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CANNABINOID-INDUCED IMMUNE SUPPRESSION IN MOUSE SPLENOCYTES INVOLVES BOTH CANNABINOID RECEPTOR-DEPENDENT AND -INDEPENDENT MECHANISMS

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ABSTRACT

CANNABINOID-INDUCED IMMUNE SUPPRESSION IN MOUSE SPLENOCYTES INVOLVES BOTH CANNABINOID RECEPTOR-DEPENDENT AND -INDEPENDENT MECHANISMS

By

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Cannabinoids are a group of structurally related compounds derived from the *Cannabis Sativa* plant. The immunosuppressive effects of cannabinoids are putatively mediated through G protein-coupled receptors (CB1 and CB2) that exhibit inhibition of adenylate cyclase activity in lymphocytes. The CB2 receptor is primarily localized to lymphoid tissue, whereas the CB1 receptor has been characterized in the CNS with lower expression levels in lymphoid tissues. The immunosuppressive effects of cannabinoid compounds include inhibition of interleukin-2 (IL-2) production in phorbol ester plus calcium ionophore (PMA/Io)-stimulated lymphocytes. The mechanism of IL-2 inhibition, including the role of the cannabinoid receptors; however, is not yet understood. The following objectives are utilized to test this hypothesis: Immune modulation by cannabinoid compounds is mediated via cannabinoid receptors (CB1 and/or CB2), resulting in disruption of activator protein-1 (AP-1) transcription factor binding in the promoter region of immune system genes, such as IL-2. The first objective was to characterize the effects of cannabinoids on AP-1 transcription factor activity in primary mouse SPLC. Cannabinoid compounds inhibited PMA/Io-stimulated AP-1 transcription factor binding to both the consensus AP-1 and proximal AP-1 sites. Cannabinoid compounds also inhibited PMA/Io-stimulated nuclear factor of activated T

cells (NF-AT) transcription factor binding activity to the distal NF-AT site, which depends on cooperative binding with AP-1 proteins. The decrease in transcription factor binding activity was due, in part, to inhibition of PMA/Io-stimulated protein expression of the AP-1 components, c-fos and c-jun. This inhibition of c-fos and c-jun protein expression was not due to inhibition of steady state mRNA expression, suggesting that cannabinoids inhibited post-translational modifications of c-fos and c-jun. Indeed, cannabinoids inhibited the activation of extracellular signal-regulated mitogen activated protein kinase (ERK MAPK). The second objective was to characterize the role of the cannabinoid receptors in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production. Cannabinoids such as cannabinol (CBN), cannabidiol (CBD) and the WIN-55212 stereoisomers inhibited PMA/Io-stimulated IL-2 production with a rank order potency of WIN-2 $\approx$ CBD > WIN-3 > CBN. This inhibition; however, was not attenuated in the presence of both cannabinoid receptor antagonists. With the demonstration that cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production was independent of the cannabinoid receptors, other T cell targets were examined. Modulation of intracellular calcium seemed a likely candidate to explain the pleiotropic effects of these compounds. Thus, the third objective was to determine the effect of cannabinoids on intracellular calcium concentration. Cannabinoid compounds elevated intracellular calcium concentration in a cannabinoid receptor-dependent manner in resting SPLC. Furthermore, the mechanism of cannabinoid-induced immune modulation is both cannabinoid receptor-dependent and –independent.

DEDICATION

To my husband, Evan, for loving me. You have been a constant source of love, support, inspiration, and fun. One of the best things about my experience at MSU is that I met you. I love you with all of my heart.

To my parents, Mike and Bev, for their unconditional love and support. Thank you for constantly inspiring me and challenging me to be a better person. You also taught me the value of working hard, being dedicated, and taking initiative. You have always been there for me when I needed you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFC	antibody forming cell
AM	acetoxymethyl
ANA	anandamide
AP-1	activator protein-1
APC	antigen-presenting cell
2-AG	2-arachidonylglycerol
APS	ammonium persulfate
ATF	activating transcription factor
BCA	bicinchoninic acid
BCS	bovine calf serum
BZIP	basic region/leucine zipper protein
CaMK	calcium calmodulin kinase
CaMKII	calcium calmodulin kinase two
CaMKIV	calcium calmodulin kinase four
CaMKK	calcium calmodulin kinase kinase
CB	cannabinoid receptor
CBD	cannabidiol
CBN	cannabinol
CBP	CREB binding protein
CD	cluster of differentiation
CD28RE	CD28 response element

$CD4^+$	helper T cells
$CD8^+$	cytotoxic T cells
CNS	central nervous system
Con A	concanavalin A
CP-55940	(-)-cis-3-[2-hydroxy-4-(1,1,-dimethylheptyl) phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol
CP-56667	(+)-cis-3-[2-hydroxy-4-(1,1,-dimethylheptyl) phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol
CRAC	calcium release-activated calcium
CRE	cAMP response element
CREB	cAMP response element binding protein
CTF	CAAT-binding transcription factor
DBcAMP	dibutyryl cAMP
Δ^8-THC	delta-8-tetrahydrocannabinol
Δ^9-THC	delta-9-tetrahydrocannabinol
dI/dC	polydeoxyinosine/polydeoxycysteine
DNP	dinitrophenyl
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated kinase
FAP	fatty acid poor
FSK	forskolin
GEF	guanine nucleotide exchange factor

HU-210	(-)-11-OH-Δ^8-tetrahydrocannabinol-dimethylheptyl
HU-211	(+)-11-OH-Δ^8-tetrahydrocannabinol-dimethylheptyl
IκB	inhibitor of NF-κB
IL	interleukin
IL-2	interleukin-2
IFN	interferon
iNOS	inducible nitric oxide synthase
Io	ionomycin
IS	internal standard
JNK	c-jun N terminal kinase
LPS	lipopolysaccharide
λ-ppase	lamba phosphatase
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MKP	MAP kinase phosphatase
mRNA	messenger RNA
NA	naïve
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor for immunoglobulin κ chain in B cells

NK cells	natural killer cells
OAP	octamer-associated protein
OCT	octamer
PD098059 (PD)	[2-(2'-amino-3'-methoxyphenyl)-oxonaphthalen-4-one]
PHA	phytohemagglutinin
pKa	acid dissociation constant
PMA	phorbol-12-myristate-13 acetate
PMA/Io	phorbol ester plus calcium ionophore;
RSK	ribosomal S6-kinase
RT-PCR	reverse-transcriptase polymerase chain reaction
SAPK	stress activated protein kinase
SB203580	4-[4-fluorophenyl]-2-[4-methyl-sulfinylphenyl]-5-[4-pyridyl]-1-H-imidazole
SIE	sis-inducible enhancer
SIF	sis-inducible factor
SP1	simian-virus-40 protein-1
SPLC	splenocytes
SR141716A	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorphenyl)-4-methyl-H-pyrazole-3-carboxyamidehydrochloride

