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Inhibition of the Cyclic AMP Signaling Cascade and Nuclear Factor Binding to CRE and κB Elements by Cannabinol, a Minimally CNS-Active Cannabinoid

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ABSTRACT. Immune suppression by cannabinoids has been widely demonstrated in a variety of experimental models. The identification of two major types of G-protein-coupled cannabinoid receptors expressed on leukocytes, CB1 and CB2, has provided a putative mechanism of action for immune modulation by cannabinoid compounds. Ligand binding to both receptors negatively regulates adenylate cyclase, thereby lowering intracellular cyclic AMP (cAMP) levels. In the present studies, we demonstrated that cannabinol (CBN), a ligand that exhibits higher binding affinity for CB2, modulates immune responses and cAMP-mediated signal transduction in mouse lymphoid cells. Direct addition of CBN to naive cultured splenocytes produced a concentration-dependent inhibition of lymphoproliferative responses to anti-CD3, lipopolysaccharide, and phorbol-12-myristate-13-acetate/ionomycin stimulation. Similarly, a concentration-related inhibition of the *in vitro* anti-sheep red blood cell IgM antibody forming cell response was also observed by CBN. Evaluation of cAMP signaling in the presence of CBN showed a rapid and concentration-related inhibition of adenylate cyclase activity in both splenocytes and thymocytes. This decrease in intracellular cAMP levels produced by CBN resulted in a reduction of protein kinase A activity, consequently leading to an inhibition of transcription factor binding to the cAMP response element and κB motifs in both cell preparations. Collectively, these results demonstrate that CBN, a cannabinoid with minimal CNS activity, inhibited both cAMP signal transduction and immune function, further supporting the involvement of CB2 receptors in immune modulation by cannabimimetic agents. BIOCHEM PHARMACOL **55**;7:1013–1023, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cannabinol; thymocytes; cAMP signaling cascade; CREB; NF-κB; immune suppression

The specific mechanism by which cannabinoids elicit their broad range of biological effects has been elusive. With the isolation and cloning of two major types of cannabinoid receptors, CB1† [1] and CB2 [2], important insight has been gained into the cellular mechanism of action by cannabimimetic agents. Both receptors are G-protein coupled and possess the characteristic seven transmembrane domains. Ligand binding to either CB1 or CB2 produces a marked inhibition of adenylate cyclase activity which is abrogated by ADP-ribosylation of G_i proteins with pertussis toxin [3, 4]. The inhibition of adenylate cyclase by cannabinoids was initially described in neuroblastoma cell lines [5] and has been identified since in several cell types including rat Sertoli cells [6], human leukemic cells [7], mouse splenocytes [8], the EL-4.IL-2 and RAW 264.7 cell lines [9, 10], and CHO cells transfected with cannabinoid receptors [11, 12]. This disruption of adenylate cyclase activity by cannabinoids strongly implicates a role by the cAMP signal transduction pathway in mediating the biological actions by these compounds.

The tissue and cell-type distribution of CB1 and CB2 have not been comprehensively characterized yet. CB1 was cloned originally from rat cerebral cortex [1] and is expressed predominantly within the CNS, whereas CB2 was isolated from the promyelocytic leukemia cell line HL60 [2], and appears to be primarily expressed on immunocompetent cells. The variation in tissue distribution for CB1 and CB2 is quite intriguing as is the fact that many cannabinoid receptor ligands exhibit similar binding affinities for both receptors in spite of the marked structural difference between the two receptor types [13]. CB1 and CB2 share only 44% identity, which increases to a modest 68% when comparing the transmembrane domains, that portion of the receptor that constitutes the putative ligand binding pocket. Interestingly, cannabinol, a cannabinoid

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† *Abbreviations:* CB, cannabinoid receptor; G protein, guanine-nucleotide-binding protein; cAMP, cyclic adenosine 3′:5′-monophosphate; Δ^9-THC, delta-9-tetrahydrocannabinol; PMA, phorbol-12-myristate-13-acetate; Io, ionomycin; sRBC, sheep red blood cells; LPS, lipopolysaccharide; CREB, cAMP response element binding protein; CRE, cAMP response element; ATF, activating transcription factor; IgM, immunoglobulin M; PKA, protein kinase A; NF-κB, nuclear factor for immunoglobulin κ chain in B cells; RIA, radioimmunoassay; IL, interleukin; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; AFC, antibody forming cell(s); and EMSA, electrophoretic mobility shift assay.

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with minimal CNS activity, is one ligand capable of discriminating between the two receptors, demonstrating greater binding affinity (approximately 10-fold) to CB2 than CB1 [2, 14, 15].

It is well established that cannabinoids inhibit immune function [16, 17]. Moreover, the identification of RNA transcripts for CB1 and CB2 in lymphoid tissues and leukocyte-derived cell lines strongly suggests the expression of cannabinoid receptors within the immune system [2, 15, 18–20]. Multiple lines of evidence further support the functional expression of cannabinoid receptors by leukocytes including: (a) inhibition of adenylate cyclase activity by Δ^9-THC [8]; (b) saturable specific binding of [^{3}H]CP-55940, a high affinity cannabinoid receptor ligand [19]; and (c) stereoselective inhibition of the anti-sRBC antibody forming cell response [19]. Together these findings established a receptor-mediated mechanism of immune suppression by cannabinoids; however, which form of the cannabinoid receptor is primarily responsible for eliciting the aforementioned profile remains unclear. Analysis of receptor expression by competitive ligand binding, northern blot analysis, and quantitative *reverse transcriptase-polymerase chain reaction* (RT-PCR) suggests that CB2 is the predominant cannabinoid receptor on immunocompetent cells [2, 15, 21]. This is further supported by our recent work demonstrating that cannabinoids inhibit signal transduction through the cAMP cascade in EL-4.IL-2 and RAW 264.7 cells, resulting in the inhibition of IL-2 and nitric oxide production, respectively, in two leukocyte-derived cell lines that express only CB2 receptor mRNA [9, 10]. Therefore, the objective of the present studies was to examine the effects of cannabinol on immune function and cAMP signal transduction in primary mouse leukocytes in order to further characterize the role of CB2 in mediating the immunomodulatory effects by cannabinoids. The predominant expression of CB2 in the immune system implicates this receptor as a putative target for immune modulation by CNS-inactive cannabimimetic agents.

