

Cannabinol Enhancement of Interleukin-2 (IL-2) Expression by T Cells Is Associated with an Increase in IL-2 Distal Nuclear Factor of Activated T Cell Activity

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ABSTRACT

It has been demonstrated previously that cannabinol (CBN) differentially modulates interleukin-2 (IL-2) protein secretion by T cells with a corresponding change in extracellular signal-regulated kinase activity. The objective of the present studies was to further investigate the molecular mechanism by which CBN enhances IL-2 gene expression using the EL4 T cell line. We demonstrate here that steady-state IL-2 mRNA expression was significantly enhanced by CBN in a concentration-dependent manner in EL4 cells activated with suboptimal concentrations of phorbol-12-myristate-13-acetate (2–10 nM). Concomitantly, a marked increase was observed in nuclear factor of activated T cells (NF-AT) DNA binding activity to the IL-2 distal NF-AT site, but not to nuclear factor for immunoglobulin κ chain in B cells or activator protein 1 motifs. Transient transfection of

EL4 cells with a reporter gene under the control of multiple IL-2 distal NF-AT motifs exhibited increased transcriptional activity by CBN in suboptimally activated cells. In addition, the CBN-mediated enhancement of IL-2 protein secretion and the transcriptional activity of the IL-2 distal NF-AT reporter gene was abrogated by the calcium/calmodulin-dependent protein kinase inhibitor KN93, but not by the CB2 receptor antagonist SR144528. Enhancement of IL-2 was also demonstrated with CP55940, Δ^9 -tetrahydrocannabinol, and cannabidiol, thus suggesting that the phenomenon is not unique to CBN. Collectively, these results suggest that increased IL-2 secretion by CBN is mediated through the enhancement of IL-2 gene transcription by activation of NF-AT in a CB1/CB2-independent manner.

Plant-derived cannabinoids, including cannabinol (CBN) and Δ^9 -tetrahydrocannabinol (Δ^9 -THC), are widely established as possessing immunomodulatory activity in a number of experimental models (reviewed by Kaminski, 1998; Klein et al., 1998). Previous studies have identified T cells as a sensitive cellular target for cannabinoids, as evidenced by decreased mitogen-induced proliferation, T-cell dependent antibody responses, and altered cytokine expression (Schatz et al., 1993; Condie et al., 1996; Klein et al., 1998). Activation of T cells results in the production of interleukin (IL)-2 and other cytokines. Because IL-2 plays an essential role in early T cell clonal expansion and differentiation, the effect of cannabinoids on IL-2 gene expression has been studied extensively (Nakano et al., 1993a; Condie et al., 1996; Yea et al., 2000). Interestingly, cannabinoids have been reported to both positively and negatively modulate IL-2 expression by T

cells (Pross et al., 1992; Nakano et al., 1993a; Condie et al., 1996; Herring et al., 1998). To decipher these seemingly paradoxical observations, we have previously demonstrated that the magnitude of T cell activation is the principal factor governing the differential regulation of IL-2 by CBN (Jan and Kaminski, 2001). CBN inhibited IL-2 secretion by T cells stimulated with strong activation stimuli, such as high concentrations of phorbol ester plus calcium ionophore (PMA/ionomycin) and immobilized anti-CD3 plus anti-CD28 monoclonal antibody. In contrast, when EL4 T cells were suboptimally activated with low concentrations of PMA (2–10 nM) or splenic T cells with soluble anti-CD3 monoclonal antibody, CBN markedly enhanced IL-2 secretion (Nakano et al., 1993a; Jan and Kaminski, 2001).

IL-2 gene expression is tightly regulated at the transcriptional level by several *cis*-acting elements in the proximal promoter/enhancer region (–326 to –52 bp) of the IL-2 gene, including NF-AT, NF- κ B, AP-1-like, Oct, and CD28 response

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ABBREVIATIONS: CBN, cannabinol; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; IL-2, interleukin-2; PMA, phorbol-12-myristate-13-acetate; NF-AT, the nuclear factor of activated T-cells; NF- κ B, nuclear factor for immunoglobulin κ chain in B cells; AP-1, activator protein-1; Oct, octamer protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; CaM kinases, calcium/calmodulin-dependent protein kinases; BCS, bovine calf serum; RT-PCR, reverse transcription-polymerase chain reaction; CBD, cannabidiol; SEAP, secreted alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; PKC, protein kinase C; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide.

elements (Jain et al., 1995; Serfling et al., 1995). Binding of multiple transcription factors, such as NF-AT, NF- κ B, AP-1, and Oct to these *cis*-acting elements is required for full activation of IL-2 gene transcription. In addition, cooperative binding between NF-AT and AP-1 has been identified to be essential for the transcriptional activity of the IL-2 distal NF-AT site, as well as for the promoter activity of IL-2 gene (Jain et al., 1995; Rao et al., 1997). The activation of NF-AT and AP-1 is primarily mediated by the calcium-dependent phosphatase calcineurin and protein kinase C-dependent activation of the MAP kinase signaling pathway, respectively. The critical role of the cooperativity between NF-AT and AP-1 in IL-2 regulation can be further evidenced by the fact that specific inhibitors for either calcineurin (i.e., cyclosporin A) or extracellular signal-regulated (ERK) MAP kinases (i.e., PD098059) are strong inhibitors for IL-2 gene expression (Dumont et al., 1998).

In light of the involvement of multiple *cis*-acting elements in IL-2 regulation, to further elucidate the molecular mechanism by which CBN enhanced IL-2 secretion by suboptimally activated T cells, the present studies aimed to investigate which regulatory elements in the IL-2 promoter are responsible for CBN-mediated enhancement of IL-2. Previous studies have demonstrated that CBN-mediated enhancement of IL-2 secretion by suboptimally activated T cells was mediated through signaling pathways, leading to an increase in ERK kinases and calcium/calmodulin-dependent protein (CaM) kinases (Jan and Kaminski, 2001). In light of these results, the present investigation focused on three potential downstream transcription factors targeted by the aforementioned kinases, which are critically involved in regulating IL-2 transcription: AP-1, NF- κ B, and NF-AT. The present investigation demonstrates that the increased IL-2 protein secretion by CBN is mediated through IL-2 gene transcription and that the distal NF-AT site, but not NF- κ B or AP-1 motifs, is closely associated with the CBN-mediated enhancement of IL-2 expression in suboptimally activated EL4 T cells. Furthermore, consistent with previous findings, CBN-mediated enhancement of both IL-2 secretion and transcriptional activity of the IL-2 distal NF-AT site is abrogated by KN93, a CaM kinase inhibitor. Conversely, the CB2 receptor antagonist, SR144528, did not attenuate the CBN-mediated enhancement of IL-2 secretion. The present studies are the first to provide a molecular mechanism for CBN-mediated enhancement of IL-2 gene expression by T cells.