SR144528	N-[(1S)-endo-1,3,3,-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-mtheylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
sRBC	sheep red blood cells
SRE	serum response element
SRF	serum response factor
STAT	signal transducer and activator of transcription
TG	thapsigargin
THMC	thymocytes
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TPA	12-O-tetradecanylphorbol 13-acetate
TRE	TPA response element
VH	vehicle
WIN-2 (WIN-55212-2)	R (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone mesylate
WIN-3 (WIN-55212-3)	S (-)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone mesylate

LITERATURE REVIEW

I. Marijuana

Marijuana is a drug derived from the *Cannabis Sativa* plant that has been used for both therapeutic and recreational uses. There are over 60 plant-derived cannabinoid compounds, of which Δ^9-THC is the primary psychoactive congener of marijuana (1). Marijuana is most often smoked and is referred to by several names, including hashish, ganja, cannabis, and pot. Recreational marijuana use is primarily social, that is, for pleasure; however, many use it like alcohol or other narcotics to escape emotional problems (2).

II. Cannabinoid pharmacokinetics

Δ^9-THC is a lipid soluble compound with a pKa of 10.6. Once absorbed, the drug is highly bound to plasma proteins, which limits its bioavailability and results in a long half-life (> 20 h). The primary route of administration of marijuana, and therefore, Δ^9-THC, is via inhalation. The amount of drug one receives from smoking marijuana is highly variable due to varying amounts of Δ^9-THC in the cigarette, puff duration, pyrolysis, loss through side stream smoke, or inefficient absorption in the lung. Smoking marijuana results in faster absorption and higher plasma levels of Δ^9-THC and its metabolites relative to oral administration. Furthermore, Δ^9-THC undergoes extensive metabolism in the liver following oral administration ("first pass effect") (3).

There are several metabolites of Δ^9-THC, many of which exhibit similar activity to the parent compound (3). Two major metabolites found in mice are 11-OH-Δ^9-THC and 8,11-OH-Δ^9-THC. Δ^9-THC is primarily metabolized in the liver, followed by spleen

and blood, implicating leukocytes as sites of metabolism. There was no metabolic conversion in the brain (4). Due to the long half life, excretion of Δ^9-THC is slow, occurring mainly via the feces and less so via the urine (3).

III. Adverse effects of marijuana use

The best characterized effects of marijuana use are those associated with the CNS. CNS effects of marijuana use include emotional and mood disturbances, sleepiness, hallucinations, alteration of time sense, and paranoia (2). High doses of cannabis might cause a toxic delirium, characterized by memory impairments, confusion and disorientation (5). Psychomotor functions are compromised including cognition alterations, distortion of perception, and diminished response times while driving (2) and some marijuana users display schizophrenia-like symptoms (5). In animal models, a "tetrad" of CNS effects has been developed in order to characterize the CNS effects and potency of various cannabinoid compounds. These include antinociception (as determined in a latency to tail-flick evaluation), hypothermia, hypomotility and catalepsy (6). Subjective rating of drug high and plasma concentrations of Δ^9-THC were positively correlated in humans (7).

Tolerance to the "high" develops within 14-21 days of daily marijuana smoking and is reversible, although there is a long duration of the tolerance following cessation of the drug (8). Tolerance does not persist with small doses or infrequent use. There is cross-tolerance with other CNS depressant drugs including alcohol. Physical dependence also occurs with marijuana abuse and cessation results in withdrawal symptoms that include irritability, sleeplessness and decreased appetite (5). Recently, with the

identification of cannabinoid receptor antagonists (discussed below), precipitated withdrawal symptoms were induced with the antagonist in animals tolerant to Δ^9-THC (9-11).

In addition to effects on the CNS, marijuana use has been associated with reproductive problems (reviewed in 5, 8). There is evidence to suggest that Δ^9-THC caused a decrease in follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Furthermore, testosterone synthesis from Leydig cells was reduced by Δ^9-THC. In pregnant rats, no viable litters were born to mothers given high doses of Δ^9-THC (100 mg/kg/day); whereas the low dose group (50 mg/kg/day) produced a reduction in weight gain during pregnancy and decreased pup weight at birth (12). There is some evidence to suggest that the reproductive effects of marijuana and other cannabinoid compounds occurred via the hypothalamic and pituitary axes rather than a direct effect on reproductive organs (8, 13).

Marijuana use also affects the cardiovascular system. Some of these effects are due to the fact that marijuana is most often smoked and thus, problems associated with smoking tobacco also occur with smoking marijuana. One such example is an increase in blood concentrations of carboxyhemoglobin (5), which decreases the oxygen carrying capacity of hemoglobin. Most often, tachycardia and hypotension were reported in humans in response to marijuana smoking, which might be due to an inhibition of vagal tone (14). The tachycardia observed in humans was verified with Δ^9-THC in animal models using isolated perfused heart preparations (15, 16).

IV. Medical use of marijuana

A. Cannabinoid pharmaceuticals

Marijuana and other cannabinoid compounds have several therapeutic applications and the psychoactive congener of marijuana is currently used medically in the United States. In 1985, a synthetic, oral form of Δ^9-THC was approved for medical use by the FDA. This capsule is known as Dronabinol (trade name Marinol) and is currently a schedule III drug. Primarily it has been used for the treatment of chemotherapy-induced nausea and for prevention of wasting syndrome in AIDS patients. Additionally, Nabilone (trade name Cesamet), a synthetic cannabinoid, is currently approved for medical use in the United Kingdom (17). A major disadvantage with Dronabinol is that the oral preparation results in low bioavailability and is difficult to absorb with constant vomiting. Therefore, several states have passed legislation to legalize marijuana smoking for medical use. The advantages of smoking marijuana include increased bioavailability, faster onset of action, and individual patient dose control.

B. Therapeutic applications

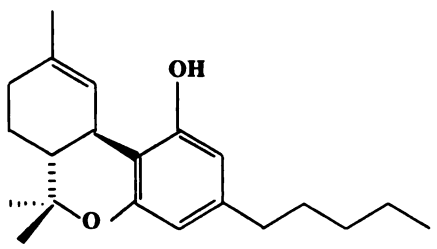
As mentioned above, the primary medical uses for marijuana or cannabinoid compounds are anti-emesis and appetite stimulation. There are several clinical studies demonstrating that Δ^9-THC or marijuana was very effective in nausea attenuation for patients receiving chemotherapy (summarized in 18). In AIDS patients, appetite was increased in response to Dronabinol in at least two different studies, although there was no significant weight gain (reviewed in 19). Marijuana or other cannabinoids have also been shown to be therapeutically effective in decreasing spasticity associated with

multiple sclerosis (20, 21), lowering intraoccular pressure in patients with glaucoma (22), as an analgesic (23, 24) and as an anti-asthmatic (25, 26). It is interesting to note that several of the diseases for which cannabinoids have been shown to be therapeutic are the result of an overactive immune system. For example, multiple sclerosis is an autoimmune disorder and asthma is, in part, due to overproduction of inflammatory cytokines (27).

