

Cannabinol-Mediated Inhibition of Nuclear Factor- κ B, cAMP Response Element-Binding Protein, and Interleukin-2 Secretion by Activated Thymocytes¹

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ABSTRACT

Cannabinol (CBN), an immunosuppressive cannabinoid and ligand for the peripheral cannabinoid receptor CB2, inhibits the cAMP signaling cascade in forskolin-stimulated thymocytes. The objective of the present studies was to further characterize the mechanism of CBN immune modulation by investigating its effects on interleukin-2 (IL-2) secretion, cAMP response element (CRE), and κ B DNA binding activity in phorbol ester (phorbol-12-myristate-13-acetate, PMA) plus calcium ionophore (PMA/Io)-activated thymocytes. PMA/Io treatment induced CRE and κ B DNA binding activity that was attenuated in the presence of CBN. A concomitant and concentration-related inhibition of IL-2 also was produced by CBN in PMA/Io-activated thymocytes. PMA/Io induced two CRE DNA binding complexes, a major complex consisting of a cAMP response element-binding protein (CREB)-1 homodimer, and a minor

CREB-1/activating transcription factor (ATF)-2 complex. Both CRE complexes were inhibited by CBN. Conversely, two κ B DNA binding complexes were observed, but only one was PMA/Io-inducible. However, the DNA binding activity of both complexes was diminished in the presence of CBN. The PMA/Io-inducible κ B complex was a p65/c-Rel heterodimer. Analysis of up-stream regulation revealed a decrease in phosphorylated CREB/ATF nuclear proteins in PMA/Io-activated thymocytes after CBN treatment. Similarly, CBN prevented the phosphorylation-dependent degradation of the nuclear factor- κ B inhibitory protein I κ B- α . These results provide a potential link between the CBN-mediated inhibition of thymocyte function, including IL-2 production, and the inhibition of two critical transcription factor families, CREB/ATF and NF- κ B/Rel.

Immune suppression by cannabinoid compounds is thought to be mediated, at least in part, through cannabinoid receptors (CB) expressed by leukocytes. CB1 and CB2, the two major types of cannabinoid receptors, belong to the G protein-coupled receptor superfamily and negatively regulate adenylate cyclase. CB1 is the predominant cannabinoid receptor expressed in the brain (Matsuda et al., 1990), whereas CB2 is primarily expressed by cells of the immune system (Munro et al., 1993; Schatz et al., 1997). Ligand binding to either CB1 or CB2 inhibits adenylate cyclase, thereby decreasing intracellular cAMP (Howlett et al., 1986; Kaminski et al., 1994). Cannabinol (CBN), a cannabinoid that possesses minimal central nervous system activity, exhibits higher binding affinity for the CB2 receptor (Munro et al., 1993;

Schatz et al., 1997) and has recently been reported to inhibit T cell and B cell proliferation and IgM antibody responses in a dose-dependent manner (Herring et al., 1998). At comparable concentrations, CBN also decreased intracellular cAMP, protein kinase A (PKA) activity, and protein binding to a cAMP response element (CRE) after forskolin stimulation (Herring et al., 1998). Increases in intracellular cAMP facilitate the release of the catalytic subunits of PKA. The cAMP response element-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors is a critical nuclear target of PKA-mediated phosphorylation and is composed of several proteins, including CREB-1, CREB-2, ATF-1, ATF-2, and CRE modulator. These transcription factors can form homodimers or heterodimers and bind to CRE motifs in the promoter region of cAMP-responsive genes.

Recent studies have found interleukin-2 (IL-2) to be sensitive to inhibition by cannabinoids (Condie et al., 1996). IL-2

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ABBREVIATIONS: CB, cannabinoid receptor; CBN, cannabinol; PKA, protein kinase A; CRE, cAMP response element; CREB, cAMP response element-binding protein; ATF, activating transcription factor; IL-2, interleukin-2; NF, nuclear factor; AT, activated T cells; AP-1, activator protein-1; PMA, phorbol-12-myristate-13 acetate; THC, tetrahydrocannabinol; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; PKAc, protein kinase A catalytic subunit.

is an autocrine/paracrine factor secreted by activated T cells (AT). IL-2 gene expression is tightly regulated, and the minimal essential promoter region possesses binding sites for several inducible transcription factors, including AP-1, nuclear factor (NF)- κ B, and nuclear factor of activated T cells (NF-AT). Although the IL-2 promoter lacks a consensus CRE site, recent reports have demonstrated a positive role for CREB in T cell activation and IL-2 expression. For example, thymocytes expressing a dominant negative form of CREB exhibited a marked inhibition of IL-2 secretion and proliferation (Barton et al., 1996). CREB/ATF proteins also have been identified in activator protein-1 (AP-1)p and CD28-response element (CD28RE) binding complexes within the IL-2 promoter of AT, further supporting the involvement of CREB in IL-2 regulation (Chen and Rothenberg, 1993; Butscher et al., 1998). Phosphorylation of CREB also has been detected after activation of T cells by a variety of stimuli, including phorbol ester (phorbol-12-myristate-13-acetate, PMA) plus calcium ionophore (PMA/Io) and anti-CD3 plus anti-CD28 (Barton et al., 1996; Hsueh et al., 1997). Furthermore, a transient increase in intracellular cAMP levels has been observed immediately after lymphocyte activation with mitogens or phorbol ester/calcium ionophore (Russell, 1978; Kaminski et al., 1994) suggesting a positive role for the cAMP pathway during T cell activation.