Materials and Methods

Reagents and Cell Cultures. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. CBN, CBD, Δ^9 -THC, CP55940, and SR144528, which were greater than 99% pure, as determined by gas chromatography/mass spectrometry, were provided by the National Institute on Drug Abuse. CBN was reconstituted in absolute ethanol, aliquoted, and stored at -80°C . Working solutions were prepared freshly just before addition to cell cultures. Water-soluble KN93 was purchased from Calbiochem (La Jolla, CA). Anti-c-jun/AP-1 and anti-c-fos rabbit polyclonal IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-c-jun/AP-1 antibody recognizes c-jun, jun-B, and jun-D, and the anti-c-fos antibody recognizes c-fos, fos-B, fra-1, and fra-2. Anti-NF-ATc1 mouse monoclonal antibody (clone 7A6) was purchased from Affinity BioReagents, Inc. (Golden, CO). The C57BL/6 mouse T cell lymphoma line EL4 was obtained from American Type Culture Col-

lection (Manassas, VA). The EL4 cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 10% BCS. In all cases, EL4 cells were cultured at 37°C in 5% CO_2 .

Measurements of Steady State IL-2, CB1, and CB2 mRNA Expression by RT-PCR. All reagents used for RT-PCR were of molecular biological grade and were purchased from Promega (Madison, WI) unless otherwise noted. Total RNA was isolated using TRI Reagent (Sigma Chemical, St. Louis, MO). All isolated RNA samples were confirmed to be free of DNA contamination as determined by the absence of product after PCR amplification in the absence of reverse transcriptase (data not shown). Steady-state mRNA expression was quantified for IL-2 by quantitative competitive RT-PCR as described previously (Condie et al., 1996) with minor modifications. Briefly, known amounts of total RNA and internal standard mRNA for IL-2 were reverse-transcribed simultaneously, in the same reaction tube, into cDNA using oligo(dT)₁₅ as primers. A PCR master mixture consisting of PCR buffer, 4 mM MgCl_2 , 6 pmol each of the forward and reverse primers, and 1.25 units of *Taq* DNA polymerase was added to the cDNA samples. Samples were heated to 94°C for 4 min and cycled 28 to 32 times at 94°C for 15 s, 60°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. Quantification was performed by assessing the optical density for both of the DNA bands (internal standard and IL-2 mRNA) using a Gel Doc 100 video imaging system (Bio-Rad, Melville, NY). The number of transcripts was calculated from a standard curve generated from the density ratio between the gene of interest (IL-2) and the different amounts of internal standard used. Qualitative RT-PCR was employed for confirmation of CB1 and CB2 expression in leukocyte preparations as described previously (Schatz et al., 1997). Briefly, for detection of CB1 and CB2 by RT-PCR, 100 ng total RNA isolated from either spleen or EL4 cells was used. RNA was reverse transcribed as described above for IL-2 determinations. The PCR annealing temperature conditions for CB1 and CB2 involved a 1°C per cycle touchup for 12 consecutive cycles, beginning at 53°C , followed by 23 cycles at 65°C . The amplicons were visualized using ethidium bromide as described above.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (Francis et al., 1995). Briefly, cells were lysed with a hypotonic buffer (10 mM HEPES and 1.5 mM MgCl_2 , pH 7.5) and the nuclei were pelleted by centrifugation at $3000g$ 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) that contained 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g/ml}$ each of aprotinin and leupeptin for 15 min on ice. After lysis, samples were centrifuged at $17,500g$ for 15 min, and the supernatant was retained for use in the DNA binding assay. Double-stranded deoxyoligonucleotides containing the mouse IL-2 distal NF-AT sequence (5'-AGAGGAAAATTTGTTTCATACAGAAGGCG-3'), consensus AP-1 sequence (5'-GATCGGCTGACTCATCAGTA-3' (Novak et al., 1990); and consensus NF- κ B sequences (5'-GGGGACTTTCC-3' (Herring et al., 1998) were synthesized and end-labeled with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$. Nuclear extracts (5 μg) were incubated with 1 μg of poly(dI-dC) and the ^{32}P -labeled DNA probe in the binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM MgCl_2 , 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g/ml}$ each of aprotinin and leupeptin) for 20 min at room temperature. DNA binding activity was separated from free probe using a 4% polyacrylamide gel in $0.5\times$ TBE ($1\times$ TBE = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried and subjected to autoradiography.

Transient Transfection Assays. The reporter gene pNFAT-SEAP (CLONTECH, Palo Alto, CA), is under the control of NF-AT; three copies of IL-2 distal NF-AT motifs were inserted into the TATA-like promoter region. EL4 cells were transfected with pNFAT-

SEAP plasmids using Cytofectene Transfection Reagent (Bio-Rad). Briefly, cells (2×10^5 cells/ml) were harvested and resuspended in RPMI 1640 medium with 2% BCS and incubated with the transfection buffer (0.3 ml RPMI 1640 medium, 7.5 μ g of plasmids, and 15 μ l of Cytofectene) for 16 to 20 h. The transfected cells were washed, resuspended with RPMI 1640 medium with 2% BCS, and received various treatments in triplicate in 48-well cell culture plates (0.2 ml/well; Corning Inc., Corning, NY). After 48 h of culture, supernatants were collected and the SEAP activity in the supernatants was assayed using Great EscAPE SEAP chemiluminescence detection kit (CLONTECH). In brief, 25 μ l of sample was added to 75 μ l of dilution buffer and incubated at 65°C for 30 min. Samples were placed on ice and 100 μ l of assay buffer was added and incubated for 5 min at room temperature. After incubation, 100 μ l of chemiluminescent substrate (25 mM) [containing 4-methylumbelliferyl phosphate (10 mM)] was added and incubated for 10 min at room temperature and luminescence was measured with a TD-20e luminometer (Turner Designs, Sunnyvale, CA). In some cases, the supernatant was also quantified for IL-2 by ELISA as described previously (Ouyang et al., 1995).

Statistical Analysis. The mean \pm S.E. was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance, and Dunnett's two-tailed *t* test was used to compare treatment groups to the vehicle control when significant differences were observed (Dunnett, 1955).

Results

Concentration-Dependent Enhancement of Steady State IL-2 mRNA Expression by CBN. In light of previous studies showing an increase in IL-2 protein secretion by CBN in suboptimally activated T cells (Jan and Kaminski, 2001), the effect of CBN on steady state IL-2 mRNA expression was examined in EL4 T cells activated with low concentrations of PMA (2–10 nM). Based on kinetics studies demonstrating the occurrence of peak steady-state IL-2 mRNA expression 4 to 8 h after T cell activation (Jain et al., 1995), total RNA from EL4 cells was isolated at 6 h after PMA treatment. The magnitude of IL-2 mRNA expression was quantified by competitive RT-PCR. Pretreatment of EL4 cells with CBN (15 μ M) for 30 min significantly enhanced the steady-state IL-2 mRNA expression induced by PMA (2–10 nM; Fig. 1A). The CBN-mediated enhancement of IL-2 steady-state mRNA expression was concentration-dependent (Fig. 1B). CBN treatment alone, in the absence of PMA, did not induce steady state IL-2 mRNA expression in EL4 cells.