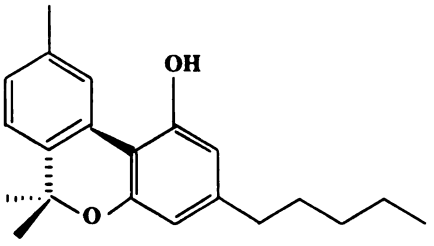
V. Cannabinoid compounds

A. Cannabinoid receptor agonists

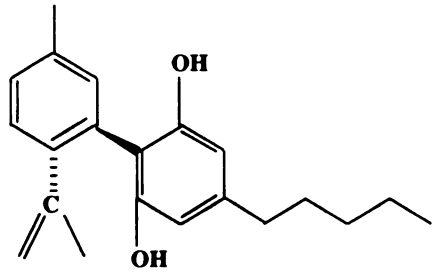
There are over 60 cannabinoid compounds (Figure 1) derived from *Cannabis Sativa*, including Δ^9-tetrahydrocannabinol (THC), cannabinol (CBN), and cannabidiol (CBD), all of which contain a bicyclic structure (28). The group of cannabinoid compounds now includes several synthetic compounds as well. One class of synthetic cannabinoids are those containing a dimethylheptyl aliphatic side chain, which increases potency of the negative stereoisomer (6, 29) and includes the stereoisomer pairs HU-210/HU-211 and CP-55940/CP-56667 (30, 31). Another class of synthetic cannabinoids are the aminoalkylindoles (i.e., WIN-55212) which are structurally very different from either the natural or other synthetic cannabinoids, but often are more potent than the naturally-occurring compounds (32, 33). It was furthermore discovered that there is a group of endogenous cannabinoid compounds, most of which are arachidonic acid derivatives. One of the most well characterized endogenous cannabinoids is anandamide (ANA, also known as arachadonylethanolamide) (34). Further investigation into the compounds that might act as endogenous cannabinoid agonists determined that



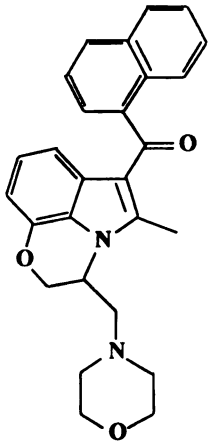
Δ^9-THC



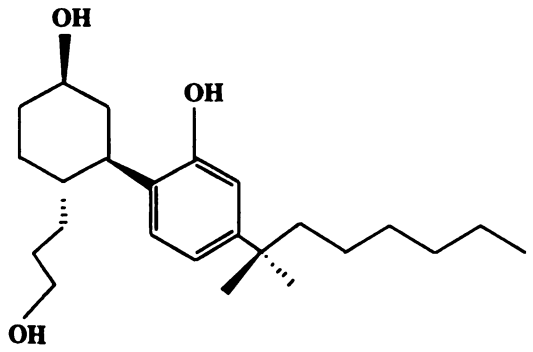
CBN



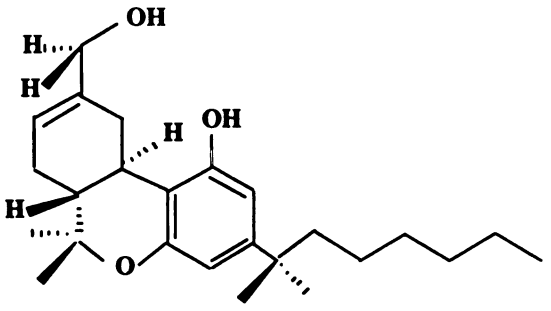
CBD



WIN-55212



CP-55,940/CP-56,667



HU-210/HU-211

Figure 1. Structures of cannabinoid compounds. Δ^9-THC, CBN and CBD are plant-derived cannabinoids. WIN-55212, CP-55,940/CP-56,667, and HU-210/HU-211 are synthetic cannabinoid stereoisomer pairs.

2-arachadonyl glycerol (2-AG) (35) and palmitoylethanolamide (36, 37) might also be cannabimimetic.

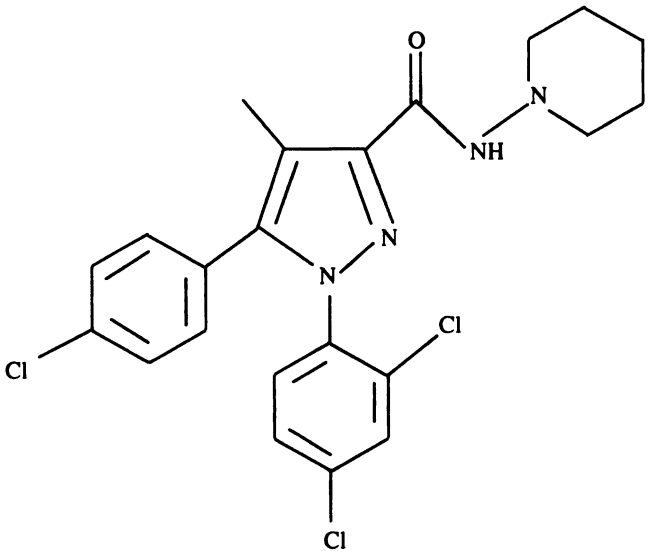
B. Cannabinoid receptor antagonists

The first cannabinoid receptor antagonist was synthesized in 1994 and exhibited high affinity binding for the CB1 receptor (SR141716A) (38). This was followed shortly thereafter by the synthesis of an antagonist for the CB2 receptor (SR144528) (39). Both of these compounds are based on a pyrazole structure and act as competitive antagonists (Figure 2). Under certain conditions, cannabinoid receptor agonists have demonstrated antagonist activity (40-42). In addition, there have been other compounds synthesized as CB1 antagonists including AM-630 and LY320135 (43, 44), although the SR compounds are the best characterized cannabinoid antagonists.