The NF- κ B/c-Rel family of transcription factors also is involved in IL-2 regulation by binding to the κ B and CD28RE motifs within the IL-2 promoter (Ghosh et al., 1993). This family of transcription factors is composed of several proteins, including p50, p65, c-Rel, and RelB that can form homo- or heterodimers with one another. These dimers are anchored in the cytosol of quiescent cells by I κ B inhibitor proteins, and I κ B- α is the best characterized of these regulatory proteins (May and Ghosh, 1998). Upon cellular activation, I κ B- α is phosphorylated on Ser32 and Ser36 leading to ubiquitination and degradation by the 26S proteasome, enabling NF- κ B translocation into the nucleus where it binds to κ B motifs in DNA (Brown et al., 1995). NF- κ B can be induced by a variety of stimuli, including cytokines, lipopolysaccharide, reactive oxygen species, cAMP elevating agents, and PMA/Io (Shirakawa and Mizel, 1989; Barnes and Karin, 1997). Recently, a large I κ B kinase complex has been identified containing two I κ B kinases (IKK α and IKK β) that phosphorylate I κ B- α after cellular activation (DiDonato et al., 1997; Regnier et al., 1997; Zandi et al., 1997).

Earlier studies with Δ^9 -tetrahydrocannabinol (THC) have characterized the T cell as a sensitive target to inhibition by cannabinoids (Schatz et al., 1993). The objective of the present studies was to further investigate the mechanism of cannabinoid-mediated modulation of T cell activation. Toward this end, experiments were performed to identify the specific CREB/ATF and NF- κ B transcription factors modulated by CBN and to examine the up-stream regulation of these transcription factors in the presence of CBN after PMA/Io activation of thymocytes.

Materials and Methods

Animals. Virus-free female B6C3F1 mice, 6 weeks of age, were purchased from the National Cancer Institute (Bethesda, MD). On arrival, mice were randomized, transferred to plastic cages containing a sawdust bedding (5 mice/cage), and given food (Purina certified

laboratory chow) and water ad libitum. Animal holding rooms were kept at 21–24°C and 40 to 60% relative humidity with a 12-h light/dark cycle.

Chemicals. CBN was provided by the National Institute on Drug Abuse (Baltimore, MD).

Culture Medium. For electrophoretic mobility shift assay (EMSA) and Western analysis, thymocytes (1×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 1% bovine calf serum (HyClone Laboratories Inc., Logan, UT), 2 mM L-glutamine, antibiotic-antimycotic (100 U penicillin and 100 μ g streptomycin) (Life Technologies, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (complete RPMI medium). For the enzyme-linked immunosorbent assay (ELISA), thymocytes (1×10^6 cells/ml) were cultured in complete medium containing 5% bovine calf serum.

Antibodies. Rabbit polyclonal antibodies for CREB-1, ATF-2, p50, p65, c-Rel, and I κ B- α were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The phospho-CREB/phospho-ATF-1 antibody was purchased from New England Biolabs (Beverly, MA).

EMSA. Thymocytes were stimulated with PMA/Io (80 nM/1 μ M) in the presence and absence of CBN (20 μ M) for 60 min. After treatment, cells were lysed with a buffer containing 10 mM HEPES, 1.5 mM MgCl₂, and the nuclei were isolated by centrifugation at 6700g for 5 min. Nuclei were lysed in hypertonic buffer (30 mM HEPES, 1.5 mM MgCl₂, 450 NaCl, 0.3 mM EDTA, and 10% glycerol) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin and leupeptin. DNA oligomers containing either the CRE (TGACGTCA) or the κ B (GGGGACTTTC) sequence were end-labeled with [γ -³²P]dATP. Nuclear proteins (5 μ g) were incubated in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin and leupeptin) with 0.5 μ g of poly(dI-dC) and the ³²P-labeled probe for 10 min on ice. DNA binding activity was separated from free probe with a 4% nondenaturing acrylamide gel in 1 $\times$ TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). For supershift analysis, antibodies for ATF-1, ATF-2, p50, p65, and c-Rel were added after nuclear protein incubation with labeled probe. CREB-1 antibody was incubated with the nuclear proteins before addition of the labeled CRE probe.

Western Analysis. Nuclear proteins (25 μ g) from the EMSA preparation were separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to nitrocellulose. Nitrocellulose was blocked for 1 h with 5% milk-Tris-buffered saline/Tween 20 and probed with 30 ng of phospho-CREB/ATF-1 antibody. For analysis of I κ B- α , thymocytes (1×10^6 cells/ml) were stimulated with PMA/Io (80 nM/1 μ M) for the indicated time points and whole-cell lysates were prepared. For the CBN studies, thymocytes were treated with CBN (1, 5, 10, 15, 20 μ M) for 15 min followed by PMA/Io (80 nM/1 μ M) stimulation for 30 min. Whole-cell lysates (25 μ g) were separated on a 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose. Nitrocellulose was blocked with 5% milk-Tris-buffered saline/Tween 20 for 1 h followed by incubation with either I κ B- α (200 ng) or I κ B- α (200 ng) plus p65 antibody (200 ng) for 2 h. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection with the enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL).

EMSA-Western. Nuclear proteins (8 μ g) from treated and untreated thymocytes were incubated with 0.5 μ g of poly(dI-dC) and either the ³²P-labeled κ B probe (30,000 cpm) or the cold κ B probe (10 pmol) in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. After electrophoresis, the ³²P-labeled samples were dried and subjected to autoradiography, and the protein complexes bound to cold κ B probe were transferred overnight at 4°C to nitrocellulose in transfer buffer (0.4% SDS, 48 mM Tris, 39 mM glycine, 20% methanol). Nitrocellulose was blocked with 1% milk-Tris-buffered saline/Tween 20 for 1 h followed by incubation with either p65 (200 ng) or c-Rel (400 ng) antibody for 2 h. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection with the ECL system (Amersham Corp.).