CBN-Mediated Enhancement of DNA Binding to the IL-2 Distal NF-AT Site, but Not NF- κ B or AP-1 Consensus Motifs. Transcription of IL-2 is tightly regulated by several *trans*-acting factors, including AP-1, NF- κ B, NF-AT, and Oct (Jain et al., 1995). In light of previous findings demonstrating a role by ERK MAP kinases and CaM kinases in CBN-mediated enhancement of IL-2 secretion (Jan and Kaminski, 2001), the present investigation focused on characterizing the effect of CBN on three potential transcription factors critically involved in the regulation of IL-2: AP-1, NF- κ B, and NF-AT. These three families of transcription factors are known to be regulated by ERKs and/or CaM kinases (Jain et al., 1995). Moreover, it has been demonstrated previously that, under optimal T cell activation conditions, cannabinoids inhibit both the activation of these transcription factors and IL-2 gene expression (Condie et al., 1996; Herring et al., 1998; Yea et al., 2000). The same experimental conditions used in measurements of steady-state IL-2 mRNA were employed to examine the DNA binding

activity of AP-1, NF- κ B, and NF-AT by electrophoretic mobility shift assay. Nuclear proteins were isolated 15, 60, or 240 min after PMA treatment. As illustrated in Fig. 2, although AP-1 binding was induced by PMA (2 nM) in a time-dependent manner, CBN pretreatment did not alter AP-1 binding activity over the 4 h period of time after activation (Fig. 2, top). Similarly, NF- κ B binding was not influenced by CBN treatment (Fig. 2, bottom). Moreover, at low concentrations, PMA (2 nM) treatment alone did not increase NF- κ B binding activity above its constitutive level. In contrast, the DNA binding activity to the IL-2 distal NF-AT motif was markedly enhanced (1.7-fold) by CBN (10 and 20 μ M) 4 h after PMA stimulation (Fig. 3). The kinetics of CBN-induced enhancement in NF-AT DNA binding was closely paralleled with CBN-mediated enhancement of IL-2 protein secretion and mRNA expression.

Supershift Analysis of CBN-Mediated Enhancement of DNA Binding to the IL-2 Distal NF-AT Site. The IL-2

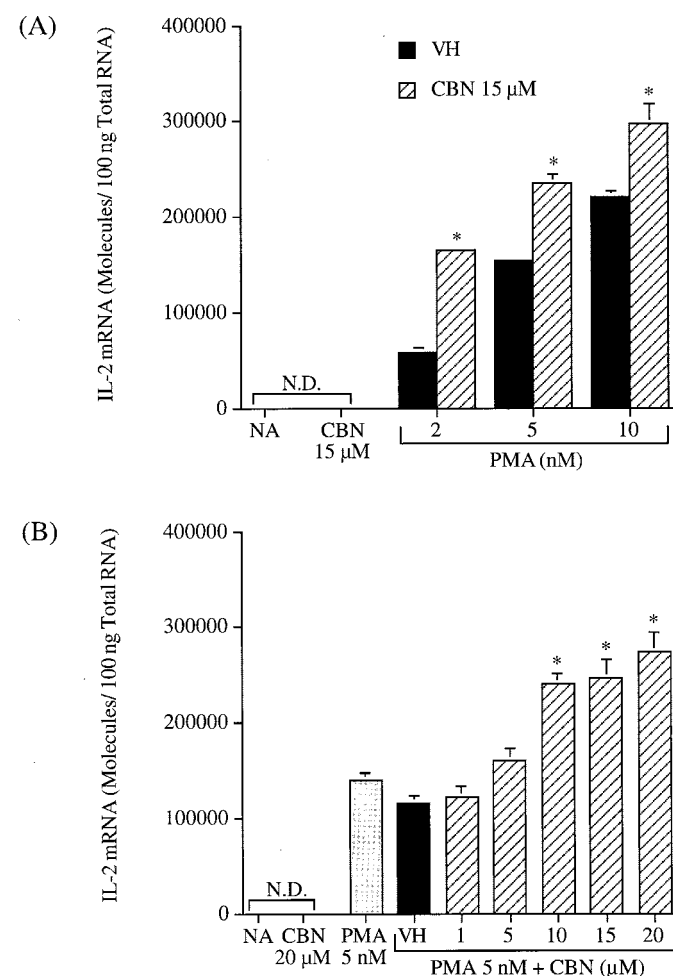


Fig. 1. CBN-mediated enhancement of steady state IL-2 mRNA expression in suboptimally activated EL4 cells. A, EL4 cells (2×10^5 cells/ml) were either untreated (NA) or pretreated with CBN (15 μ M) and/or VH (0.1% ethanol) for 30 min followed by stimulation with the suboptimal activation stimulus PMA (2–10 nM) for 6 h at 37°C. B, EL4 cells (2×10^5 cells/ml) were either untreated (NA) or pretreated with CBN (1–20 μ M) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 nM) for 6 h at 37°C. Total RNA was isolated and IL-2 mRNA determined by competitive RT-PCR. The data are expressed as the mean \pm S.E. of triplicate cultures. *, *p* < 0.05 compared with the VH control group. N.D., IL-2 mRNA was below the level of quantification. Results are representative of three independent experiments.

distal NF-AT site is a composite site to which both AP-1 and NF-AT transcription factors bind cooperatively (Rao et al., 1997). To confirm the presence of AP-1 and/or NF-AT as the CBN-modulated proteins bound to the IL-2 distal NF-AT element, supershift assays were performed. Antibodies di-

rected against components of AP-1 (i.e., c-fos and c-jun family members) or NF-ATc1 (NF-AT2) were employed. c-fos and c-jun family member proteins and NF-ATc1 were all identified in the NF-AT binding complex induced by CBN pretreatment using low activating concentrations of PMA, as evidenced by the fact that anti-c-fos polyclonal rabbit IgG inhibited the NF-AT binding whereas anti-c-jun/AP-1 polyclonal rabbit IgG and anti-NF-ATc1 mouse monoclonal antibody supershifted the binding (Fig. 4). In contrast, control rabbit IgG and control mouse ascites fluid did not alter the DNA binding to the IL-2 distal NF-AT site.