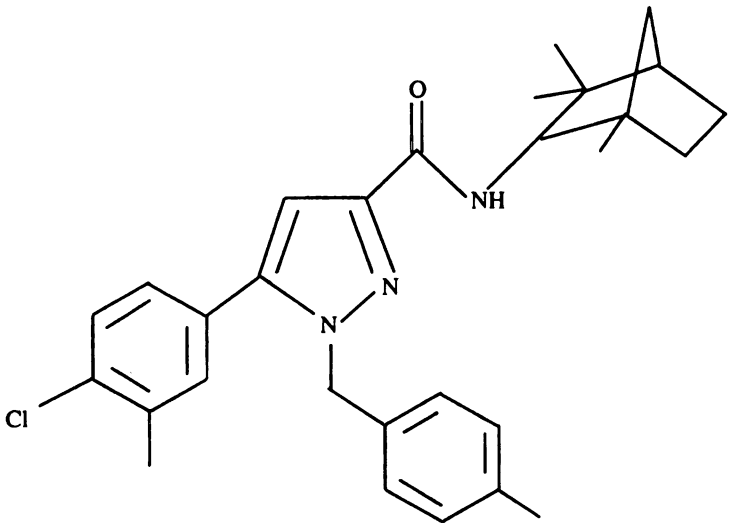
C. Cannabinoid receptors

Two cannabinoid receptors have been cloned, CB1 in 1990 (45) and CB2 in 1993 (46). In addition, a splice variant of CB1, CB1A, was discovered (47). Both receptors are G protein-coupled receptors with seven transmembrane domains. Human CB2 shares approximately 44% identity with human CB1, which increases to 68% when comparing residues that make up the receptor binding pocket (46).

The tissue distribution varies between the two receptors. While CB1 is primarily localized to the CNS, CB2 has been primarily characterized in cells of the immune system. Specifically, in the brain, CB1 is expressed in the substantia nigra pars reticulata, globus pallidus, hippocampus and cerebellum (48). CB1 is also found in testis (49), pituitary, adrenals, heart, lung, uterus, ovary, prostate, pancreas (50) and several immune system cells (51). In the immune system, CB1 mRNA can be detected in thymus, bone



SR141716A



SR144528

Figure 2. Structures of cannabinoid receptor antagonists. The CB1 receptor antagonist is SR141716A and the CB2 receptor antagonist is SR144528.

marrow, spleen and tonsils, usually at levels approximately 10% of those for CB2 (51, 52). In lymphocytes specifically, the amount of CB1 mRNA expressed is highest in B cells followed by NK cells, monocytes, PMNs, $CD8^+$ T cells, and $CD4^+$ T cells (51). Again, the levels of CB1 are approximately 10% of the levels of CB2.

CB2 mRNA has not been detected in the brain as assessed by RT-PCR (51, 52). It has however, been detected in murine cerebellar granule cells using *in situ* hybridization (along with CB1) and two cannabinoid binding sites were detected in this preparation as well (53). Other tissues in which CB2 mRNA has been detected include lung, uterus, pancreas, and most immune tissues (bone marrow, thymus, spleen and tonsils). In lymphocytes, the levels of CB2 mRNA expressed are highest in B cells, followed by NK cells, monocytes, PMNs, $CD8^+$ T cells, and $CD4^+$ T cells (51). It is interesting that this rank order of CB2 mRNA is the same as that for CB1 mRNA in lymphocytes.

D. Cannabinoid ligand binding affinities

Cannabinoid ligand binding affinities have been determined using displacement of tritiated compounds, usually either ^{3}H-CP-55940 or ^{3}H-WIN-55212-2. Specifically in rat brain, the rank order of displacement of ^{3}H-CP-55940 was CP-55940 > Δ^9-THC > CBN > CBD with K_i values of approximately 15, 420, 3200 and 53,000 nM, respectively (48). Thus, CBN and CBD are low affinity ligands at the CB1 receptor. WIN-55212-2 and HU-210 however, are high affinity ligands for CB1 receptors (K_d 15 nM and 0.060 nM, respectively) (54, 55). The selective CB1receptor antagonist, SR141716A, binds with high affinity to the CB1 receptor (K_i 1.98 nM) (38). With respect to the endogenous cannabinoids, ANA exhibits CB1 selectivity with K_i values ranging from 61-89 nM in the

presence of PMSF to inhibit hydrolysis of this endogenous cannabinoid (reviewed in 56). In general, the rank order potency of binding to the CB1 receptor is as follows: HU-210 > SR141716A > CP-55940 > WIN-55212-2 > Δ^9-THC > ANA > CBN > CBD (reviewed in 56, 57).

Many of the compounds mentioned above which bind with high affinity to CB1 also bind with high affinity to CB2, including HU-210, WIN-55212-2 and CP-55940 (K_i 0.524, 0.28 and 1.8 nM, respectively as determined in CB2 receptor transfected cells) (55, 58, 59). The affinity of Δ^9-THC for CB2 as determined in mouse spleen (K_i 11.8 nM) is similar to that for CB1 as determined in rat brain as mentioned above (48, 52). CBN binds with 10-fold higher affinity to CB2 than to CB1 (46), and thus, is one of the few natural cannabinoids to exhibit selectivity for a cannabinoid receptor. The endogenous cannabinoids ANA and 2-AG are relatively low affinity ligands for the CB2 receptor (K_i for both exceed 100 nM as determined in CB2 receptor transfected cells; reviewed in 56). As with the CB1 selective antagonist, the CB2 selective antagonist SR144528 binds to the CB2 receptor with high affinity (Ki 0.3 nM) (39). Therefore, the rank order potency for binding to the CB2 receptor is slightly different than that for CB1: SR144528 > HU-210 > CP-55940 > WIN-55212-2 > CBN > Δ^9-THC > ANA > CBD (reviewed in 56, 57).

E. Cannabinoid receptor cellular signaling

Many cannabinoid compounds inhibit FSK-stimulated cAMP accumulation in several cell types. In N18TG2 neuroblastoma cells, Δ^9-THC inhibited FSK-stimulated cAMP accumulation, whereas CBN and CBD had little or no effect, indicating these cells were sensitive to primarily the psychoactive cannabinoid. In addition, the inhibition was

stereoselective (60). These results collectively suggest that CB1 mediated the inhibition of FSK-stimulated cAMP accumulation in these cells. In fact, in CHO cells transfected with either the CB1 or CB2 receptor, CP-55940 inhibited FSK-stimulated adenylyl cyclase activity and this inhibition was prevented by treatment with either SR141716A or SR144528, respectively (38, 39). Under certain conditions, cannabinoids have been shown to stimulate basal (61) or FSK-stimulated (62, 63) cAMP accumulation, which has been associated primarily with the CB1 receptor. Specifically in the immune system, CBN and Δ^9-THC inhibited FSK-stimulated cAMP accumulation in EL-4 T cells (64). In addition, CBN inhibited FSK-stimulated cAMP accumulation in both murine SPLC and THMC (65). Although mRNA for both cannabinoid receptors was detected in the immune system, it was not determined which, if either, receptor mediated the FSK-stimulated cAMP accumulation.