ELISA. Thymocytes were cultured in triplicate (1×10^6 cells/ml) in 12-well culture plates for 24 h. Supernatants were collected post-stimulation and quantified for IL-2 with the sandwich ELISA method as described previously (Ouyang et al., 1995). The IL-2 standard (mouse recombinant IL-2), purified rat anti-mouse IL-2 antibody, and biotinylated anti-mouse IL-2 antibody were purchased from PharMingen (San Diego, CA).

Densitometry. The optical density of each treatment group was obtained by using the Multi-Analyst program and a GS-700 imaging densitometer (Bio-Rad, Hercules, CA). With the density values, the ratio between the control and treated samples was calculated. The control group was designated with the value of 1.0 to assess qualitative changes between treatments.

Statistical Analysis. The mean $\pm$ S.E. was determined by a parametric analysis of variance for each treatment group within the ELISA. When significant differences were detected, treatment groups were compared with the PMA/Io-stimulated control with Dunnett's two-tailed *t* test.

Results

Inhibition of IL-2 Protein Secretion by CBN. Thymocytes were treated with CBN (1, 5, 10, 15, 20, and 25 μ M) before activation with PMA/Io for 24 h. After activation, supernatants were collected and analyzed for IL-2 activity by ELISA. Maximal induction of IL-2 protein by PMA/Io in the absence of CBN was ~ 16.1 U/ml (Fig. 1). In the presence of CBN, IL-2 secretion by thymocytes was inhibited in a concentration-dependent manner. At 20 μ M, CBN produced a 55% reduction in IL-2 protein compared with the activated control. No effect on cell viability was observed in any of the treatment groups.

CBN Inhibits CRE and κ B Binding in PMA/Io-Activated Thymocytes. In the current studies, EMSA was used to further characterize the effect of CBN on CRE and κ B binding activity in phorbol ester-activated thymocytes. Nuclear proteins were prepared from thymocytes activated with PMA/Io (80 nM/1 μ M) for 1 h in the presence or absence of CBN (20 μ M). PMA/Io treatment induced the formation of two CRE binding complexes, a major complex (lower band)

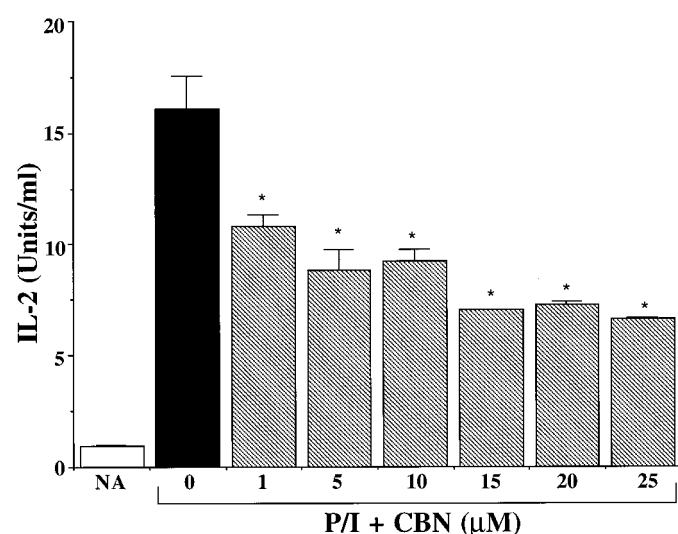


Fig. 1. Inhibition of IL-2 protein secretion by CBN in thymocytes. Thymocytes (1×10^6 cells/ml) were pretreated with CBN (1, 5, 10, 15, 20, 25 μ M) for 15 min followed by activation with PMA/Io (80 nM/1 μ M) for 24 h at 37°C. IL-2 levels were determined by ELISA. Data are expressed as means $\pm$ S.E. for triplicate cultures. **p* < .05 with comparison to the PMA/Io group.

and a minor complex (upper band), both of which were inhibited by CBN (Fig. 2A). The specificity of CRE binding was demonstrated by the addition of excess unlabeled CRE probe.

With culture conditions identical to those for the CRE EMSA studies, the effect of CBN on NF- κ B/c-Rel DNA binding was examined in PMA/Io-activated thymocytes. Two distinct κ B DNA complexes were detected in naive thymocytes. PMA/Io strongly induced only the upper κ B binding complex which was significantly inhibited in the presence of CBN (Fig. 3A). Interestingly, the constitutive lower κ B binding complex also was inhibited by CBN. Addition of excess unlabeled κ B probe inhibited the binding of both protein-binding complexes. The percentage of bovine calf serum in the medium also exhibited some influence on the ability of CBN to inhibit NF- κ B binding activity. Specifically, the inhibition of NF- κ B binding by CBN was marked in the presence of 1% serum, whereas no inhibition was observed when cells were cultured in 5% serum (data not shown).

Identification of Specific CRE and κ B Binding Proteins Regulated by CBN. To identify the specific CREB/ATF proteins in the CRE complexes induced by PMA/Io that were inhibited by CBN, supershift analysis was performed. In these experiments, nuclear proteins were isolated from thymocytes 1 h after PMA/Io treatment. As shown in Fig. 2B, CREB-1 was identified in both the upper and lower binding complexes as evidenced by the loss of CRE binding activity in the presence of anti-CREB-1. Anti-CREB-1 recognizes epitopes within the DNA binding domain of CREB-1 to block DNA binding. ATF-2 was identified only in the upper CRE complex. An anti-ATF-1 antibody had no effect on either CRE binding complex in the supershift assays. Thus, the lower CRE complex consisted of a CREB-1 homodimer, whereas the upper CRE complex was a CREB-1/ATF-2 heterodimer.

