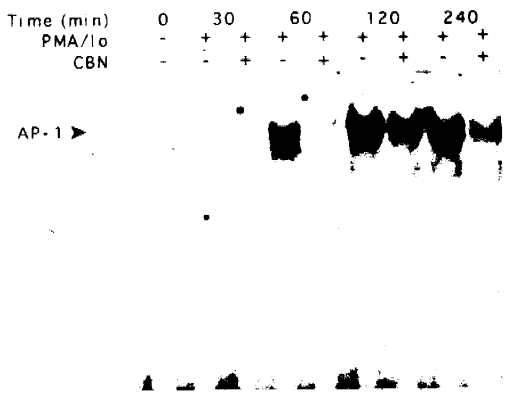

FIG. 9. **Inhibition of AP-1 binding with cannabinol treatment in forskolin-stimulated EL4.IL-2 cells.** Nuclear extract (3 μg) from cannabinol (20 μM) treated and untreated EL4.IL-2 cells stimulated with PMA/ionomycin were incubated with 0.5 μg of poly(dI-dC) and ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. *Lane 1* represents free probe. Results are representative of two separate experiments.

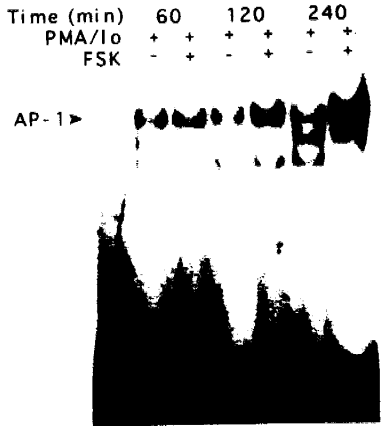

FIG. 10. **Enhancement of PMA/ionomycin-stimulated AP-1 binding by forskolin treatment in EL4.IL-2 cells.** Nuclear extract (3 μg) from forskolin-treated and untreated EL4.IL-2 cells stimulated with PMA/ionomycin were incubated with 0.5 μg of poly(dI-dC) and ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. *Lane 1* represents free probe. Results are representative of two separate experiments.

parison with either the mouse or EL4.IL-2 genome.

Signal transduction through the adenylate cyclase pathway is regulated by the formation of cAMP which binds to the regulatory subunits of PKA resulting in the release and activation of PKA catalytic subunits. These catalytic subunits go on to phosphorylate a variety of intracellular proteins including the CREB/ATF family of transcription regulators which consist of CREB, ATF, and CREM. CREB, which is the best characterized member of this family, is activated by PKA-mediated phosphorylation at Ser residue 133 (41) and forms either homo- or heterodimers with a variety of other transcription factors capable of binding cAMP regulatory element (CRE) DNA sequences present in the promoter region of a variety of genes. In the present studies, adenylate cyclase was activated in EL4.IL-2 cells by forskolin in the presence or absence of either Δ^9-THC or cannabinol to assess the influences of cannabinoids on various constituents of this signaling pathway. Both cannabinol, and to a lesser extent, Δ^9-THC produced an inhibition of forskolin-stimulated adenylate cyclase confirming the expression of functional cannabinoid receptors on EL4.IL-2 cells. The difference exhibited by these two cannabinoid congeners in EL4.IL-2 cells is consistent with the relative binding affinity for CB2 by the respective agents (*i.e.* cannabinol exhibits modestly greater binding affinity for CB2 than Δ^9-THC and has significantly lower affinity for CB1) (10)[2] and is further supported by the RNA analysis showing only expression of CB2 transcripts in EL4.IL-2 cells. The conclusion that EL4.IL-2 cells express functional CB2 receptors is also supported by recent findings showing that the human T-cell line, Jurkat, which expresses aberrant sized CB2 mRNA transcripts and no transcripts for CB1, did not exhibit adenylate cyclase inhibition at an identical concentration of either Δ^9-THC or cannabinol.[2] In accordance with diminished cAMP formation, PKA activity was also found to be markedly inhibited by both Δ^9-THC and cannabinol. It is important to emphasize that inhibition of PKA was not due to a direct effect of cannabinoids on kinase activity as demonstrated by a robust stimulation of PKA in the presence of high concentrations of Δ^9-THC by the addition of exogenous cAMP.