In addition to modulation of cAMP levels, the CB1 receptor has been shown to couple to other signaling pathways including inhibition of N- and Q-type voltage dependent calcium channels (66-68), and stimulation of inwardly rectifying potassium channels (66) in neuronal cells. Cannabinoid effects on both potassium and calcium conductance were determined to be mediated via the CB1 receptor (69, 70). Transient increases in calcium in response to several different cannabinoid agonists was determined to be mediated via CB1 and CB2 in NG108-15 cells or HL-60 cells, respectively (71, 72). It was not determined in either study whether the mechanism of increased calcium was mediated via calcium channels.

Both CB1 and CB2 have also been shown to couple to the activation of ERK MAP kinases (73, 74) in CB1 and CB2 receptor-transfected cells, respectively. This

effect has also been demonstrated in cells that express endogenous cannabinoid receptors as well (75-77). In addition, CB1 has been shown to couple to activation of JNK and PKB/Akt in CB1-transfected CHO cells (78, 79).

VI. Cannabinoid effects on the immune system

A. T cells

T cells are one of the major types of lymphocytes that participate in an adaptive immune response. The adaptive immune response is characterized by the idea of immunological memory; that is, once an individual encounters a particular antigen, the immune response to that antigen upon subsequent exposures is faster and more robust. In order for T cells to become immunologically functional following development in the thymus, they must undergo several changes. T cells circulate in the body until they encounter antigen. Following exposure to antigen and appropriate signals from antigen presenting cells (APCs), T cells become "activated" and begin to secrete cytokines, including IL-2, an autocrine, paracrine T cell growth factor. T cell activation is a series of biochemical changes that results in production and secretion of IL-2, initiation of cellular proliferation, interaction with other cells and cytoskeletal rearrangement. The T cells then undergo clonal expansion or proliferation in order to increase the amount of T cells that are required to combat the antigen to which it was initially exposed. Finally, the T cells differentiate into effector T cells, usually helper T cells or cytotoxic T cells (designated T_H or T_C). Some of these antigen-specific effector T cells will remain in circulation in the event of subsequent exposures (immunological memory) (80).

It is interesting that while T cells exhibit a relatively low expression level of CB1 and CB2 mRNA (52), there are profound effects by cannabinoids on this population of cells. The T cell was determined to be a target of cannabinoids using the AFC response, a particularly sensitive immune response to cannabinoids that is actually a measure of humoral immunity (see next section). This assay is a measure of the ability of B cells to produce antibody-forming cells, secrete antibodies and lyse target cells. Using various pharmacological agents, the assay is either T cell-dependent (stimulated with sRBC) or T cell-independent (stimulated with DNP-Ficoll or LPS). It was determined that Δ^9-THC inhibited the T cell-dependent AFC response, but not the T cell-independent AFC response (81). CBN also inhibited the sRBC AFC response (82), indicating that while CBN exhibited minimal CNS activity, it possessed immunosuppressive effects similar to Δ^9-THC. Confirming that the T cell was a sensitive target of these compounds, cannabinoids inhibited proliferation in response to agents that specifically activate T cells. In mouse SPLC, Δ^9-THC inhibited Con A-, PHA- and α-CD3-stimulated proliferation in a concentration dependent manner (81, 83). These studies were again confirmed using CBN in SPLC stimulated with either α-CD3 or PMA/Io (82) and with 2-AG in SPLC stimulated with α-CD3 (84).

With the demonstration that cannabinoids inhibited proliferation in T cells, it was tempting to examine effects on the T cell growth cytokine, IL-2. Interestingly, the effects of cannabinoids on IL-2 is variable depending on age, concentration of cannabinoid and method of stimulation (85, 86). For instance, in mouse SPLC that were stimulated with an optimal activation signal to produce maximal IL-2, CBN inhibited the response; whereas in the same cells that were sub-optimally activated, CBN enhanced the minimal

IL-2 production induced by the sub-optimal stimulus (85). There have been other studies demonstrating that cannabinoid compounds inhibit IL-2 production in response to PMA/Io. In mouse SPLC and EL-4 T cells, Δ^9-THC and CBN inhibited IL-2 production at both the mRNA and protein levels in cells that were stimulated with PMA/Io (64). Similarly, CBN inhibited IL-2 protein secretion in PMA/Io-stimulated THMC (65). Thus, cannabinoid-induced inhibition was demonstrated in at least two different pure T cell populations and confirmed in a mixed lymphocyte population as well.

The mechanism of inhibition of IL-2 is partially understood to occur through inhibition of transcription factors that bind in the minimal essential promoter region of the gene (87). In T cells, for example, CBN inhibited transcription factor binding to the distal NF-AT site and the proximal AP-1 site (88) and the NF-κB site (65). Furthermore, Yea, *et. al.* demonstrated using reporter gene assays that CBN inhibited the transcriptional activity of the IL-2 promoter and, specifically, the distal NF-AT site from the IL-2 promoter. In fact, NF-AT has been determined to be a sensitive target of several cannabinoids in several cell systems. The endogenous cannabinoid 2-AG also inhibited IL-2 at the protein and mRNA level, in part due to inhibition of NF-AT transcription factor binding and transcriptional activity (89). The role of the cannabinoid receptors in these effects is not yet known.

IL-2 is one of the main cytokines secreted by T cells, but there are several others including IFN-γ, IL-3, IL-4, IL-5, IL-6, IL-10 and TGF-β (80). Cannabinoids (Δ^9-THC) have also been shown to produce inhibitory effects on IFN-γ in SPLC stimulated with Con A or PHA (90). In addition, both CBD and Δ^9-THC inhibited constitutive expression of IL-10 in a T cell line (91). Thus, cannabinoid induced immune suppression

is, in part, due to direct effects on T cells, and this might perturb the interaction between T cells and other important immune cells, such as B cells.

B. B cells

B cells are the other major lymphocyte in the immune system and they are responsible for humoral immunity; that is, the immune response mediated by antibodies. B cells mature in the bone marrow, after which they circulate in the body until they encounter antigen. B cells, similar to T cells, after exposure to antigen, undergo a series of biochemical changes, including activation, proliferation and differentiation to an effector cell. Often the activation of the B cell requires signals from the T_H cell that has previously recognized the same antigen. Thus, a robust immune response to a particular antigen often requires cooperation between B cells and T cells. The effector B cell (plasma cell) secretes antibodies specific to the antigen to which the B cell was initially exposed and these antibodies destroy pathogens in several ways. Binding of antibodies to a particular pathogen might prevent its interaction with a cellular target, promote phagocytosis and destruction by macrophages, or activate the complement system (a series of cleavage enzymes that destroy the pathogens). Again, like T cells, a small population of antigen-specific memory B cells remain in circulation in the event of subsequent antigen exposure (80).