CBN-Mediated Enhancement of IL-2 Distal NF-AT Transcriptional Activity. To further evaluate the significance of the increased DNA binding to the IL-2 distal NF-AT site by CBN, the effect of CBN on the transcriptional activity of a reporter gene under the control of multiple IL-2 distal NF-AT motifs was assessed. In this series of studies, EL4 cells were transiently transfected with the pNFAT-SEAP reporter gene, pretreated with CBN (1–20 μ M) for 30 min, and then activated with low concentrations of PMA (5 or 10 nM). The SEAP activity in the supernatants was measured

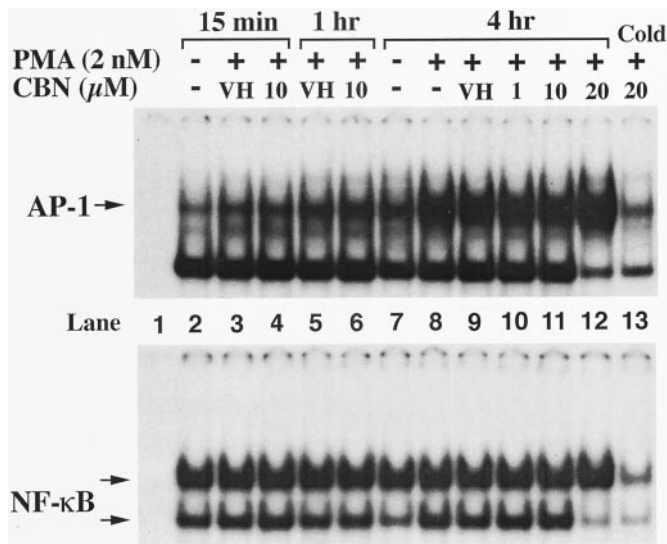


Fig. 2. Effect of CBN on AP-1 and NF- κ B binding in suboptimally activated EL4 cells. EL4 cells (2×10^5 cells/ml) were either untreated or pretreated with CBN (1–20 μ M), and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (2 nM). EL4 cells were cultured for 15 min, 1 h, or 4 h at 37°C and nuclear proteins isolated as described under *Materials and Methods*. Nuclear proteins (5 μ g) were incubated with 1 μ g of poly(dI-dC) and 32 P-labeled AP-1 or NF- κ B consensus DNA probe in binding buffer at room temperature for 20 min followed by separation on a 4% polyacrylamide gel. Lane 1, free probe; lane 2 and 7, naïve cells. The competitor lane (lane 13) included 50-fold molar excess of the unlabeled DNA probe as cold competitors. Results are representative of three independent experiments.

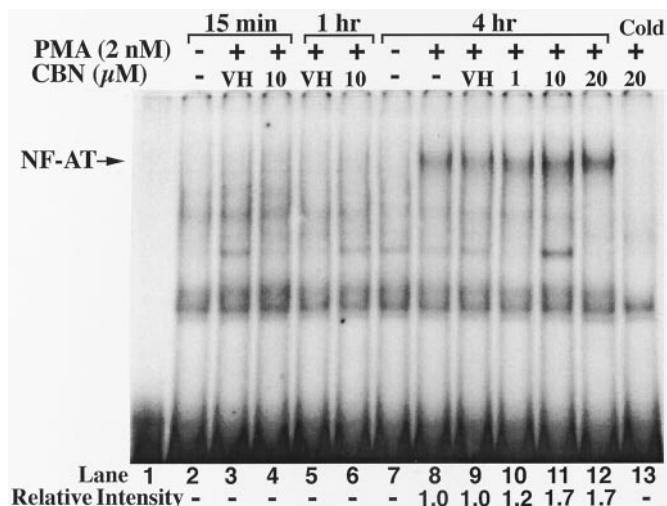


Fig. 3. Effect of CBN on murine IL-2 distal NF-AT binding in suboptimally activated EL4 cells. EL4 cells (2×10^5 cells/ml) were either untreated or pretreated with CBN (1–20 μ M) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (2 nM). EL4 cells were cultured for 15 min, 1 h, or 4 h at 37°C and nuclear proteins isolated. Nuclear proteins (5 μ g) were incubated with 1 μ g of poly(dI-dC) and 32 P-labeled murine IL-2 distal NF-AT DNA probe in binding buffer at room temperature for 20 min followed by separation on a 4% polyacrylamide gel. Lane 1, free probe; lane 2 and 7, naïve cells. The competitor lane (lane 13) included 50-fold molar excess of the unlabeled DNA probe as competitors. Results are representative of three independent experiments.

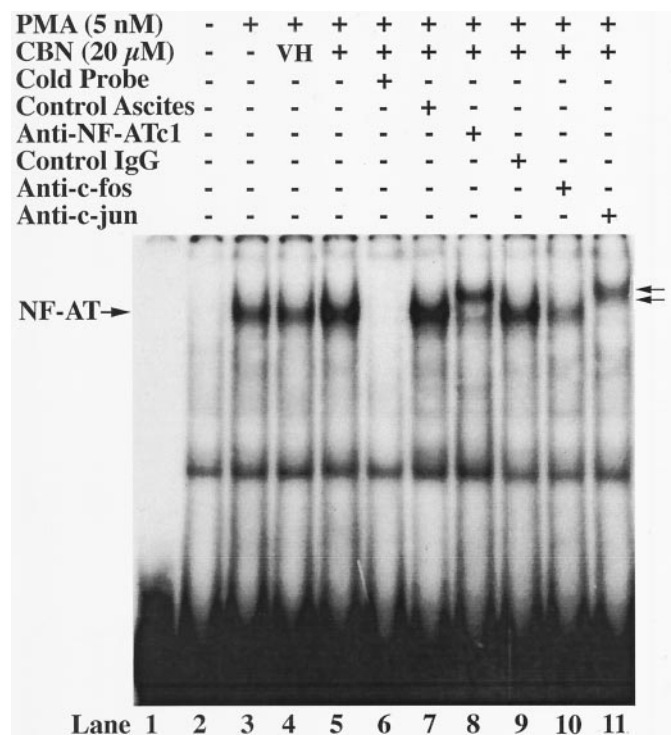


Fig. 4. Supershift assay analysis of CBN-mediated enhancement of NF-AT binding. EL4 cells (2×10^5 cells/ml) were either untreated or pretreated with CBN (20 μ M) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 nM) for 4 h at 37°C. The nuclear proteins were isolated and 5 μ g of nuclear proteins were incubated with 1 μ g of poly(dI-dC) and 32 P-labeled murine IL-2 distal NF-AT DNA probe in binding buffer at room temperature for 20 min, and then incubated with supershift antibodies (2 μ l at 2 μ g/ μ l) for another 20 min followed by separation on a 4% polyacrylamide gel. Lane 1, free probe; lane 2, naïve cells; lane 3, PMA-stimulated cells; lanes 4, VH-pretreated and PMA-stimulated cells; lanes 5 to 11, CBN-pretreated and PMA-stimulated cells incubated with 50-fold molar excess of cold probe (lane 6); incubated with control ascites fluid (lane 7); incubated with anti-NF-ATc1 monoclonal antibody (lane 8); incubated with control rabbit IgG (lane 9); incubated with anti-c-fos rabbit IgG (lane 10); and incubated with anti-c-jun/AP-1 rabbit IgG (lane 11). The arrows on the right indicate the supershifted complexes induced by anti-NF-ATc1 and anti-s-fos. Results are representative of two independent experiments.