MATERIALS AND METHODS

Animals

Virus-free female B6C3F1 mice, 5–6 weeks of age, were purchased from the NCI. On arrival, mice were randomized, transferred to plastic cages containing a saw dust bedding (5 mice/cage) and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad lib.* and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were kept at 21–24° and 40–60% relative humidity with a 12-hr light/dark cycle.

Chemicals

Cannabinol and Δ^9-THC were both provided by the National Institute on Drug Abuse.

Culture Medium

Splenocytes were cultured in RPMI 1640 supplemented with 5% bovine calf serum (Hyclone), 2 mM of L-glutamine, antibiotic–antimicotic (100 units penicillin and 100 μg streptomycin) (GIBCO), and 5×10^{-5} M of 2-mercaptoethanol (complete RPMI medium). For EMSA, 1% bovine calf serum was used.

In Vitro *Proliferative Responses to Anti-CD3, LPS, or PMA/Io*

Lymphoproliferation assays were performed as previously described [4, 22]. Splenocytes were cultured in a 96-well culture plate at 1×10^5 cells/well in the presence of either vehicle (0.1% ethanol), cannabinol (1, 5, 10, 15, 20 μM), or Δ^9-THC (22 μM) and stimulated with either anti-CD3 (1.0 μg/mL), LPS (100 μg/mL), or PMA (80 nM) plus Io (1 μM). Splenocytes were cultured at 37° and 5% CO_2 for 48 hr (PMA/Io) or 72 hr (anti-CD3 and LPS) and pulsed with 1.0 μCi/well of [^{3}H]thymidine for the last 12 hr of the culture period. Cells were harvested onto glass fiber filters with a PHD Cell Harvester (Cambridge Technology, Inc.), and tritium incorporation was measured using a Packard 460C liquid scintillation counter. Results are expressed as mean counts per minute ± SEM of quadruplicate samples.

In Vitro *Antibody Forming Cell Response*

The T-dependent AFC response was performed as described previously [22]. Briefly, splenocytes were cultured at 1×10^7 cells/mL in a 48-well Costar culture plate, and Δ^9-THC (22 μM), cannabinol (1, 5, 10, 15, 20 μM), or vehicle (0.1% ethanol) was added directly to spleen cell cultures. Each well was sensitized with 6.5×10^6 sRBC and cultured for 5 days in a Bellco stainless steel tissue culture chamber pressurized to 6.0 psi with a gas mixture consisting of 10% O_2, 7% CO_2, and 83% N_2. The culture chamber was rocked continuously on a rocking platform for the duration of the culture period. Enumeration of the AFC response and cell viability was performed as previously described [23].

cAMP Determination and Analysis of PKA Activity

Intracellular cAMP levels were assessed as previously described [4]. Briefly, splenocytes were incubated with either vehicle (0.1% ethanol), Δ^9-THC (22 μM), or cannabinol (1, 5, 10, 15, 20 μM) for 10 min followed by stimulation with forskolin (50 μM) (Calbiochem) for 15 min at 37°. Levels of cAMP in reconstituted cell lysates were determined using a cAMP RIA assay kit (Diagnostic Products Inc.). The concentration of cAMP in each sample was determined by comparison with a standard curve. The PKA assay was performed according to Condie *et al.* [9]. Splenocytes were lysed in ice-cold lysis buffer (0.25 M of sucrose, 5 mM of Tris base, 5 mM of EGTA, 1 mM of PMSF, 0.1 mM of DTT, 0.1% Trition X-100, 10 μg/mL of leupeptin,

TABLE 1. Effect of cannabinol (CBN) on mitogen-stimulated proliferation

	Mitogenic stimuli					
Treatment	Anti-CD3 (cpm)	% Control	LPS (cpm)	% Control	PMA/Io (cpm)	% Control
No mitogen	1,200 ± 70*	7	1,020 ± 100*	3	1,370 ± 170*	2
NA	18,370 ± 780	102	34,540 ± 1,280	94	90,020 ± 2,860	101
VH	17,930 ± 1,010	100	36,900 ± 1,610	100	89,180 ± 3,430	100
THC, 22 μM	8,060 ± 450*	45	8,960 ± 650*	24	44,590 ± 3,650*	50
CBN, 1 μM	20,020 ± 490	112	29,970 ± 220*	81	76,300 ± 860*	86
CBN, 5 μM	22,680 ± 1,300*	126	25,410 ± 340*	69	63,870 ± 1,550*	72
CBN, 10 μM	20,790 ± 1,250	116	21,390 ± 1,250*	58	41,340 ± 1,940*	46
CBN, 15 μM	12,470 ± 900*	70	19,150 ± 1,390*	52	37,720 ± 2,510*	42
CBN, 20 μM	4,680 ± 790*	26	14,000 ± 900*	38	16,620 ± 760*	19

Naive (NA) female B6C3F1 mouse spleens were isolated and made into a single cell suspension. Splenocytes were cultured in a 96-well plate at 1×10^5 cells/well in the presence of either vehicle (VH; 0.1% EtOH), CBN, or Δ^9-THC and stimulated with either anti-CD3 (0.5 μg/mL), LPS (100 μg/mL), or PMA (80 nM) and Io (1 μM). Splenocytes were pulsed with [^{3}H]thymidine for the last 12 hr of the culture period. Data are expressed as the mean cpm ± SEM for quadruplicate samples.

*$P < 0.05$ (determined by Dunnett's *t*-test) with comparison to the vehicle group.

10 mg/mL of aprotinin) followed by gentle sonication. Aliquots of the cell extract (100 μL) were incubated with appropriate concentrations of cannabinol or Δ^9-THC for 5 min in triplicate for use in the PKA assay (GIBCO BRL).