B cells express the highest relative level of CB1 and CB2 mRNA in immune system cells (51) and cannabinoids affect B cell function in many ways. Humoral immunity was adversely affected by Δ^9-THC as determined using the AFC response. As mentioned above, the AFC response, which measures the ability of B cells to produce antibodies, was inhibited in the presence of Δ^9-THC when stimulated with the T-

dependent antigen, sRBC, but not the T-independent antigens, LPS or DNP-Ficoll (81). This inhibition of the AFC response to sRBC was stereospecific (using the cannabinoid stereoisomer pairs HU210/HU-211 and CP-55940/CP-56667) and could be reversed with cAMP analogs, suggesting that the cannabinoid receptors might be involved in the inhibition of the sRBC AFC response (92, 93). Thus, cannabinoids inhibited a B cell response that is dependent on T cells, demonstrating the impact of cellular cooperation in the immune system.

B cells however, are also directly modulated by cannabinoids. B cell proliferation, for example, was inhibited in SPLC by CBN when co-treated with the polyclonal mouse B cell activator, LPS (82). In addition, LPS-induced B cell proliferation was more sensitive to inhibition by Δ^9-THC than was Con A- or PHA-induced T cell proliferation in mouse SPLC (94). Furthermore, the endogenous cannabinoid, 2-AG, inhibited LPS-stimulated B cell proliferation in mouse SPLC (84). It was not determined in these studies if the inhibition of B cell proliferation was mediated via cannabinoid receptors. While there were several reports demonstrating that cannabinoids inhibited B cell proliferation, a few studies reported enhancement of B cell proliferation at low cannabinoid concentrations (95, 96). CP-55940, WIN-55212-2 and Δ^9-THC stimulated human B cell proliferation when co-stimulated with the cannabinoid and either anti-Ig cross-linking or CD40 ligand, both of which stimulate B cell proliferation. One difference between these studies and the aforementioned studies in which inhibition of B cell proliferation was observed was not only the concentration of cannabinoid but that the studies in which B cell proliferation was enhanced was conducted in serum-starved cells. The authors did report that the CB1 antagonist,

SR141716A did not reverse the cannabinoid-induced enhancement of B cell proliferation (the CB2 antagonist was not yet synthesized for this study) (96). However, in a later study by the same group, CP-55940-induced enhancement of B cell proliferation was determined to be mediated via the CB2 receptor using the CB2 antagonist, SR144528 (97).

The cannabinoid receptors, in addition to mediating the cannabinoid-induced enhancement of B cell proliferation, also were determined to play a role in B cell differentiation. Comparing B cells of different maturity levels as determined by differential expression of extracellular markers, it was determined that the CB2 receptor expression was highest in virgin and memory B cells and relatively lower in centroblast and germinal center B cells (proliferating B cells). The change in CB2 receptor expression was detected at both the mRNA and protein level (97). Interestingly, in both virgin and germinal center B cells, following stimulation with CD40 ligand, CB2 receptor expression was increased at both the protein and mRNA level with maximal expression at 24 h (97). Thus, CB2 receptor expression is upregulated following stimulation with CD40 ligand irregardless of the maturity level of the B cells. This result was also confirmed in mouse SPLC with CD40 ligand (98). Thus, CB2 receptor expression was relatively high in resting B cells and was induced in response to CD40 ligation. This was followed by a downregulation of CB2 expression in proliferating B cells and finally, a return to high levels of CB2 expression in memory B cells (97), indicating that the CB2 receptor plays a role in B cell differentiation.

C. Macrophages

Macrophages mature from monocytes, which are derived from a myeloid progenitor cell in the bone marrow, and they serve many different roles in the immune system. Macrophages are a critical part of the innate immune system, they can be activated by the TH_1 cells to aid in acquired immunity, and they process and present antigens to T cells. Innate immunity differs from acquired immunity in that there is no immunological memory and the response is non-specific. For example, macrophages engulf and digest many substances detected as non-self, including a wide range of pathogens. This process does not require proliferation of an antigen-specific cell, nor will the macrophage establish memory cells, as with B cells and T cells. Once a macrophage engulfs a pathogen, for example, it can process and present an antigenic peptide on the cell surface for specific recognition by T cells. In this case, the macrophage acts as an APC and recruits help from T cells, which can subsequently mount a specific immune response and provide activation signals back to the macrophage. The activated macrophage secretes cytokines (TNF-α and IFN-γ) and several other factors, such as nitric oxide and oxygen radicals, intended to destroy the pathogen. The cross-talk between T cells and macrophages again demonstrates the cellular cooperation that occurs during an immune response (80).

The ingestion of pathogens by macrophages is one of the primary defenses of the innate immune system and cannabinoid compounds have been shown to inhibit phagocytosis. In mouse peritoneal macrophages, Δ^9-THC inhibited the ability of these cells to phagocytize yeast (99). In addition, phagocytic activity of human peripheral macrophages and the macrophage cell line P388D1 was inhibited by Δ^9-THC (100, 101).

In addition, the production of various cellular factors by macrophages was also inhibited in the presence of cannabinoid compounds. Specifically, in LPS-stimulated RAW cells, a macrophage cell line, Δ^9-THC inhibited the expression of iNOS, and subsequently, the production of nitric oxide, which was mediated, in part, by an inhibition of NF-κB DNA binding activity (102). Similar studies were conducted in rat microglial cells and CB1 was determined to mediate the LPS/IFN-γ-stimulated inhibition of nitric oxide by CP-55940 (103). Furthermore, KCl-induced activation of nitric oxide synthase was inhibited by WIN-55212-2, CP-55940 and HU-210 and this effect was determined to be mediated via CB1 as determined using SR141716A (104).

Cytokine production by macrophages was also inhibited by cannabinoid compounds, particularly TNF-α (105-108). In only one of the above studies was the cannabinoid receptor involvement addressed, and it was determined that the inhibition of LPS-stimulated cytokine production (IL-1α, IL-1β, IL-6 and TNF-α) by Δ^9-THC and CP-55940 in rat microglial cells was not mediated via either cannabinoid receptor (107). In addition, the inhibition of TNF-α by Δ^9-THC in macrophage cell lines was shown to be a post-translational event; that is, via inhibition of the conversion of the presecretory form to the secretory form (108).