In light of the CBN-mediated inhibition of the inducible κ B DNA binding complex, similar experiments were performed to identify which specific NF- κ B proteins were being modulated by CBN. For the supershift studies, nuclear proteins isolated from thymocytes activated for 1 h with PMA/Io were incubated with antibodies specific for p50, p65, or c-Rel (Fig. 3B). The p50 antibody produced a shift (lane 3) that was predominantly from the lower κ B complex. By comparison, anti-p65 and anti-c-Rel appeared to primarily shift the upper κ B complex. Due to the difficulty in determining which κ B binding complexes were being supershifted, an EMSA/Western analysis was conducted to confirm the identity of the DNA binding proteins. In these experiments, the protein/ κ B complexes were subjected to Western analysis with either p65 or c-Rel antibody and compared with the EMSA. EMSA/Western analysis identified both p65 and c-Rel proteins as components of the upper κ B complex that verified the supershift results (Fig. 4). Therefore, the lower κ B complex was identified as a p50 homodimer, whereas the inducible (upper) κ B complex consisted of a p65/c-Rel heterodimer. These findings also demonstrated that CBN primarily inhibits the DNA binding of p65 and c-Rel in PMA/Io-activated thymocytes (Fig. 4B, lane 3).

Inhibition of CREB Phosphorylation and I κ B- α Degradation by CBN. The phosphorylation of CREB/ATF proteins facilitates protein dimerization and DNA binding to CRE motifs. In light of the above-mentioned inhibition of CRE DNA binding, the effect of CBN on PMA/Io-induced phosphorylation of CREB and ATF-1 was examined. Activa-

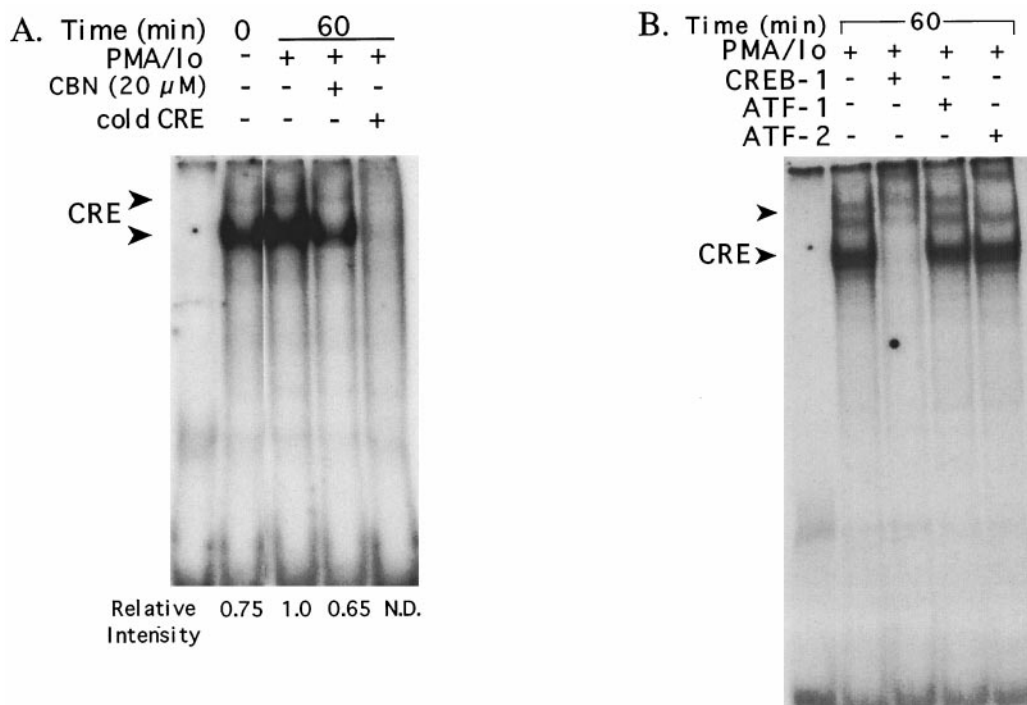


Fig. 2. CBN inhibits protein binding of a CREB-1 homodimer in PMA/Io-activated mouse thymocytes. A, nuclear proteins (5 μ g) from treated and untreated thymocytes were incubated with 0.5 μ g of poly(dI-dC) and the 32 P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1×10^6 cells/ml) activated for 60 min with PMA/Io (80 nM/1 μ M). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μ M) for 15 min followed by PMA/Io activation (80 nM/1 μ M) for 60 min. Cold competitor studies were done by adding 1 pmol of unlabeled CRE to nuclear proteins isolated from the 60-min PMA/Io sample. B, CREB-1 antibody (1 μ g) was incubated with nuclear proteins at 4°C for 45 min before addition of the CRE probe. ATF-1 or ATF-2 antibody (1 μ g) was incubated with the protein/CRE complex at 4°C for 45 min. Lane 1 represents free probe and lane 2 indicates PMA/Io-stimulated thymocytes. Lanes 3, 4, and 5 contain CREB-1, ATF-1, and ATF-2 antibody, respectively. One of three representative experiments is shown.

tion of thymocytes for 60 min strongly induced the phosphorylation of CREB and modestly increased the phosphorylation of ATF-1 (Fig. 5). Conversely, thymocytes that were activated in the presence of CBN exhibited a marked decrease in nuclear phosphorylated CREB and ATF-1, which was concordant with the inhibition in CRE binding activity. Although a minor point, the modest amount of phosphorylated ATF-1 in PMA/Io-activated cells is most likely the reason why ATF-1 was not detected in the supershift experiments.