EMSA

Nuclear proteins were isolated as described previously [24]. Briefly, lymphocytes were stimulated with forskolin (50 μM) in the presence and absence of cannabinol (20 μM) for 15, 30, 60, 90, and 120 min. Following treatment, cells were lysed with HB buffer (10 mM of HEPES, 1.5 mM of $MgCl_2$), and the nuclei were pelleted by centrifugation at 6700 *g* for 5 min. Nuclei were lysed in hypertonic buffer (30 mM of HEPES, 1.5 mM of $MgCl_2$, 450 mM of NaCl, 0.3 mM of EDTA, and 10% glycerol) supplemented with 1 mM of DTT, 1 mM of PMSF, and 1 μg/mL of aprotinin and leupeptin. Following nuclear lysis, the samples were centrifuged at 17,500 *g* for 15 min, and the supernatant was retained for use in the DNA binding assay. DNA oligomers containing either the CRE (TGACGTCA) or the κB (GGGGACTT TCC) consensus sequence were end-labeled with [γ-^{32}P] dATP. Nuclear extract (3 μg) was incubated in binding buffer (100 mM of NaCl, 30 mM of HEPES, 1.5 mM of $MgCl_2$, 0.3 mM of EDTA, 10% glycerol, 1 mM of DTT, 1 mM of PMSF, and 1 μg/mL of aprotinin and leupeptin) with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled probe for 10 min on ice. DNA binding activity was separated from free probe using a 4% acrylamide gel (National Diagnostics) in 1 × TBE buffer (89 mM of Tris, 89 mM of boric acid, and 2 mM of EDTA). After electrophoresis, the gel was dried and autoradiographed for analysis.

Statistical Analysis

The mean ± SEM 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 [25].

RESULTS

Inhibition of Functional Immune Responses by Cannabinol

To determine the immunomodulatory activity of cannabinol, mitogen-induced proliferation of mouse splenocytes was measured. As presented in Table 1, cannabinol inhibited anti-CD3-induced proliferation by 30 and 74% at 15 and 20 μM, respectively. Cannabinol also produced a concentration-dependent inhibition of LPS-induced proliferation at all concentrations (1, 5, 10, 15, 20 μM) tested (Table 1). It is notable that the magnitudes of inhibition by Δ^9-THC (22 μM), a positive control, and cannabinol (20 μM) were very comparable. Cannabinol was also found to alter antigen receptor independent lymphocyte activation, as shown by a marked inhibition of PMA/Io-induced proliferation. Interestingly, as reported previously with Δ^9-THC, of these three proliferative stimuli tested, the PMA/Io response appeared to be the most sensitive to inhibition by cannabinol [4, 22].

The effects of cannabinol on humoral immunity were investigated by employing the hemolytic plaque assay. The IgM T-dependent AFC humoral response has been shown previously to be highly sensitive to inhibition by cannabinoids [22]; therefore, similar studies were performed with cannabinol. In the present studies, cannabinol exhibited a concentration-dependent inhibition of the *in vitro* T-dependent AFC response at 10, 15, and 20 μM, as compared with the vehicle control (0.01% ethanol) shown in Table 2. No effect on cell viability was observed at any of the cannabinol concentrations tested.

Inhibition of Forskolin-Stimulated Adenylate Cyclase Activity by Cannabinol in Mouse Lymphoid Cells

The binding of Δ^9-THC to cannabinoid receptors has been widely established to negatively regulate adenylate cyclase activity in a variety of leukocyte preparations [4, 8–10]. Due to the similarity in structure of cannabinol to Δ^9-THC and their comparable binding affinities in mouse spleno-

TABLE 2. Effect of cannabinol (CBN) on the *in vitro* IgM AFC response to sRBC

Treatment	AFC/10^6 splc	% Control	Viability (%)
NA	1046 ± 65	118	70 ± 0.5
VH	884 ± 28	100	78 ± 3
THC, 22 μM	265 ± 22*	30	80 ± 2
CBN, 1 μM	643 ± 105	73	78 ± 2
CBN, 5 μM	672 ± 33	76	81 ± 3
CBN, 10 μM	590 ± 45*	67	87 ± 2
CBN, 15 μM	545 ± 27*	62	79 ± 3
CBN, 20 μM	386 ± 51*	44	91 ± 3

Spleens from naive (NA) female B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes (1×10^7 cells/mL) were added to a 48-well culture plate and treated with either vehicle (VH; 0.1% EtOH), CBN, or Δ^9-THC. The cultures were sensitized with sRBC (1×10^9 cells/mL). On day 5, the number of AFC were determined. Results are expressed as means ± SEM for quadruplicate samples.

*$P < 0.05$ (determined by Dunnett's *t*-test) with comparison to the vehicle group.

cytes [15], studies were conducted to determine if cannabinol, presumably acting through the CB2 receptor, would likewise inhibit adenylate cyclase. Mouse splenocytes treated with forskolin for 15 min exhibited stimulation of adenylate cyclase as demonstrated by approximately a 4-fold increase in intracellular cAMP as compared to the unstimulated naive and vehicle-treated cells (Fig. 1). Pretreatment of splenocytes with cannabinol prior to forskolin stimulation decreased intracellular cAMP by 25% at 15 and 20 μM. The magnitude of inhibition by 20 μM of cannabinol was again comparable to that by 22 μM of Δ^9-THC, the positive control. We also investigated the effects of

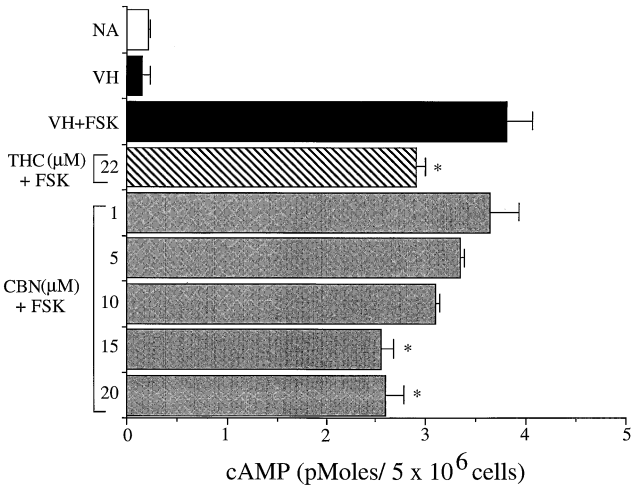

FIG. 1. Inhibition of cAMP production by cannabinol (CBN) in forskolin-stimulated mouse splenocytes. Spleens were isolated and made into a single cell suspension of 5×10^6 cells/mL. Splenocytes were treated with either vehicle (0.1% ethanol), CBN, or Δ^9-THC for 10 min followed by a 15-min forskolin stimulation (50 μM). Intracellular cAMP values from one of three independent experiments are expressed as the means ± SEM for triplicate samples as determined for each group. NA = naive; VH = vehicle. *$P < 0.05$ (determined by Dunnett's *t*-test) in comparison to the forskolin-stimulated vehicle group.