As with T cells, there are also conflicting reports of cannabinoid effects on cytokines produced from macrophages. As mentioned above, Δ^9-THC and CP-55940 inhibited IL-1α in LPS-stimulated microglial cells (107). However, it was also demonstrated that Δ^9-THC increased the production of IL-1 from macrophages that were treated with LPS (109). Interestingly, the major difference between these studies was that the order of treatments was reversed. In the study in which inhibition was

demonstrated, the cells were treated with Δ^9-THC before LPS; while in the study in which inhibition was demonstrated, the cells were treated with LPS before Δ^9-THC (107, 109). This suggests that the time of addition of cannabinoids with respect to the activation signal might impact the effect by cannabinoids. In fact, the time of addition was determined to be critical in the inhibition by Δ^9-THC in the sRBC AFC assay. It was determined that Δ^9-THC inhibited an early event because Δ^9-THC inhibited the response only if it was added within 2 h of the stimulation with sRBC (110).

Several studies have demonstrated that cannabinoids inhibit antigen processing and presentation to T cells. In T cells that were incubated with macrophages and antigen (hen egg lysozyme or hen egg lysozyme peptide fragments), Δ^9-THC inhibited hen egg lysozyme-induced T cell proliferation. The magnitude of inhibition by Δ^9-THC was greater in the studies in which the entire antigen was utilized, indicating that Δ^9-THC inhibited antigen processing to a greater degree than antigen presentation. These effects were determined to be mediated by a direct effect of Δ^9-THC on macrophage function and not due to a decrease in the interaction between the macrophages and the T cells (111). Furthermore, the effect on macrophage antigen processing was determined to be CB2 mediated as assessed using the CB2 receptor antagonist and CB2 receptor knockout mice (112, 113).

D. Host resistance studies

Several studies demonstrated that cannabinoid compounds exhibit suppressive actions on immune cells as described in the previous sections. These effects on various cell types contribute to suppression of the immune system, which can be measured using host resistance studies. Early studies demonstrated decreased host resistance to *Listeria*

monocytogenes and Herpes Simplex Virus in mice treated with Δ^9-THC (114). Host resistance to *Legionella pneumophilia* was decreased in mice (115), and in macrophages that were treated with Δ^9-THC, the growth of *Legionella pneumophilia* was enhanced (116). Furthermore, host resistance to *Corynebacterium parvum* was also decreased in mice treated with WIN-55212-2 and HU-210 (117). In addition, other immune responses are inhibited in response to various pathogens. Specifically, in mice inoculated with Herpes Simplex Virus 2 (HSV2), Δ^9-THC inhibited *in vitro* proliferation of SPLC which were challenged with HSV2 (118). Similarly, Δ^9-THC inhibited IFN-γ and IL-12 cytokine production in mice that were treated with *Legionella pneumophilia* and this effect was determined to be mediated through both CB1 and CB2 (115). Finally, in LPS-treated mice that were primed with *Corynebacterium parvum*, WIN-55212-2 and HU-210 inhibited production of TNF-α and IL-12. These effects were prevented with pretreatment with the CB1 antagonist (117).

VII. Signaling in T cells

Cannabinoid compounds modulate immune function through various effects on different immune cells. The T cell however, was determined to be a sensitive target of inhibition by these compounds as assessed by effects on the sRBC AFC response (81). Moreover, production of IL-2 in activated T cells is also subject to inhibition by cannabinoids (64). Thus, the review of intracellular signaling is restricted to T cells.

A. cAMP

cAMP is a critical second messenger in almost every cell type. It is converted from ATP by adenylate cyclase, of which there have been nine different forms cloned to

date. These enzymes are differentially regulated by other cellular signals, including calcium, PKA, PKC and G protein-coupled receptors (reviewed in 119). For example, cAMP production is induced by the α subunit of G_s protein-coupled receptors and inhibited by the α subunit of $G_{i/o}$ protein-coupled receptors via activation or inhibition of adenylate cyclase, respectively (reviewed in 119). The intracellular cAMP level is also regulated by phosphodiesterases, which hydrolyze cAMP (120).

The signaling cascade associated with cAMP has been well characterized and involves (with respect to the cannabinoid receptors) negative regulation of adenylate cyclase by the α subunit of G_i protein-coupled receptors, which inhibits cAMP production (Figure 3). This subsequently inhibits PKA activity, which requires cAMP binding to its regulatory subunit to release catalytic subunits (121, 122). A primary target of phosphorylation by the catalytically active PKA is CREB, a transcription factor which binds in the promoter region of cAMP-responsive genes (123). While PKA has been traditionally identified as a target of cAMP activation, there are other intracellular targets as well. In human T cells, cAMP increased the open probability of potassium channels (124), which might affect the membrane potential of the T cell and subsequently, calcium influx. In addition, there are cAMP-responsive GEFs, which might activate B-raf and, in turn, MEK to active ERK MAPK (125, 126). Finally, cAMP has been shown to activate ras in a PKA-independent manner in thyroid cells and melanocytes (127, 128), thereby providing another link to the ERK MAPK pathway.

Inhibition of the cAMP signaling cascade by cannabinoid compounds has been demonstrated in lymphocytes following stimulation with FSK (64, 82, 129). Specifically, Δ^9-THC and CBN inhibited FSK-stimulated cAMP accumulation, PKA activity and