To determine the effects of cannabinoids on the activation of CRE-binding proteins, electrophoretic mobility shift assays were employed using a CRE DNA fragment. Forskolin treatment induced CRE binding in EL4.IL-2 cells beginning at 30

min but was noticeably diminished in the presence of cannabinol and would be consistent with a decrease in the activation of CRE-binding proteins by PKA. The time course for CRE binding by members of the CREB/ATF family of transcription factors is well characterized in a number of different cell types, all of which exhibited peak CRE binding around 30–60 min following forskolin stimulation and a subsequent decline over the next 10–12 h (48). Further, these kinetics are also very consistent with the fact that immune suppression by cannabinoids, as measured by the antibody-forming cell response, could be reversed by low concentrations of dibutyryl-cAMP (10–100 μM) through presumably activating PKA, however, only if added to culture within the first 30 min following antigen activation (16).

Activation of EL4.IL-2 cells or primary T-cells by PMA/ionomycin initiates IL-2 gene transcription and a marked secretion of IL-2 protein. In the presence of either cannabinol or Δ^9-THC, IL-2 transcription as well as protein secretion was significantly inhibited in EL4.IL-2 cells and in mouse splenocytes. IL-2 gene transcription is highly regulated through a number of well characterized recognition sites in the promoter region for inducible and noninducible regulatory factors. In light of the fact that PMA-induced IL-2 transcription was decreased by the inhibition of PKA with cannabinoids, it is most probable that this effect is mediated through a regulatory factor(s) which is both inducible and cross-regulated by both PKC and PKA. This possibly implicates AP-1, NF-AT, NF-κB, or CD28RE DNA sites each of which is present in the IL-2 promoter. Forskolin has been shown by gel mobility shift assays to act synergistically as does IL-1, in conjunction with phorbol ester/calcium ionophore stimulation of EL4 cells, to enhance the bound amount of transcriptional-regulatory proteins to the AP-1 proximal site of the IL-2 promoter (45). This forskolin enhancement implicates a positive signal through the cAMP pathway at the AP-1 site in helping to induce IL-2 transcription. In addition, although no CRE sites are present within the IL-2 promoter, recent studies have demonstrated CREB binding to the AP-1 regulatory sites in the IL-2 gene (44). In fact, CREB binding to AP-1 regulatory sites within the IL-2 promoter was observed in mouse-derived $CD4^-CD8^-$ and $CD4^+CD8^+$ immature T-cells and $CD4^+CD8^-$ mature T-cells. Although the best characterized regulation at AP-1 sites is through protein dimers of the Fos and Jun family members, recent studies employing anti-CREB and anti-Fos/Jun gel shifts identified all three protein types bound to AP-1, suggesting that CREB family proteins help to regulate IL-2 transcription through the formation of heterodimers with Fos and Jun (44). This is not completely unexpected in light of the fact that CREB has been shown to form heterodimers with both Fos and Jun proteins (42, 43). In the present studies we demonstrate a decrease in the binding of *trans*-acting factors to the AP-1 proximal site of the IL-2 promoter when EL4.IL-2 cells were stimulated by PMA/ionomycin in the presence of cannabinol and an increase in binding in the presence of forskolin. This observation further implicates a regulatory role for the CREB/ATF family of DNA-binding proteins in the regulation of IL-2. However, since we have not yet identified which specific AP-1 nuclear binding factors are negatively regulated by cannabinoid treatment, an alternative mechanism for the decrease in AP-1 binding may be through the inhibition of upstream signaling events which regulate the activation of Jun family members. It is equally notable that the c-*fos* gene possesses CRE sites in its promoter suggesting an additional site of IL-2 regulation by CREB/ATF family member proteins (49–51). Further, this may explain why AP-1 binding following PMA/ionomycin stimulation was not evident until 60 min, and concomitant treatment with forskolin did not markedly enhance AP-1 binding until 4 h post-treatment in the present studies.