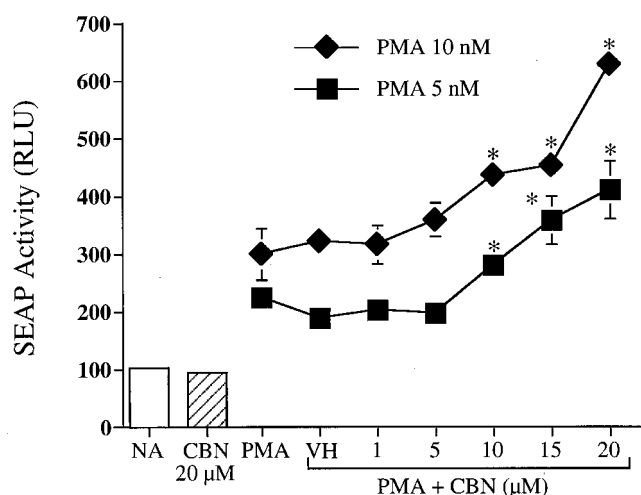
48 h after PMA activation. Consistent with increased NF-AT DNA binding activity observed in the electrophoretic mobility shift assay studies, transcriptional activity of pNFAT-SEAP reporter gene, induced by PMA (5 and 10 nM), was clearly enhanced in a concentration-dependent manner by CBN pretreatment (Fig. 5A). Additional confirmatory studies identified a concomitant CBN-mediated increase in IL-2, as measured by ELISA, in the same supernatants that were used for determinations of SEAP activity in the transfected EL4 cells (Fig. 5B).

Evidence for the Involvement of CaM Kinases in CBN-Mediated Enhancement of IL-2 Distal NF-AT Transcriptional Activity and IL-2 Secretion. We have

previously reported that the CaM kinase inhibitor KN93 attenuated CBN-mediated enhancement of IL-2 secretion (Jan and Kaminski, 2001). The putative involvement of CaM kinases on CBN-mediated enhancement of the IL-2 was investigated employing the pNFAT-SEAP reporter gene in transiently transfected EL4 cells. Complete attenuation of the CBN-mediated enhancement in pNFAT-SEAP reporter gene activity and IL-2 secretion was produced by 5 μ M KN93, a concentration of the inhibitor that produced no inhibitory effect on either responses in the absence of CBN (Fig. 6).

Neither CBN-Mediated Enhancement of IL-2 Distal NF-AT Reporter Activity nor IL-2 Secretion Is Attenuated by KN93.

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion

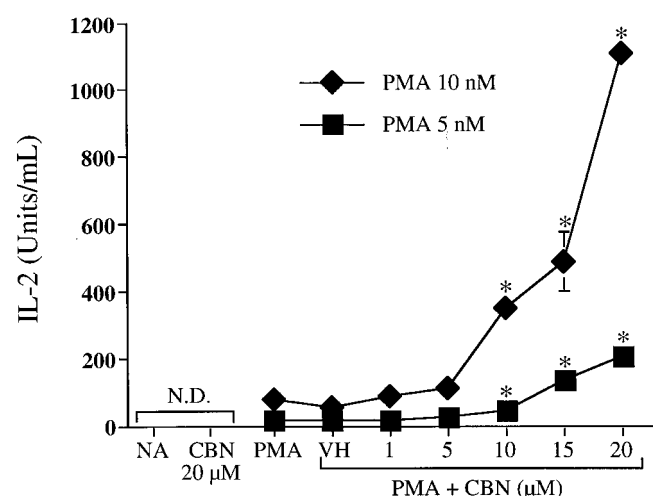
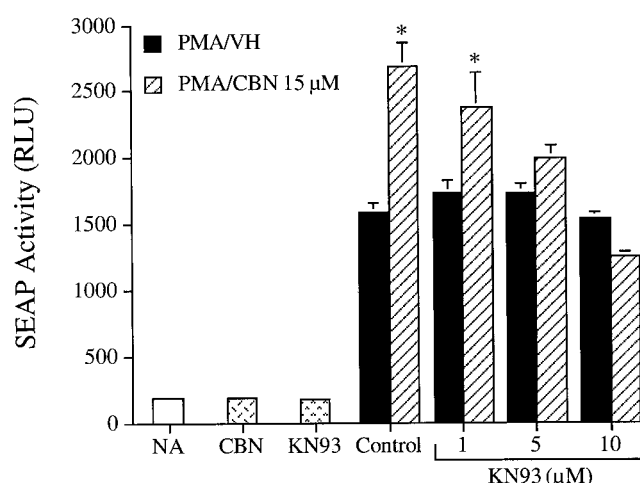


Fig. 5. Effect of CBN on IL-2 distal NF-AT reporter gene activity (A) and IL-2 protein secretion in suboptimally activated EL4 cells (B). EL4 cells (2×10^5 cells/ml) were transiently transfected with pNFAT-SEAP reporter plasmids as described under *Materials and Methods*. The transfected cells were either untreated (NA) or pretreated with CBN (1–20 μ M) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 or 10 nM) for 48 h at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means \pm S.E. of triplicate cultures. N.D., the IL-2 activity was below the level of quantification. *, $p < 0.05$ compared with the matched VH group. Results are representative of three separate experiments.

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion

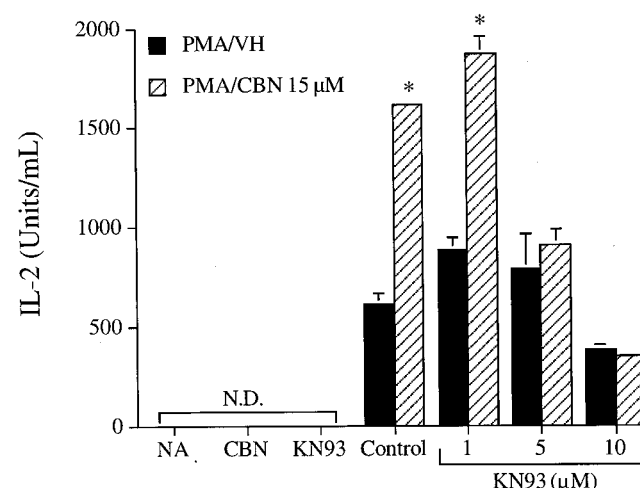


Fig. 6. Reversal by KN93 of CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity and IL-2 protein secretion in suboptimally activated EL4 cells. EL4 cells (2×10^5 cells/ml) were transiently transfected with pNFAT-SEAP plasmids. The transfected cells were either untreated (NA) or pretreated with CBN (15 μ M) for 30 min in the absence (control) or presence of KN93 (0.1–10 μ M). After the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 h at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means \pm S.E. of triplicate cultures. N.D., the IL-2 activity was below the level of quantification. *, $p < 0.05$ compared with the matched PMA/VH group. Results are representative of three separate experiments.