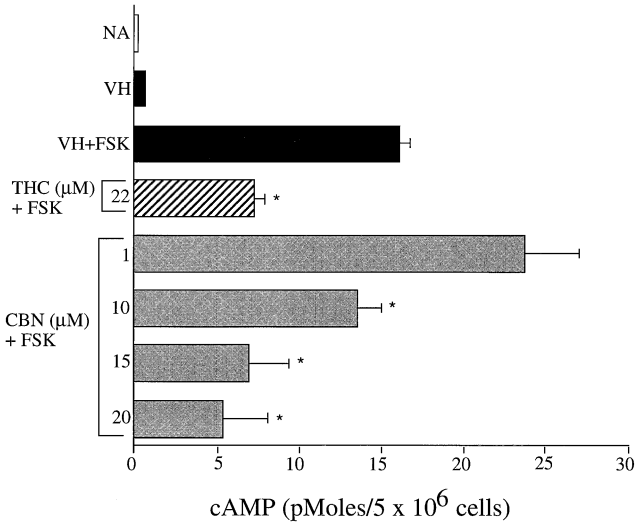

FIG. 2. Cannabinol (CBN)-mediated inhibition of forskolin-stimulated adenylate cyclase activity in mouse thymocytes. Thymocytes were freshly isolated, adjusted to 5×10^6 cells/mL, and incubated with either vehicle (0.1% ethanol), CBN, or Δ^9-THC for 10 min followed by a 15-min stimulation with forskolin (50 μM). Intracellular cAMP values from one of three independent experiments are expressed as the means ± SEM for triplicate samples as determined for each group. NA = naive; VH = vehicle. *$P < 0.05$ (determined by Dunnett's *t*-test) in comparison to the forskolin-stimulated vehicle group.

cannabinol on adenylate cyclase activity in thymocytes because our past studies have shown T-cells to be markedly sensitive to inhibition by cannabinoid compounds [9, 19, 22]. Consistent with this observation, cannabinol concentration-dependently inhibited forskolin-stimulated adenylate cyclase activity in mouse thymocytes (Fig. 2). Interestingly, the increase in adenylate cyclase activity by forskolin was significantly greater in thymocytes than in splenocyte preparations. Scherer and coworkers [26] have demonstrated recently a similar difference in intracellular cAMP levels following forskolin stimulation of thymocytes, suggesting that cAMP may play a critical role in T-cell differentiation. Moreover, the magnitude of adenylate cyclase inhibition by cannabinol was significantly greater in thymocytes than in splenocytes, further supporting the sensitivity of T-cells to cannabinoids.

Effect of Cannabinol on PKA Activity

PKA is immediately downstream from adenylate cyclase and is comprised of regulatory and catalytic subunits. Increases in intracellular cAMP activate PKA by binding to the regulatory subunit, resulting in the dissociation and activation of the kinase catalytic subunit. We have shown in EL-4 cells that inhibition of adenylate cyclase activity by Δ^9-THC consequently leads to a reduction in PKA activity [9]. Considering these findings together with the inhibition of adenylate cyclase by cannabinol, splenocyte PKA activity was evaluated in the presence of cannabinol. As shown in Fig. 3, cannabinol produced a concentration-dependent

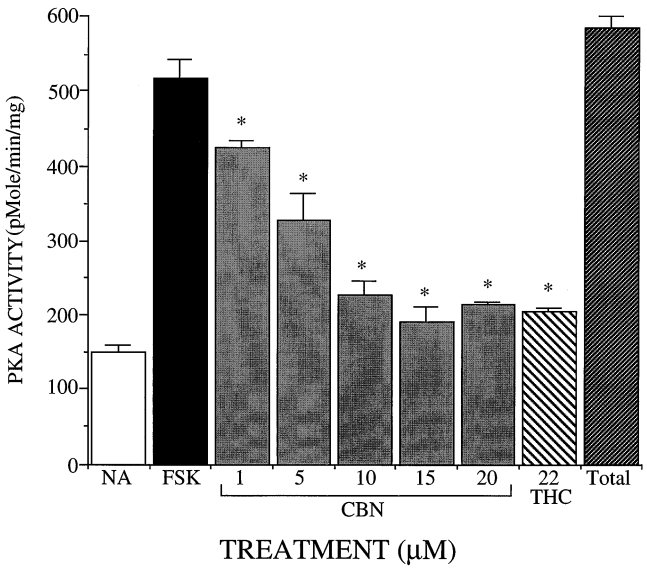

FIG. 3. Inhibition of PKA activity in splenocytes by cannabinol (CBN). Cell extracts were preincubated with CBN or Δ^9-THC for 5 min and then placed in reaction mixture in the presence or absence of forskolin (50 μM) for 10 min. Values from one of two independent experiments are expressed as the means ± SEM for triplicate samples as determined for each group. NA = naive. *$P < 0.05$ (determined by Dunnett's *t*-test) as compared with the forskolin control group.