To gain further insights into the mechanism by which CBN modulates NF- κ B proteins, we examined the effects of CBN on I κ B- α . PMA/Io activation of thymocytes produced a rapid degradation of I κ B- α during the first 60 min that was then followed by an increase in I κ B- α at 90 and 120 min (Fig. 6A). Because maximal degradation of I κ B- α was detected 30 min after PMA/Io activation, this time point was chosen to examine the effects of CBN on I κ B- α . As shown in Fig. 6B, CBN prevented the degradation of I κ B- α , presumably through an inhibition of I κ B- α phosphorylation. The level of p65 protein also was examined under these conditions to investigate possible direct effects of CBN on p65 expression. The p65 protein levels were relatively unchanged in the presence of increasing concentrations of CBN, suggesting that the decrease of NF- κ B DNA binding activity by CBN occurs at the level of I κ B- α and not p65 (Fig. 6B). This was further demonstrated by examining the cellular localization of p65 in the presence of CBN. Nuclear levels of p65 were induced after PMA/Io activation (30 min) and this induction was suppressed by CBN at 15 and 20 μ M (Fig. 7A). Cytosolic levels of p65 were

increased in the presence of 15 and 20 μ M CBN, which correlated with the decrease in nuclear p65 (Fig. 7B). These results suggest that the decrease in NF- κ B DNA binding in the presence of CBN is due to an inhibition in I κ B- α phosphorylation and subsequent degradation that precludes NF- κ B translocation into the nucleus.

Discussion

In the present studies, we demonstrate that CBN inhibits IL-2 production in PMA/Io-activated thymocytes while concomitantly inhibiting the binding of transcription factors to CRE and κ B motifs. The major CRE binding complex inhibited by CBN in PMA/Io-activated thymocytes was a CREB-1 homodimer, whereas the κ B complex was a heterodimer of c-Rel and p65. In addition, CBN was found to inhibit PMA/Io-induced phosphorylation of CREB and the phosphorylation-dependent degradation of I κ B- α thereby decreasing nuclear localization of NF- κ B proteins.

It is now well established that cannabinoid receptors are expressed on immune cells, and ligand binding to CB1 or CB2 inhibits the cAMP signaling pathway in leukocytes. Previous studies demonstrated that CBN, a ligand with higher binding affinity for the CB2 receptor, inhibits forskolin-induced cAMP accumulation, PKA activity, and CRE binding activity in thymocytes and EL-4.IL-2 cells (Condie et al., 1996; Herring et al., 1998). Although these findings provided insight into the effects of CBN on the cAMP cascade in T cells, the effects of CBN following a T-cell activation signal have not

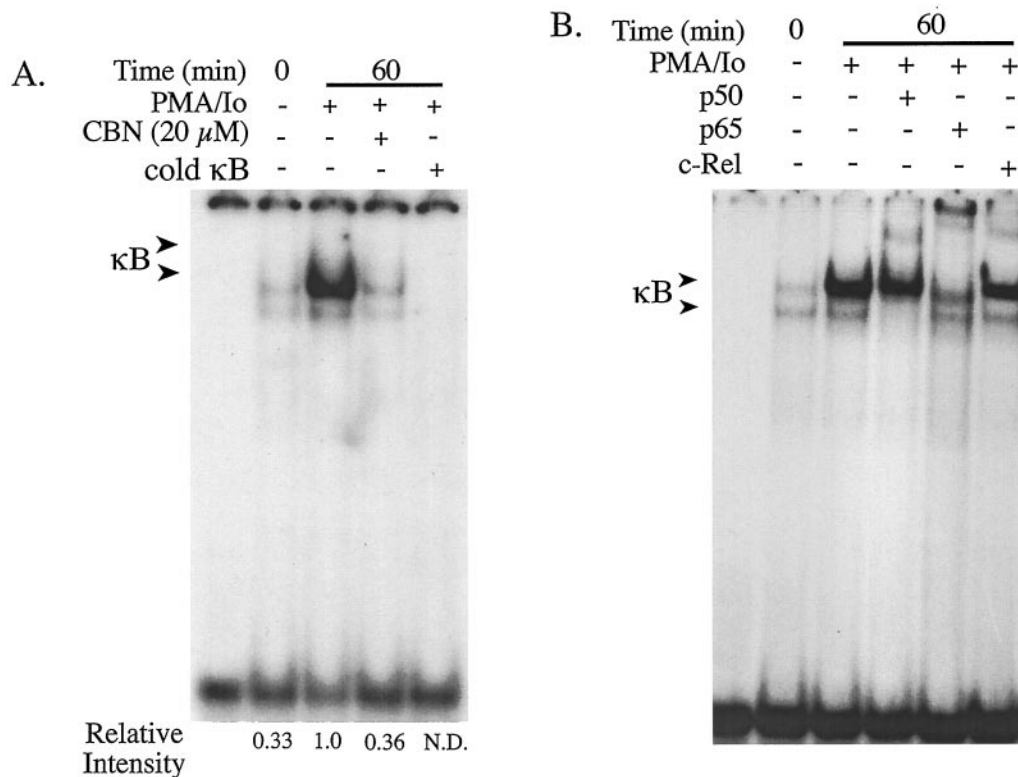


Fig. 3. Inhibition of a p65/c-Rel heterodimer by CBN in PMA/Io-activated mouse thymocytes. A, nuclear proteins (5 μ g) from treated and untreated thymocytes were incubated with 0.5 μ g of poly(dI-dC) and the 32 P-labeled κ B probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1×10^6 cells/ml) activated for 60 min with PMA/Io (80 nM/1 μ M). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μ M) for 15 min followed by PMA/Io (80 nM/1 μ M) for 60 min. Cold competitor studies were done by adding 1 pmol of unlabeled κ B probe to nuclear proteins isolated from the 60-min PMA/Io sample. B, p50, p65, or c-Rel antibody (1 μ g) was incubated with the protein/ κ B complex for 30 min at room temperature. Lane 1 represents free probe, lane 2 indicates basal binding activity, and lane 3 indicates PMA/Io-activated thymocytes. Lanes 4, 5, and 6 contain p50, p65, and c-Rel antibody, respectively. One of three representative experiments is shown.