ated by the CB2 Receptor Antagonist SR144528. To examine the putative role of cannabinoid receptors on CBN-mediated IL-2 enhancement, the effect of SR144528 on cannabinoid modulation of NF-AT report activity and IL-2 secretion was investigated. This laboratory has previously reported the identification of CB2 and absence of CB1 mRNA transcripts in EL4 cells by Northern blot (Schatz et al., 1997). Here, RT-PCR was used to confirm the expression of CB2 and absences of CB1 mRNA transcripts in EL4 cells (Fig. 7). Mouse spleen was used as a positive control for both CB1 and CB2 mRNA expression. It is notable that CB1 expression in spleen was modest, as previously reported (Schatz et al., 1997). Based on these findings, the CB2 antagonist SR144528 (0.1–5 μ M) was employed over a range of concentrations that were selected on the basis of previously reported binding affinity for CB2 (Rinaldi-Carmona et al., 1998). CBN-mediated enhancement of the IL-2 distal NF-AT transcriptional activity, as well as the enhancement of IL-2 secretion in transfected EL4 cells, was not attenuated in the presence of SR144528 at any of the concentrations tested (Fig. 8).

Enhancement of IL-2 Secretion by Δ^9 -THC, CBD, and CP55940. To determine whether the CBN-mediated elevation of IL-2 production is unique to this specific cannabinoid, three other cannabinoids were investigated: Δ^9 -THC, CBD, and CP55940. Previously reported radioligand binding analysis (reviewed in Pertwee, 1999) showed that none of the three cannabinoids exhibited significant selectivity for either CB1 or CB2 but differed greatly in CB1 and CB2 binding affinity [CP55940, $K_i \sim 1$ nM; Δ^9 -THC, $K_i \sim 50$ nM; and CBD, $K_i \sim 2000$ nM (Pertwee, 1999)]. Using mouse splenic T cells suboptimally activated with soluble anti-CD3/anti-CD28, conditions under which CBN also produced marked enhancement of IL-2 (Jan and Kaminski, 2001), we assayed IL-2 modulation by the CB1/CB2 high-affinity ligand CP55940 and the low-affinity ligand CBD. Both cannabinoids pro-

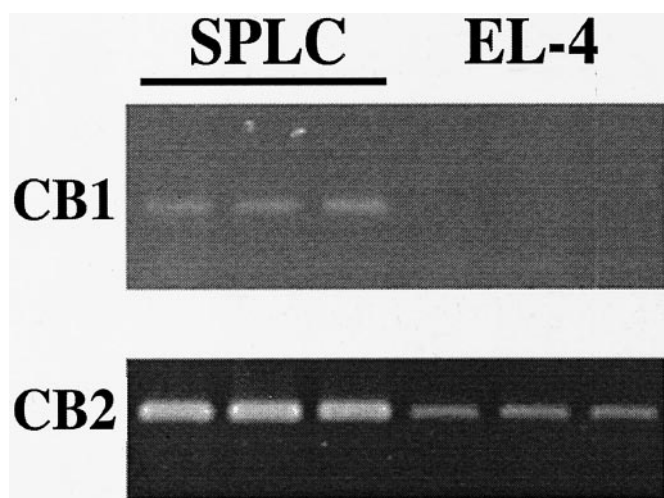
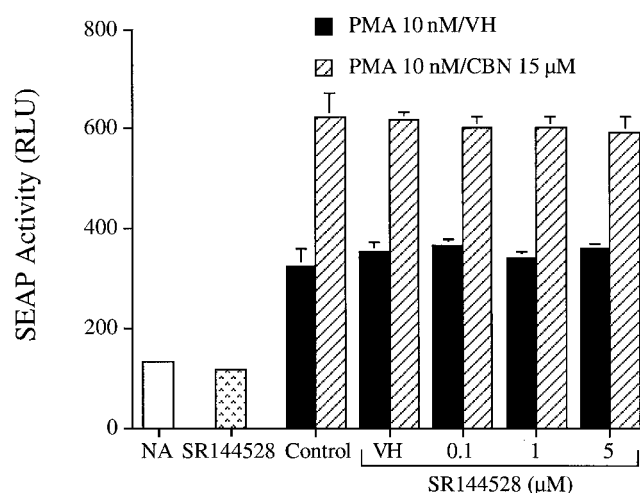


Fig. 7. RT-PCR analysis of CB1 and CB2 in EL4 cells and mouse spleen. Total RNA was isolated from EL4 cells and mouse spleen. Three separate RNA isolates were assayed for expression of CB1 and CB2 mRNA transcripts by RT-PCR. Each RNA sample was confirmed to be free of DNA contamination as determined by the absence of product after PCR amplification in the absence of reverse transcriptase (data not shown). RNA (100 ng) was reverse transcribed and then amplified using 35 cycles (conditions described under *Materials and Methods*) for detection of CB2 and CB1.

duced a concentration-dependent enhancement of IL-2 secretion that was similar in magnitude over a comparable concentration range (Fig. 9). An additional series of experiments was performed to investigate the IL-2 enhancing activity of CP55940 (10 μ M) and Δ^9 -THC (10 μ M) in suboptimally activated (10 nM PMA) EL4 cells. To more directly examine the putative role of CB2 on cannabinoid-mediated IL-2 modulation, measurements were made in the presence and absence of increasing concentrations of SR144528. Both CP55940 and Δ^9 -THC induced a similar magnitude of enhancement on IL-2 secretion by EL4 cells (Fig. 10). SR144528 did not attenuate the cannabinoid-mediated enhancement of IL-2 secretion by

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion

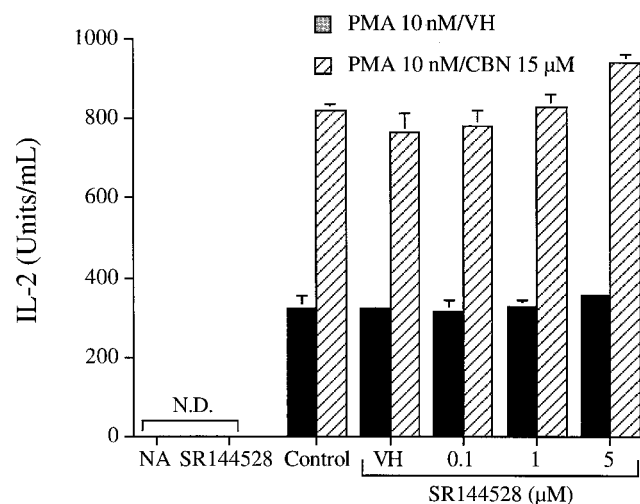


Fig. 8. No reversal by SR144528 of CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity and IL-2 protein secretion in suboptimally activated EL4 cells. EL4 cells (2×10^5 cells/ml) were transiently transfected with pNFAT-SEAP plasmids. The transfected cells were either untreated (NA) or pretreated with CBN (15 μ M) for 30 min in the absence (VH; 0.05% DMSO) or presence of SR144528 (0.1–5 μ M). After the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 h at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means \pm S.E. of triplicate cultures. N.D., the IL-2 activity was below the level of quantification. *, $p < 0.05$ compared with the matched PMA/VH group. Results are representative of three separate experiments.

either CP55940 or Δ^9 -THC. Although there was a modest trend toward attenuation of the cannabinoid-induced IL-2 enhancement by SR144528 at the highest concentration employed (5 μ M), the effect was not statistically different from the comparative control (i.e., cannabinoid-mediated enhancement of IL-2 in the absence of SR144528). SR144528 produced no effect on IL-2 secretion in the absence of CP55940 or Δ^9 -THC. There was no effect on cell viability in any of the treatment groups.