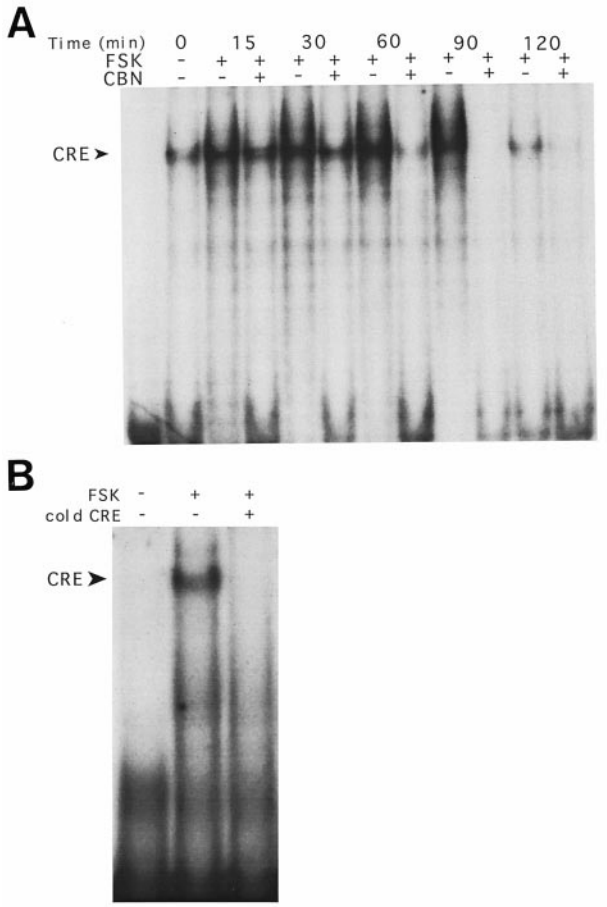

FIG. 4. Inhibition of protein/DNA binding to the CRE consensus motif in mouse splenocytes by cannabinol (CBN). (A) Nuclear extracts (3 μg) from treated and untreated splenocytes were incubated with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated splenocytes. Lanes 3, 5, 7, 9, and 11 represent forskolin-stimulated splenocytes, while lanes 4, 6, 8, 10, and 12 indicate forskolin-stimulated/CBN-treated splenocytes. (B) Cold competitor studies were done by adding 1 pmol of unlabeled CRE to the nuclear extract isolated from the 90-min forskolin sample. Results are representative of four separate experiments.

inhibition of PKA activity at all concentrations (1, 5, 10, 15, 20 μM) tested. Again, the magnitude of inhibition between cannabinol and Δ^9-THC was comparable at 20 and 22 μM, respectively. It is notable that similar experiments were performed in the presence of exogenous cAMP, and no direct inhibition of PKA activity was observed with cannabinoids (data not shown). These studies indicate that the inhibition of PKA activity is mediated through an inhibition of cAMP formation.

Inhibition of Transcription Factor Binding to a CRE and κB Consensus Motif by Cannabinol

Upon activation of PKA, the catalytic subunits translocate to the nucleus to phosphorylate target proteins including the CREB/ATF family of transcription factors [27, 28]. Due to the inhibition of cAMP formation and PKA activity by cannabinol, the binding of PKA-activated transcription factors to DNA consensus motifs was investigated. These studies demonstrated that forskolin treatment alone induced binding to the CRE in spleen cells at 15, 30, 60 and 90 min (Fig. 4A compare lane 2, no forskolin stimulation, to lanes 3, 5, 7, and 9), which returned to basal level at 120 min. The observed time course is typical for forskolin-stimulated CRE protein binding following activation of the cAMP signaling cascade as measured by gel shift assays in a variety of cell types [29, 30]. Conversely, protein/CRE binding was decreased markedly in nuclear extracts isolated from cannabinol-treated splenocytes as evident at 15, 30, 60, 90, and 120 min (Fig. 4A; lanes 4, 6, 8, 10, and 12, respectively). The specificity of protein binding was demonstrated by the addition of excess unlabeled CRE oligonucleotide (Fig. 4B). CRE binding was also investigated in thymocytes under identical conditions to those used in splenocyte preparations. A distinct protein complex was induced by forskolin treatment at 15, 30, 60, 90, and 120 min (Fig. 5A; lanes 3, 5, 7, 9, and 11) with maximum binding detected at 90 min. The kinetics of CRE binding appear to be slightly delayed in the thymocytes as compared with splenocytes in that protein binding was still detected in thymocytes at 120 min, whereas the binding activity returned to basal levels by 120 min in the splenocytes. Similarly, stimulation of thymocytes with forskolin in the presence of cannabinol resulted in a marked inhibition of CRE binding at all time points assayed (Fig. 5A; lanes 4, 6,