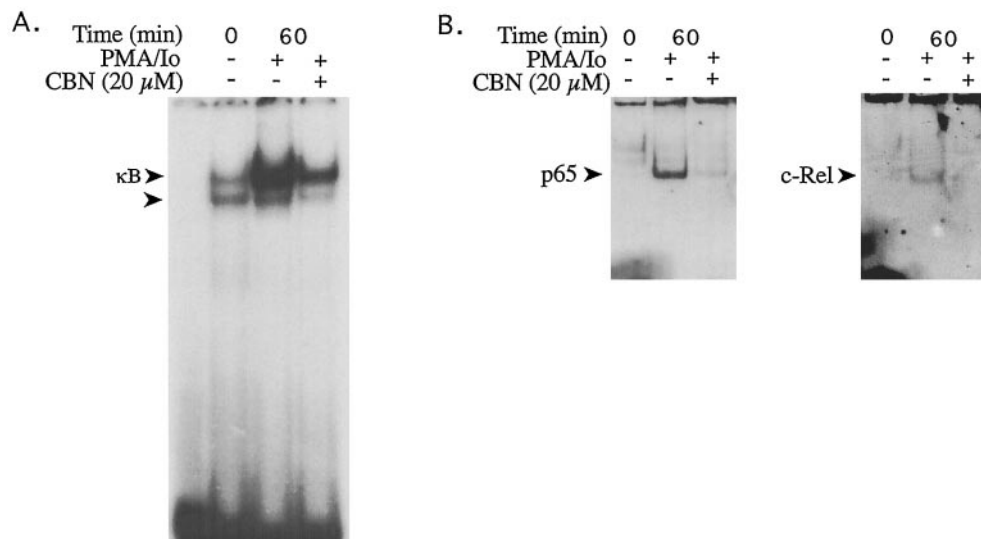


Fig. 4. Identification of the components of the upper κ B binding complex induced by PMA/Io in thymocytes. A, nuclear proteins (8 μ g) from treated and untreated thymocytes were incubated with 0.5 μ g of poly(dI-dC) and the 32 P-labeled κ B probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1×10^6 cells/ml) activated for 60 min with PMA/Io (80 nM/1 μ M). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μ M) for 15 min followed by PMA/Io stimulation (80 nM/1 μ M) for 60 min. B, identical nuclear protein samples were incubated with 0.5 μ g of poly(dI-dC) and 10 pmol of cold κ B probe for 10 min on ice and separated on a 4% acrylamide gel. After electrophoresis, the protein complexes were transferred to nitrocellulose and incubated with either p65 (200 ng) or c-Rel (400 ng) antibody for 2 h. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection with the ECL system.

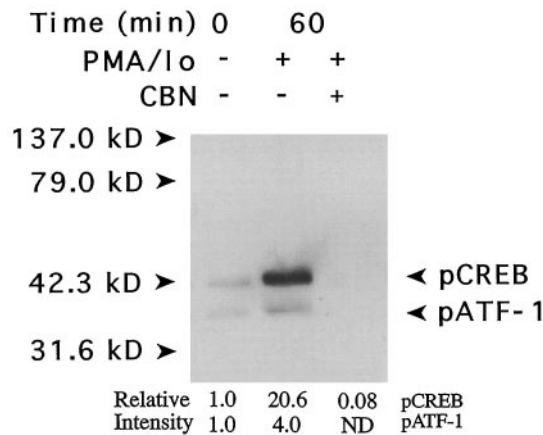


Fig. 5. Phosphorylation of CREB and ATF-1 is inhibited by cannabinal in PMA/Io-activated thymocytes. Nuclear proteins (25 μ g) from treated thymocytes were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated for 2 h with 30 ng of a rabbit polyclonal antibody that recognizes the phosphorylated Ser133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection with the ECL system. CREB-1 is 43 kD and ATF-1 is 38 kD.

been examined in primary T lymphocytes. In the present studies, PMA/Io was used as a T cell activator because it mimics signaling induced through the T cell antigen receptor. A role for the cAMP cascade in leukocyte activation is supported by the observed rapid and transient increase in intracellular cAMP levels after PMA/Io stimulation of splenocytes (Kaminski et al., 1994). In addition, phorbol ester activation of PKC was reported to enhance adenylate cyclase activity, indicating cross-talk between the cAMP and PKC signaling pathways (Yoshimasa et al., 1987). Our present results demonstrate that PMA/Io activation of thymocytes induced a CRE DNA binding complex consisting of a CREB-1 homodimer that was markedly inhibited by CBN. The detection of CREB-1 in this CRE complex is consistent with recent findings that CREB-1 is a major component of the CRE complexes induced after T cell activation through the antigen receptor or with Con A plus 12-*O*-tetradecanoylphorbol-13-acetate (Wollberg et al., 1994; Feuerstein et al., 1996). Although CREB can be phosphorylated at Ser133 by a variety of protein kinases, including casein kinase, PKC, CaM kinase II and IV, and the RSK family of kinases (Gonzalez et al., 1989; Means et al., 1997; Tamai et al., 1997), it is presently tempting to speculate that the decrease in CREB phosphorylation in the presence of CBN is primarily due to the inhibition of PKA. However, it is notable that recent studies have suggested that CREB phosphorylation in AT occurs by a cAMP-independent mechanism (Barton et al., 1996; Hsueh et al., 1997). Thus, the modulation of additional kinases and signaling pathways by CBN may contribute to the inhibition of CREB phosphorylation.