Discussion

Plant-derived cannabinoids are known to differentially modulate IL-2 secretion by T cells (Pross et al., 1992; Nakano et al., 1993a; Condie et al., 1996; Jan and Kaminski, 2001). Recently, we demonstrated that the principal factor that influenced whether CBN treatment enhanced or inhibited IL-2 production was the magnitude of T cell activation. In the presence of strong activation stimuli, CBN markedly inhibited IL-2. Conversely, CBN markedly potentiated IL-2 production under conditions of suboptimal T cell activation (Jan and Kaminski, 2001). Here, we extend our characterization of the underlying molecular mechanism by which CBN enhances IL-2 expression through several key observations.

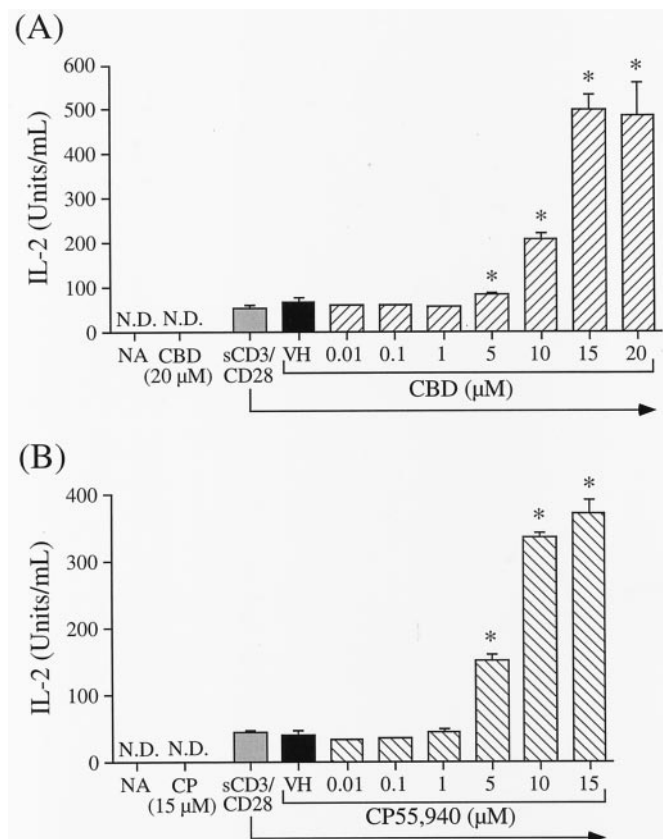


Fig. 9. Concentration-dependent enhancement by CBD and CP55,940 of IL-2 secretion by sCD3/CD28-activated splenocytes. Splenocytes (2×10^6 cells/ml) were either untreated (NA), or pretreated for 30 min with CBD (0.01–20 μ M) and/or VH (0.1% ethanol) (A) or CP55940 (0.01–15 μ M) and/or VH (0.15% DMSO) (B). Splenocytes were activated with sCD3/CD28 (2 μ g/ml of each antibody). After 48 h of culture, the supernatants were assayed for IL-2 protein by ELISA. Data are expressed as the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with the VH control group. N.D., IL-2 protein was below the level of quantification. Results are representative of three independent experiments.

Most significantly, the present studies suggest that the enhancement of IL-2 production by CBN 1) is caused, at least in part, by an increase in NF-AT DNA binding and transcriptional activity at the IL-2 NF-AT element; 2) is associated with a concomitant enhancement of steady state IL-2 mRNA expression; 3) occurs through a CB1- and CB2-independent mechanism; 4) involves increased CaM kinase activity; and 5) is not unique to CBN because several other cannabinoids tested in this study exerted similar potentiating activity.

Regulation of IL-2 is primarily mediated at the level of transcription, as evidenced by the fact that resting T cells exhibit virtually no background IL-2 mRNA expression; however, there is rapid onset of IL-2 transcription in response to activation stimuli. Even in the case of the EL4 cell line, which can be induced to secrete large quantities of IL-2, IL-2 mRNA expression in nonactivated EL4 cells was below the level of quantification (<100 transcripts/100 ng RNA) as determined by quantitative RT-PCR. Consistent with regulation at the

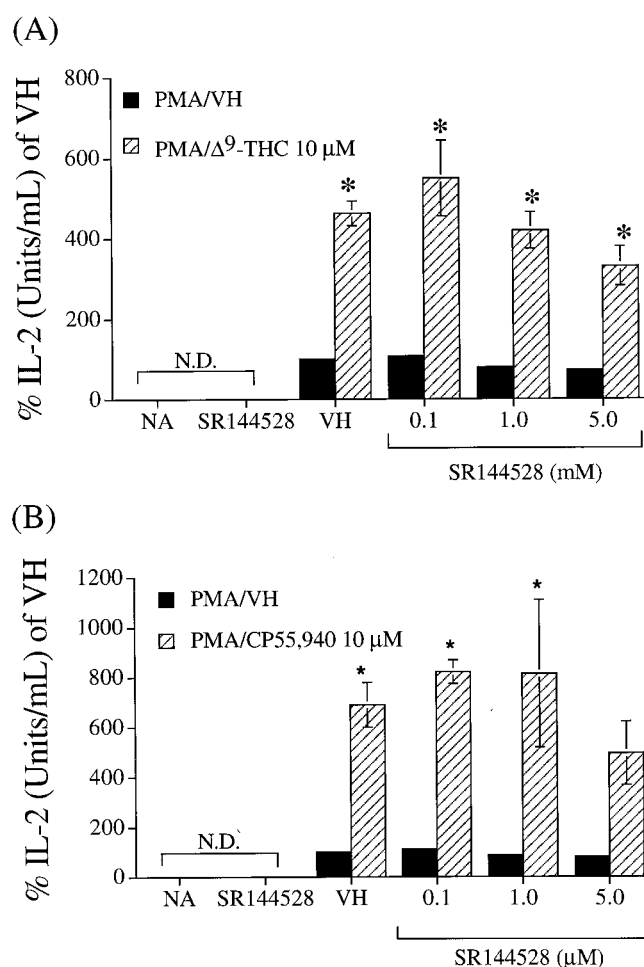


Fig. 10. No antagonism by SR144528 of Δ^9 -THC and CP55940 mediated enhancement of IL-2 protein secretion in suboptimally activated EL4 cells. EL4 cells (2×10^5 cells/ml) were either untreated (NA) or pretreated with CP 55,940 (10 μ M) (A) or Δ^9 -THC (B) for 30 min in the absence (VH; 0.05% DMSO) or presence of SR144528 (0.1–5 μ M). After the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 h at 37°C. The supernatants were assayed for IL-2 protein by ELISA. Results are expressed as the means percent of IL-2 (Units/ml) in the vehicle control \pm S.E. of triplicate cultures. N.D., the IL-2 activity was below the level of quantification. *, $p < 0.05$ compared with the matched PMA/VH group. Results represent pooled data from three (A) or two (B) separate experiments.