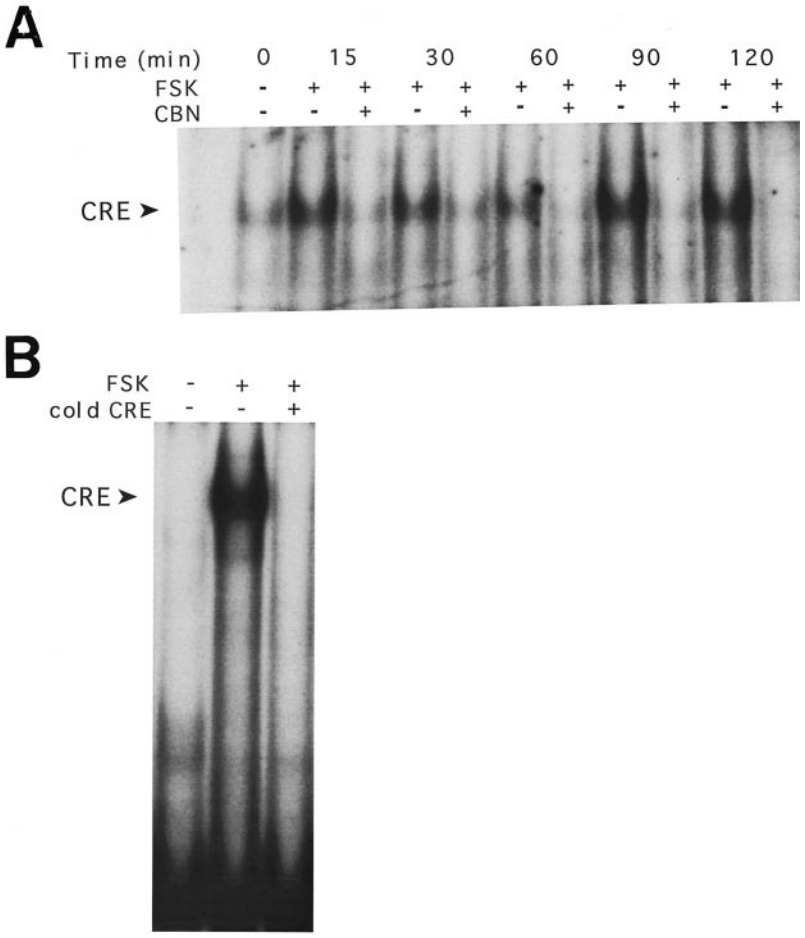

FIG. 5. Inhibition of protein/DNA binding to the CRE consensus motif in mouse thymocytes by cannabinol (CBN). (A) Nuclear extracts (3 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lanes 3, 5, 7, 9, and 11 represent forskolin-stimulated thymocytes while lanes 4, 6, 8, 10, and 12 indicate forskolin-stimulated/CBN-treated thymocytes. (B) Cold competitor studies were done by adding 1 pmol of unlabeled CRE to the nuclear extract isolated from the 90-min forskolin sample. Results are representative of three separate experiments.

8, 10, and 12). Protein binding to the CRE consensus motif was specific, as determined by unlabeled (cold) competitor studies (Fig. 5B). In general, the diminution of CRE binding by cannabinol is indicative of a marked decrease in the activation of the CREB/ATF family of transcription factors.

PKA is also involved in the activation of NF-κB/c-Rel transcription factors as demonstrated by the induction of κB binding following stimulation with cAMP elevating agents such as LPS, forskolin, and IL-1 [31–33]. Additionally, we reported recently the involvement of the cAMP signaling cascade in the activation of NF-κB/c-Rel transcription factors in RAW 264.7 cells [10]. In light of these findings, the DNA binding activity of NF-κB/c-Rel proteins was examined in primary spleen cells and thymocytes. Incubation of nuclear extracts from forskolin-stimulated splenocytes with a ^{32}P-labeled κB oligomer resulted in the formation of two distinct DNA binding complexes (Fig. 6A). More importantly, cells stimulated in the presence of cannabinol exhibited an attenuation of NF-κB binding activity at 30 and 60 min (Fig. 6A; lanes 6 and 8, respectively). Studies in thymocytes revealed two major protein complexes and a minor upper complex in forskolin-stimulated nuclear extracts (Fig. 7A). Similarly, stimulation of cells in the presence of cannabinol resulted in a marked inhibition of κB binding at 60, 90, and 120 min (Fig. 7A; lanes 8, 10, and 12). The formation of all protein complexes was inhibited by excess unlabeled κB oligonucleotide in both cell preparations (Fig. 6B, splenocytes; Fig. 7B, thymocytes).

DISCUSSION

In the present studies, we demonstrated that cannabinol, a plant-derived cannabinoid which exhibits modest CNS activity, inhibited both immune function and cAMP signal transduction in mouse lymphoid cells. These results strongly implicate the involvement of CB2 receptors in mediating the immunosuppressive effects by cannabinoid compounds. Although a systematic evaluation of cell-type receptor distribution has not been performed yet, previous studies have identified RNA transcripts for both cannabinoid receptors, CB1 and CB2, in a number of lymphoid tissue preparations [2, 15, 19], purified leukocytes [18, 34], and immune system-derived cell lines [9, 10, 15, 34]. Northern analysis and quantitative RT-PCR of splenic RNA determined a greater expression of CB2 than CB1 in mouse spleen and expression of only CB2 in thymus [15]. Furthermore, competition binding analysis in mouse splenocytes demonstrated that cannabinol exhibited modestly greater binding affinity than Δ^9-THC [15], which is similar to previous results in CB2 transfected cells [2, 14].

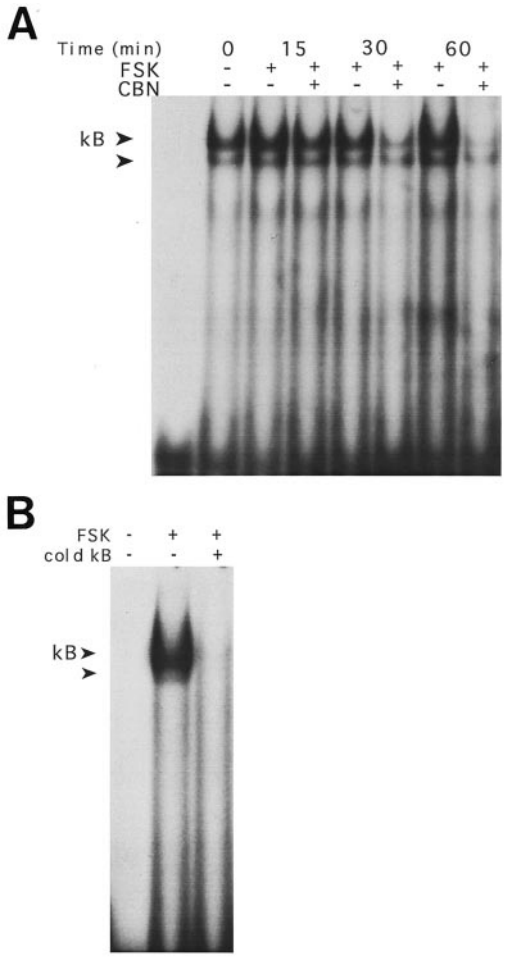

FIG. 6. Inhibition of NF-κB/c-Rel binding to the κB consensus motif in spleen cells by cannabinol (CBN). (A) Nuclear extracts (3 μg) from treated and untreated spleen cells were incubated with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe, and lane 2 indicates unstimulated splenocytes. Lanes 3, 5, and 7 represent forskolin-stimulated splenocytes, while lanes 4, 6, and 8 indicate forskolin-stimulated/CBN-treated spleen cells. (B) Cold competitor studies were done by adding 1 pmol of unlabeled κB to the nuclear extract isolated from the 90-min forskolin sample. One of three representative experiments is shown.