Several studies have shown the activation of NF- κ B after increases in intracellular cAMP, which was thought to be mediated by PKA phosphorylation of I κ B- α (Shirakawa and Mizel, 1989; Shirakawa et al., 1989; Muroi and Suzuk, 1993; Herring et al., 1998). Recently, a large cytoplasmic I κ B kinase complex has been characterized, and two I κ B kinases (IKK α and IKK β) that can phosphorylate I κ B- α in response to activating stimuli have been identified (DiDonato et al., 1997). Interestingly, an increase in p65 binding activity has

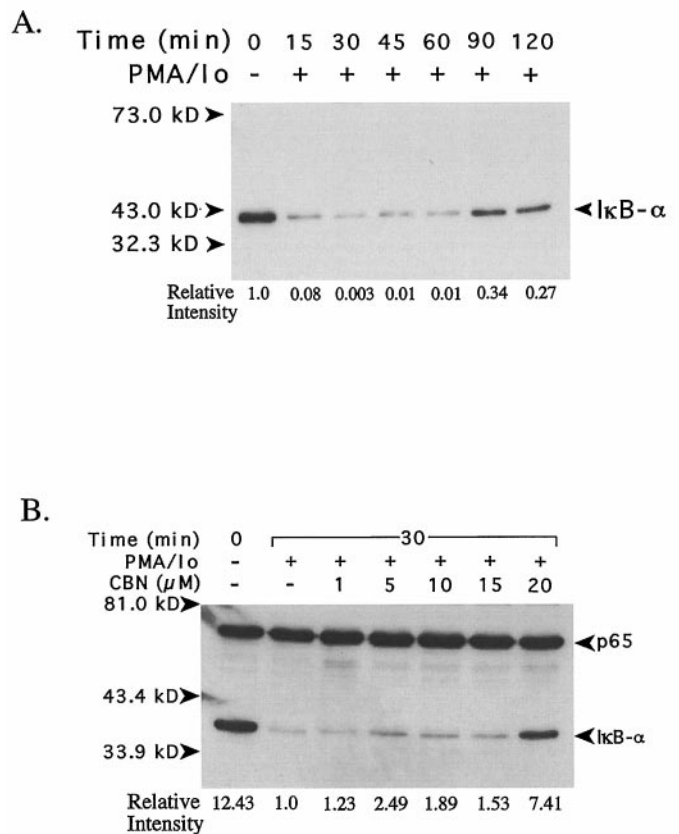


Fig. 6. Inhibition of I κ B- α degradation by cannabinal. A, thymocytes (1×10^6 cells/ml) were stimulated with PMA/Io for 0 to 120 min and whole-cell lysates (25 μ g) were resolved on a 10% SDS-PAGE gel. B, whole-cell lysates were prepared from thymocytes (1×10^6 cells/ml) treated with CBN (1, 5, 10, 15, 20 μ M) for 15 min followed by PMA/Io (80 nM/1 μ M) activation for 30 min. Proteins were transferred to nitrocellulose and incubated with 200 ng of a rabbit polyclonal antibody for I κ B- α for 2 h. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for detection with the ECL system. The whole-cell lysates (25 μ g) also were examined for changes in p65 protein expression with 200 ng of p65 antibody and the anti-rabbit Ig horseradish peroxidase secondary antibody. I κ B- α is a 38-kD protein.

been reported after phosphorylation by a PKA catalytic subunit (PKAc) found associated with the cytosolic NF- κ B-I κ B complex (Zhong et al., 1997, 1998). This PKAc is inactive when bound to the NF- κ B-I κ B complex and becomes activated upon degradation of I κ B- α . The phosphorylation of p65 by PKAc also has been shown to potentially increase the transactivating activity of NF- κ B (Zhong et al., 1998). Therefore, the inhibition of NF- κ B activation and nuclear translocation by CBN may occur at several levels. First, CBN may inhibit the phosphorylation of I κ B- α either through inhibition of I κ B kinase or by inhibiting key regulatory signals necessary for I κ B kinase activation. This initial decrease in phosphorylation retains NF- κ B in the cytosol and prevents the degradation of I κ B- α . As a result, the PKAc associated with the NF- κ B-I κ B complex remains inactive and unable to phosphorylate p65, thereby inhibiting its DNA binding and activation of target gene expression. The lack of I κ B- α degradation also may explain the CBN-induced inhibition of constitutive NF- κ B binding activity because I κ B- α has been shown to remove bound κ B complexes from DNA. These conclusions are based on the premise that CBN alters the phosphorylation of I κ B- α ; however, inhibition of ubiquitina-