transcriptional level, the present studies show that under the conditions in which CBN potentiated IL-2 secretion, there was a parallel increase in steady state IL-2 mRNA expression. The regulation of IL-2 is complex and involves multiple transcription factors, including NF-AT, NF- κ B, AP-1, and Oct, which are in turn regulated through distinct but yet overlapping signaling pathways (Novak et al., 1990; Jain et al., 1995; Serfling et al., 1995). Results in the present study suggest that the modulation of the NF-AT signaling pathway is a critical component of the molecular mechanism by which CBN treatment enhances IL-2 production in suboptimally activated T cells. Several lines of evidence supported this conclusion. First, under experimental conditions in which CBN enhanced IL-2 protein secretion and steady-state mRNA expression, there was a parallel increase in the DNA binding activity at the IL-2 NF-AT site. Moreover, the increase in NF-AT binding activity seems to be a highly specific effect because a concomitant enhancement in DNA binding activity was not observed for either NF- κ B or AP-1. Super-shift assays confirmed that NF-AT was a component of the NF-AT-induced protein complex. Second, transient transfection experiments showed that the increase in NF-AT DNA binding activity by CBN correlated closely with enhanced NF-AT reporter gene activity and IL-2 secretion. Lastly, there was a parallel attenuation by KN93 treatment of CBN-mediated NF-AT reporter gene activity and IL-2 secretion. Concentration response studies with the CaM kinase inhibitor revealed that although a high concentration of KN93 (10 μ M) alone inhibited IL-2 production, at lower concentrations, KN93 completely attenuated CBN-mediated enhancement of IL-2 production and NF-AT reporter gene activity but alone produced no effect on IL-2 secretion by EL4 cells. Collectively, these results strongly suggest that the increase in NF-AT activity is a critical component of the mechanism responsible for enhancement of IL-2 by CBN.

A major objective of the present studies was to critically evaluate the putative involvement of cannabinoid receptors on the IL-2 enhancing activity by CBN. Although the specific cellular target with which CBN interacts to enhance IL-2 production in suboptimally activated T cells remains to be elucidated, our results seem to exclude both CB1 and CB2. The fact that EL4 cells do not express CB1 receptors, as suggested previously by Northern blotting and presently by RT-PCR, greatly simplified this analysis by ruling out the involvement of CB1. In turn, several different strategies were employed to investigate a putative involvement by CB2. The most direct approach involved the employment of the CB2 selective antagonist, SR144528. Our studies clearly show that SR144528 failed to attenuate CBN-mediated enhancement of NF-AT reporter gene activity or IL-2 secretion over a broad concentration range (0.1–5 μ M). Another critical observation supporting a lack of CB2 involvement was the striking similarity in the potency of CP55940, CBN, Δ^9 -THC, and CBD in enhancing IL-2 secretion despite the marked differences in CB1/CB2 binding activity between these cannabinoids. In this respect, the most remarkable comparison was between the CB1/CB2 high-affinity ligand CP55940 and the CB1/CB2 low-affinity ligand CBD. These two cannabinoids differ by more than 1000-fold in cannabinoid receptor binding affinity, yet both exhibited a similar degree of potency in enhancing IL-2. The findings are also significant because they suggest that there is something inherent in the

cannabinoid structure that is critical to the IL-2 enhancing activity yet it does not follow a structure activity consistent with either CB1 or CB2 binding affinity. This observation is equally critical in that although in the present study relatively high concentrations of individual cannabinoids were required to produce IL-2 enhancement, marijuana smoke contains more than 60 different structurally related cannabinoids. The cumulative cannabinoid concentration contained in marijuana smoke, especially within the lungs of marijuana smokers, are probably comparable with the concentration levels used with individual cannabinoid congeners in the present study.

That the same concentration of CBN could enhance or inhibit IL-2 expression, depending on the magnitude of T cell activation, suggests that cannabinoid treatment may actually provide a positive signal for T cell activation that is supraoptimal under normal activation conditions. It is notable that we have previously reported that under those conditions in which CBN treatment enhanced IL-2 there was a concomitant increase in the activated form of the ERK MAP kinases p42/p44 (Jan and Kaminski, 2001). Likewise, under optimal T cell activation conditions, the same concentration of CBN produced a marked decrease in activated ERK MAP kinases (Faubert and Kaminski, 2000; Jan and Kaminski, 2001). These previous studies also implicated the involvement of PKC, an upstream regulator of ERK MAP kinases, and the involvement of CaM kinases as staurosporin and KN93, respectively, attenuated the CBN-mediated enhancing activity. The possibility that the CBN-mediated enhancing activity may involve positive regulation of PKC is consistent with at least one report showing that CBN, CBD, and Δ^9 -THC all increased PKC activity at concentrations similar to those used in these studies (Hillard and Auchampach, 1994). An equally important point is that it is unlikely that changes in calcium homeostasis are involved in the cannabinoid-mediated enhancement of IL-2 because PMA treatment, in the absence of a calcium ionophore, robustly induced IL-2 secretion in EL4 cells. Moreover, PMA concentrations that induce EL4 cells to secrete IL-2 do not induce a rise in intracellular calcium (G. K. Rao, unpublished observations). Collectively, these findings implicate the modulation of PKC and downstream targets, including ERK MAP kinases and CaM kinases, but exclude changes in calcium homeostasis.

In summary, the present studies demonstrate that the enhancement of IL-2 secretion by CBN is mediated through a positive influence on the NF-AT signaling cascade, which correlates closely with an increase in IL-2 steady-state mRNA expression. Although the precise molecular mechanism for this effect remains to be elucidated, these and previous studies suggest that the mechanism is CB1/CB2-independent and involves increased activity by PKC, ERK MAP kinases, and CaM kinases, all of which are widely established as upstream regulators of NF-AT. In light of the critical role NF-AT plays in the regulation of a large number of cytokine genes, in addition to IL-2, these findings are important because they have potentially identified a common intracellular target that can account, at least in part, for the diverse effects cannabinoids exert on immune function.

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