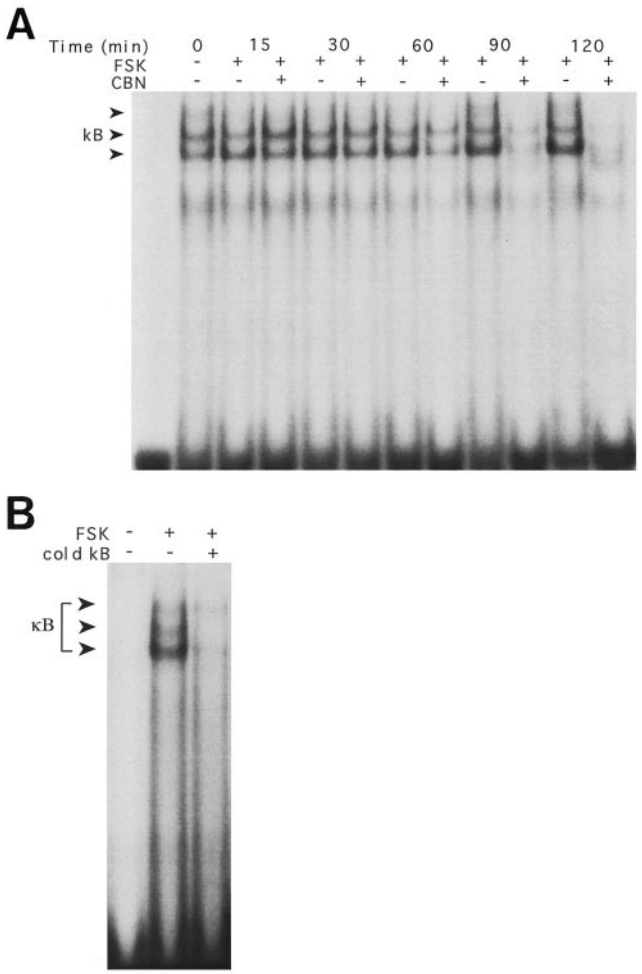

FIG. 7. Inhibition of NF-κB/c-Rel binding to the κB consensus motif in thymocytes by cannabinol (CBN). (A) Nuclear extracts (3 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lanes 3, 5, 7, 9, and 11 represent forskolin-stimulated thymocytes, while lanes 4, 6, 8, 10, and 12 indicate forskolin-stimulated/CBN-treated thymocytes. (B) Cold competitor studies were done by adding 1 pmol of unlabeled κB to the nuclear extract isolated from the 90-min forskolin sample. One of three representative experiments is shown.

Taken together, these previous findings suggest that CB2 is the predominant cannabinoid receptor expressed on primary mouse leukocytes.

Although Δ^9-THC and the synthetic bicyclic cannabinoid CP-55940 are two of the most widely utilized cannabinoids experimentally, they are incapable of distinguishing between CB1 and CB2. Conversely, cannabinol, which is similar in structure to Δ^9-THC, is one of the first cannabinoid receptor ligands identified that exhibits higher binding affinity for CB2 than CB1 [2, 14, 15]. In light of this property, we utilized cannabinol in the present studies as a biological probe to examine the functional role of CB2 on immune modulation by cannabinoids in primary mouse leukocytes. Direct addition of cannabinol to mouse spleen cell cultures produced a significant inhibition of proliferative responses to anti-CD3, LPS, and PMA plus Io as well as to the *in vitro* anti-sRBC IgM AFC response. It is important to emphasize that cannabinol produced no effect on cell viability at the concentrations utilized in the present studies even after 5 days of culture. Interestingly, cannabinol exhibited a similar profile of immunomodulatory activity in the B6C3F1 mouse as previously described for Δ^9-THC [22].

Based on the fact that cannabinoid receptors negatively regulate adenylate cyclase activity through a pertussis toxin sensitive G-protein [5, 8], one of the major focuses of our studies was to evaluate the status of the cAMP signaling

pathway in splenocyte and thymocyte preparations in the presence of cannabinol. Forskolin was used to activate the cAMP cascade through direct stimulation of adenylate cyclase in the presence and absence of cannabinol. Forskolin stimulation of either splenocytes or thymocytes in the presence of cannabinol resulted in a significant inhibition of intracellular cAMP levels, indicating the functional expression of CB2 receptors in both of these cell preparations. In addition to the fact that thymocytes express virtually no CB1 mRNA, the thymocyte studies are particularly interesting for two reasons: (1) intracellular cAMP levels were approximately 4-fold greater in thymocytes than splenocytes following forskolin stimulation, and (2) the magnitude of adenylate cyclase inhibition by cannabinol was significantly greater in thymocytes. A similar difference in intracellular cAMP levels following forskolin stimulation has been shown in thymocytes and peripheral T-cells, suggesting an important role of cAMP in T-cell development and differentiation [26]. We further examined the effect of cannabinol on downstream components of the cAMP signaling cascade, specifically the activation of cAMP-dependent kinase (PKA) and the induction of PKA-regulated transcription factors. These studies showed that cannabinol produced a marked inhibition of PKA activity in forskolin-stimulated splenocytes. It is important to note that the inhibition of PKA activity was not due to a direct effect by cannabinoids on PKA. Rather, PKA inhibition was mediated indirectly through a decrease in cAMP formation, as demonstrated by the ability of exogenous cAMP to activate PKA in the presence of cannabinol (data not shown). Interestingly, although the inhibition of adenylate cyclase activity by cannabinol was moderate (approximately a 30% decrease at 20 μM), changes at the level of PKA were more profound, as evidenced by a greater that 50% decrease in kinase activity at the same cannabinol concentration. This is most likely due to the amplification of the signal as it is transduced from the plasma membrane to the nucleus. The EMSA was used to evaluate the effect of cannabinol on the terminal event of the cAMP cascade, the binding of CREB/ATF transcription factors to the CRE consensus motif. It is important to emphasize that although PKA is the most extensively investigated kinase by which CREB/ATF proteins are regulated, this family of transcription factors has also been found to be regulated by PKC, casein kinase, and calmodulin kinase II [35–37]. In the present studies, forskolin treatment alone (0–120 min) induced a CRE binding complex in both splenocytes and thymocytes that was inhibited markedly by cannabinol at every time point tested. The kinetics of DNA binding and the sensitivity to inhibition by cannabinol correlate with previous findings demonstrating that reversal of cannabinoid-mediated inhibition with cAMP analogs only occurs within the first 60 min after antigen sensitization [4], supporting the hypothesis that cannabinoids inhibit an early leukocyte activation event. Cannabinol was also found to inhibit the activation of NF-κB/c-Rel binding complexes in primary mouse splenocyte and thymocyte nuclear extracts. The regulation of the NF-κB/c-Rel transcription factors has also been shown to be, at least in part, under the control of PKA in leukocytes [10, 31–33]. Recent studies from our laboratory have identified a direct association between the inhibition of cAMP signaling, a decrease in NF-κB/c-Rel DNA binding, and the inhibition of inducible nitric oxide synthase (iNOS) in the presence of Δ^9-THC by macrophages [10].