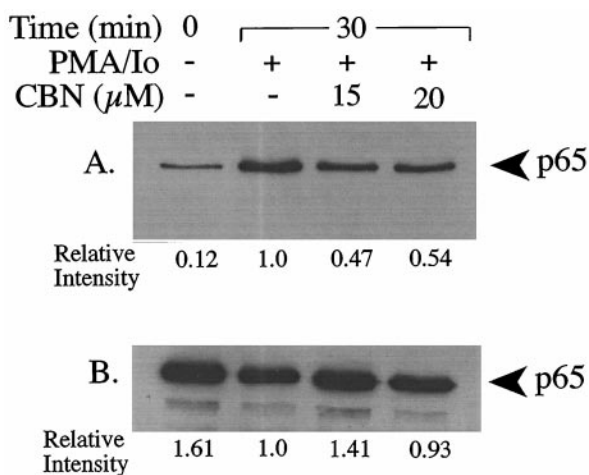


Fig. 7. Cellular localization of p65 in the presence of CBN. A, nuclear proteins were isolated from thymocytes (1×10^6 cells/ml) treated with CBN (15 or 20 μ M) for 15 min followed by PMA/Io (80 nM/1 μ M) activation for 30 min. B, cytosolic proteins isolated from thymocytes treated as described in A. Proteins (25 μ g) were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated for 2 h with 200 ng of p65 antibody. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection with the ECL system.

tion or activation of a phosphatase would produce similar effects and cannot be excluded at this time. In contrast to our findings, Δ^9 -THC was reported to enhance NF- κ B activity in NKB61A2 cells, a natural killer-like cell line (Daaka et al., 1997) and may reflect differences between cell types, activation stimuli, and/or culture conditions.

The minimal essential promoter region of the IL-2 gene contains binding sites for NF-AT, AP-1, and NF- κ B; however, no specific CRE binding sites are present in the IL-2 promoter region. Despite the lack of a CRE in the IL-2 promoter, several reports have described a critical role for the CREB/ATF proteins in IL-2 regulation after T cell activation (Barton et al., 1996; Hsueh et al., 1997; Butscher et al., 1998). An essential role for CREB in IL-2 regulation was initially demonstrated with a dominant negative form of CREB that revealed a drastic inhibition of IL-2 production in PMA/Io-activated thymocytes (Barton et al., 1996). This effect was attributed to a decrease in the CREB-dependent expression of fos and jun proteins in the transgenic thymocytes; however, a direct role for CREB at the IL-2 promoter has been proposed. Additionally, CREB has been identified as part of the protein complex binding to the AP-1p site of the IL-2 promoter in thymocytes (Chen and Rothenberg, 1993). It is important to note that this particular AP-1 site is critical for AP-1 induction of the IL-2 gene (Jain et al., 1992). In addition, stimulation of EL-4.IL-2 cells with PMA/Io plus forskolin enhanced binding to the AP-1p site, further suggesting the direct involvement of the cAMP cascade in IL-2 regulation (Condie et al., 1996). CREB also has been shown to bind to the CD28RE within the IL-2 promoter, which is further supported by the observation that activation of a CD28 response element-TPA response element chloramphenicol transferase (CD28RE-TRE CAT) reporter construct was inhibited by dominant-negative CREB expression vectors (Butscher et al., 1998). In light of this, the present findings strongly suggest that inhibition of CREB-1 binding by CBN in PMA/Io-activated thymocytes is involved in the CBN-mediated inhibition of IL-2 in these cells. NF- κ B/c-Rel transcrip-

tion factors also play an important role in IL-2 regulation by binding to the κ B and CD28RE sequences in the IL-2 promoter (Ghosh et al., 1993; Lai et al., 1995; Butscher et al., 1998). The p65/c-Rel heterodimer was specifically found to be a potent activator of the CD28RE (Ghosh et al., 1993; Lai et al., 1995). Therefore, the inhibition of p65/c-Rel binding to the κ B motif produced by CBN in the current study may be modulating IL-2 levels through both the κ B and CD28RE of the IL-2 promoter. It is also notable that despite the strong inhibition by CBN of NF- κ B and CRE DNA binding that was observed in the EMSA, the overall inhibition of IL-2 secretion was $\sim$ 50% compared with the control thymocytes. These findings are consistent with the fact that although NF- κ B and CREB family proteins contribute to the maximal expression of the IL-2 gene, they clearly are not the only transcription factors that regulate IL-2 gene expression. Due to the complex regulation of the IL-2 gene involving multiple response elements, it is not surprising that the strong inhibition of CREB and NF- κ B did not completely suppress IL-2 expression.

In addition to the cAMP signaling cascade, two other signaling pathways for cannabinoid receptors have been reported. Activation of CB1 receptors has been shown to inhibit Q-type calcium channels and inward rectifying potassium channels, yet ligand binding to the CB2 receptor did not affect either of these channels (Felder et al., 1995). Coupling to the mitogen-activated protein kinase (MAPK) pathway also has been described after ligand binding to unstimulated Chinese hamster ovary cells transfected with either the CB1 or CB2 receptor (Bouaboula et al., 1995, 1996). Clearly, MAPK is a critical regulator of both CREB/ATF and NF- κ B proteins and may therefore significantly contribute to the inhibition of IL-2. This premise is further supported by recent studies from our laboratory that demonstrate that CBN inhibited MAPK activity in a concentration-dependent manner after PMA/Io activation of mouse splenocytes (unpublished observations). Due to the complexity of signaling cascades that regulate IL-2 expression, it is unlikely that inhibition of the cAMP cascade can completely account for the inhibitory effects cannabinoids exert on T cells or CREB/ATF and NF- κ B/c-Rel activation. The present findings contribute significant insights into the mechanism of CREB and NF- κ B inhibition by cannabinoids during T cell activation and also suggest that CBN, a minimally CNS-active cannabinoid, may be a prototype for cannabinoid-based immune modulators.

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