Although the role of the cAMP signaling cascade in lymphocyte function is poorly understood and historically perceived as a negative regulatory pathway, there is significant evidence suggesting that this is likely an oversimplified view. Almost without exception, those studies which have reported decreased T-cell function by cAMP, especially with respect to IL-2 expression, have been performed using very high concentrations of membrane-permeable cAMP analogs (≥500 μM) (52–54). In contrast, these same analogs are immunostimulatory at low concentrations (<100 μM) as demonstrated in a variety of assay systems (16, 55, 56). The critical role of cAMP in lymphoid cell function is further supported by the fact that there is a rapid transient burst in adenylate cyclase activity within the first 5 min following lymphocyte activation by mitogens or phorbol ester plus calcium ionophore (16, 40, 57–60) implicating positive lymphocyte regulation through this mechanism. This positive role by modest transient increases in cAMP during T-cell activation are consistent with the recent finding that unlike for a variety of cell types including fibroblasts, adipocytes, and muscle cells in which cAMP antagonizes the Raf-mitogen-activated protein kinase pathway (61–67), in T-cells this pathway is resistant to short-term increases in cAMP (54). This is evidenced by the previously reported finding that cAMP did not inhibit ERK2, the dominant form of mitogen-activated protein kinase in T-cells (54). Similarly, the newly identified c-Jun N-terminal kinase which defines a T-cell antigen receptor independent activation pathway was also shown to be resistant to short-term increases (<30 min) in cAMP (54). Conversely, sustained (2 h) treatment of T-cells with 0.5 mM dibutyryl-cAMP resulted in an antagonism of c-Jun N-terminal kinase which was not due to a decrease in the synthesis of the kinase but was dependent on protein synthesis (54). Previous results from our own laboratory indicate that the immunoinhibitory effects by cannabinoids are associated with an early lymphocyte activation event. This is supported by temporal addition studies which demonstrate that cannabinoids inhibit the T-cell-dependent IgM antibody forming cell response only when added within the first 120 min following antigen stimulation with the greatest inhibition occurring when the drug was added to culture within the first 30 min (5). Likewise, reversal of this inhibition can be achieved with the addition of low concentration of dibutyryl-cAMP (10 μM) to culture, however, only within the first 20 min following antigen stimulation (16). Additional insight into the role of the cAMP cascade in T-cell function has come from the finding that high concentrations of cAMP (1 mM) activate the binding of nuclear factors to GATA-3 and the conserved lymphokine element (CLE0) to enhance the expression of the B cell differentiation factor, IL-5 (68, 69). Since IL-5 is primarily secreted by the Th2 subset of helper cells taken together with the finding that high sustained concentrations of cAMP inhibit IL-2 expression by Th1 cells, it has been proposed that cAMP may act as a Th1/Th2 switch factor. Based on the results described in this report, we propose that cAMP is essential for Th1 and Th2 lymphokine gene expression, with low and perhaps transient intracellular cAMP concentrations favoring the activation of Th1 lymphokines, whereas high and sustained cAMP concentration favors the induction of Th2-associated cytokines. Further, the apparent requirement for high cAMP concentrations for IL-5 expression provides an explanation for the marked sensitivity of T-cell-dependent humoral responses to inhibition by cannabinoid compounds since these compounds are potent inhibitors of adenylate cyclase.

In summary, these studies demonstrate that cannabinoid

compounds inhibit adenylate cyclase/cAMP-associated signaling in T-cells as demonstrated by a decrease in cAMP formation, PKA activity, and CREB binding to CRE DNA regulatory sites. Additionally, a decrease was observed in nuclear factor binding to the AP-1 proximal motif of the IL-2 promoter following PMA/ionomycin stimulation of EL4 cells in the presence of cannabinoids. Concordantly, cannabinoid treatment also produced a decrease in IL-2 transcription and protein secretion in EL4.IL-2 and spleen cells. We believe that the cannabinoid-mediated alteration in IL-2 regulation may be mediated through the inhibition of nuclear factor binding at AP-1 sites possibly by CREB/Fos and/or CREB/Jun heterodimers. The significance of the present studies is that they support the recent findings from a number of laboratories implicating a positive regulatory role for the modest activation of the cAMP cascade in the modulation of lymphocyte function, in this case the regulation of IL-2. Studies are presently underway to further characterize which specific AP-1 binding proteins within this diverse family are modulated by cannabinoid compounds to inhibit IL-2 expression.

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