The role of cAMP signaling in immune regulation is not well defined; however, numerous studies suggest a positive/stimulatory role for cAMP in mediating certain leukocyte cellular responses. Evidence supporting this premise includes a rapid and transient increase in intracellular cAMP following mitogenic stimulation of splenocytes [4, 38–40] and enhancement of proliferative and T-cell dependent AFC responses by cAMP analogs [4, 41]. Additionally, inhibition of adenylate cyclase activity by cannabinoids is closely correlated with the suppression of certain cell-mediated and humoral immune responses [4]. A cause-and-effect relationship between the inhibition of intracellular cAMP and decreased immune function is further supported by the ability of exogenous cAMP or glucagon, a hormone that elevates intracellular cAMP levels, to reverse the inhibition of immune function by cannabinoids [4, 42]. Studies investigating the inhibitory effects of Δ^9-THC on humoral immune responses have shown that only immunoglobulin production to T-cell dependent antigens (i.e. sheep erythrocytes) is suppressed by cannabinoids [22], suggesting that helper T-cells are a sensitive target for inhibition by cannabinoid compounds. Additional evidence supporting the sensitivity of helper T-cells to cannabinoids includes disruption of cAMP signal transduction and IL-2 production by Δ^9-THC in the murine T-cell line EL-4.IL-2 [9]. These studies also demonstrated an inhibition of IL-2 transcription in splenocytes by Δ^9-THC and cannabinol. Consistent with these results are several recent findings indicating a positive role for cAMP signal transduction during T-cell activation. These include the observation that stimulation of T lymphocytes through the antigen receptor or with mitogen induced distinct CRE complexes [43–45] and Ser-133 phosphorylation of CREB [46, 47]. We have also demonstrated induction of CRE binding in mouse splenocytes following anti-CD3 or PMA/Io stimulation [48]. Moreover, expression of a dominant-negative form of CREB clearly showed decreased mitogen-stimulated proliferation and IL-2 production in thymocytes and induced cell cycle arrest [49]. Collectively, these findings strongly suggest that inhibition of cAMP signaling induces T-cell dysfunction. Based on T-cell sensitivity, splenocyte preparations that were a mixture of T-cells, B-cells, and macrophages were utilized in combination with thymocytes in the present studies. It is notable that not all immune responses appear to be sensitive to inhibition by cannabinoid compounds; however, this differential sensitivity does not appear to be due to the lack of cannabinoid receptors in certain subpopulations of cells. We and others have detected cannabinoid receptor expres-

sion in all three major leukocyte cell types present in the spleen: B-cells, T-cells, and macrophages [2, 9, 10, 15, 18]. A more likely explanation for the differential sensitivity of immune responses to cannabinoids pertains to whether the cAMP signaling cascade is critical to a specific effector function. For example, cAMP positively regulates iNOS expression in macrophages [10, 50–52], and Δ^9-THC has been shown to inhibit iNOS transcription in these cells [10, 53]. Conversely, B-cells do not appear to be as dependent on cAMP signaling as suggested by the fact that the secretion of immunoglobulin in response to T-cell-independent antigens (LPS or DNP-Ficoll) is refractory to inhibition by cannabinoids [22] despite a marked decrease in B-cell adenylate cyclase activity [15]. This premise is further supported by recent evidence demonstrating that phosphorylation of CREB, which is most often mediated by PKA, may be mediated primarily by PKC in B lymphocytes [54, 55]. Interestingly, the present studies show that cannabinol inhibits LPS-induced proliferation by B-cells, which is likely due to the critical role PKA plays in cell-cycle control [56]. Considering the evidence discussed above, we believe that the inhibition of adenylate cyclase, and consequently cAMP formation, is a critical biochemical change induced by cannabinoids that leads to a decrease in certain immune responses.

In summary, these studies suggest that functional CB2 receptors are expressed on mouse splenocytes and thymocytes based on the ability of cannabinol to inhibit adenylate cyclase, PKA, and CREB/ATF DNA binding activity. Additionally, NF-κB/c-Rel protein/DNA binding was inhibited by cannabinol in both cell preparations. We also report that cannabinol can modulate immune function, as demonstrated by an inhibition of lymphocyte proliferation and T-cell-dependent humoral immune responses. These results are significant because cannabinol has been regarded historically as a relatively inactive cannabinoid compound, based largely on experimental models assessing changes in CNS activity. The present findings, however, clearly demonstrate that cannabinol possesses immunomodulatory activity that supports the involvement of CB2 receptors in cannabinoid-mediated immune suppression. Immune modulation by selectively targeting the CB2 receptor on lymphoid cells using agents such as cannabinol may be potentially useful therapeutically. Lastly, these results further support a positive role for the cAMP signaling pathway in leukocyte function.

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