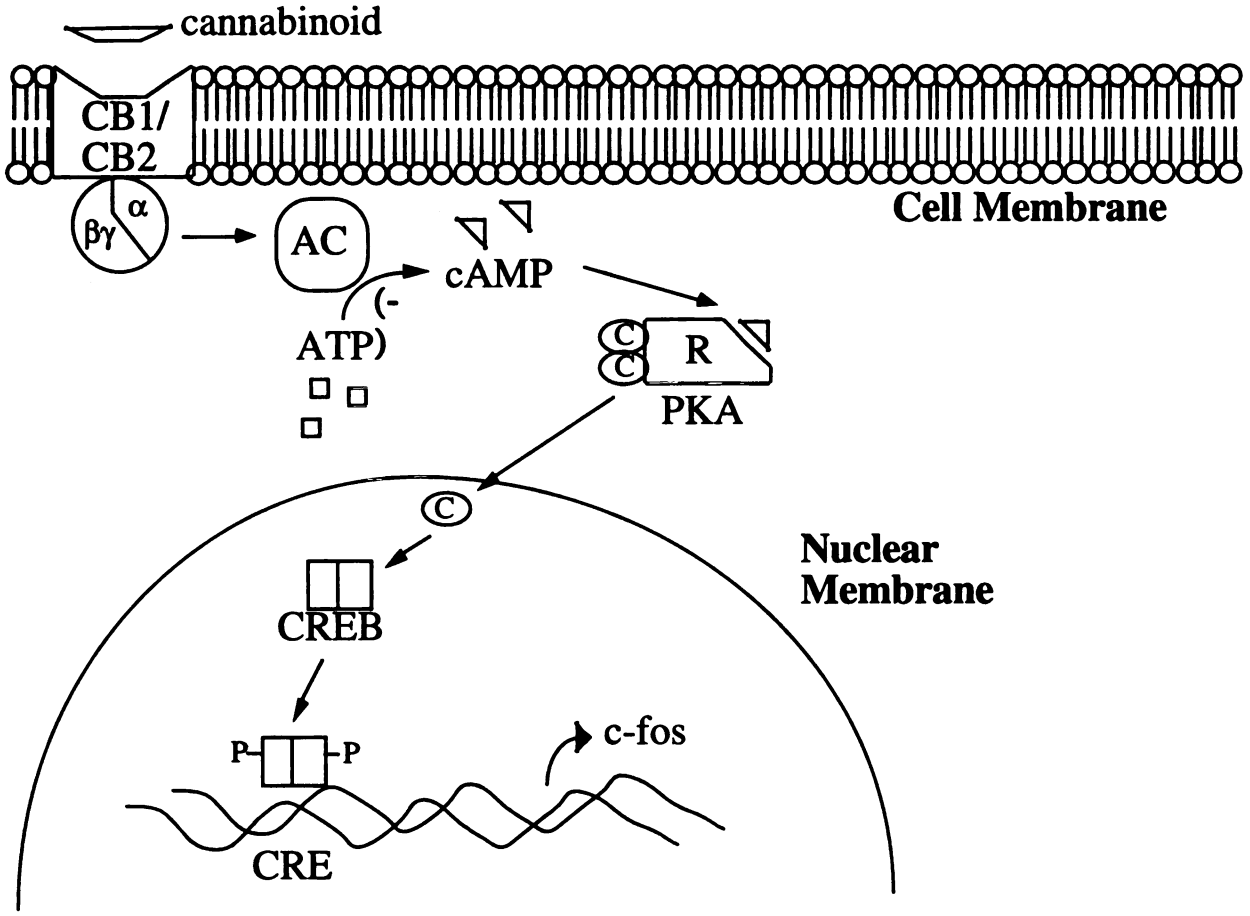


Figure 3. Inhibition of the cAMP signaling cascade by cannabinoids in T lymphocytes. Cannabinoids inhibit cAMP production and subsequently, PKA activity, CREB phosphoryation, and CREB binding to the CRE DNA element in cAMP-responsive genes such as c-fos. It is not yet known if these effects occur via either CB1 or CB2.

CREB binding to a CRE consensus sequence (64, 82, 129). It was not determined in these studies whether or not the inhibition of the cAMP signaling cascade was mediated via the cannabinoid receptors.

The inhibition of the cAMP signaling cascade by cannabinoids and the demonstration that cannabinoids inhibit T cell function lead to the hypothesis that cAMP plays a critical role in T cells. However, there have been several studies demonstrating that increased cAMP levels inhibit T cell activation and IL-2 production. Specifically, high intracellular cAMP levels (>250 μM) were historically thought to be immunoinhibitory as demonstrated by decreased IP_3 and calcium mobilization following cholera toxin treatment (130), decreased IL-2 production post treatment with PGE1, DBcAMP, or FSK (131), and suppression of IL-2 promoted T cell cycle progression following cholera toxin or DBcAMP treatment (132). Nevertheless, even in these studies which demonstrate an inhibitory effect of cAMP on T cell function show either no inhibition or an immunostimulatory effect at lower concentrations of cAMP (131). In addition, some of these inhibitory effects were not seen until later times (i.e., >90 min) following T cell activation (130). This suggests that there might be a differential role of cAMP on T cell activation depending on intracellular levels and when the cAMP signal is generated with respect to T cell activation. Indeed, this has been confirmed in several studies. In mouse SPLC, cAMP was readily induced, with a peak level of production at 3 min post cell activation, with PMA/Io, a stimulus which mimics activation via the T cell antigen receptor (93). In addition, the PKA I isozyme has been shown to be activated within 1 min following T cell activation with either PMA/Io or α-CD3 plus recombinant IL-1, which returned to baseline by 60 min. The PKA I isozyme exhibits peak

phosphotransferase activity at 5 min and phosphorylates many membrane-associated proteins (133). Furthermore, T cells that express a dominant-negative form of CREB, resulting in a protein which is no longer PKA-responsive due to a mutation at serine 119, demonstrated decreased IL-2 production associated with a decrease in expression of c-jun, c-fos, fra-2 and fos-B (134). The use of membrane permeable cAMP analogs (i.e. DBcAMP or 8-bromo-cAMP) provides further evidence for a positive role for cAMP in immune function. There are differential effects on the T cell-dependent AFC assay and PMA/Io-induced proliferation following treatment with DBcAMP: DBcAMP concentrations up to 50 μM were immunostimulatory, whereas increasing the concentration of DBcAMP further had no stimulatory effect (135). There is also evidence that agents that increase levels of intracellular cAMP are able to reverse the immunoinhibitory effects produced by cannabinoid treatment. In SPLC, the inhibition of the T cell dependent AFC assay by Δ^9-THC could be reversed with glucagon, which upon binding to the glucagon receptor couples positively to adenylate cyclase (136). Furthermore, the inhibition by Δ^9-THC of the AFC response in mouse SPLC could be reversed by DBcAMP treatment (up to 100 μM; 250 μM demonstrated an inhibitory effect) (93).

The differential role of cAMP in immune function can be explained in many ways. First, there might be a time and concentration dependence of cAMP on T cell activation as suggested by the "burst" in cAMP following cellular activation, and immunostimulatory and immunoinhibitory effects of membrane permeable analogs at lower and higher concentrations, respectively (93, 133, 135). Second, the role of cAMP might depend on which PKA isozyme is activated, PKA I or PKA II (133). Third, the

PKA targets might result in opposing effects of cAMP. For example, phosphorylation of IκB in the cytosol targets its degradation and allows subsequent release of NF-κB to the nucleus to activate transcription of IL-2, for instance (137, 138). Conversely, phosphorylation of PLC-γ by PKA results in inhibition of T cell activation (139). Therefore, the role of cAMP in immune function is complex, yet crucial, and the inhibition of cAMP production by cannabinoids might mediate the immunosuppressive actions of these compounds.

B. IL-2

IL-2 is an autocrine, paracrine T cell growth factor required for proliferation and differentiation into either T_H or T_C. In response to specific antigen and a co-stimulatory signal via CD28 on the T cell, T cells enter the cell cycle and begin to produce IL-2 (in resting, naive cells, IL-2 is not produced). Secreted IL-2 then binds to IL-2 receptors to induce signaling pathways for proliferation and differentiation. In SPLC, IL-2 production depends on two critical signals, calcium and PKC, both of which are generated following T cell receptor ligation (reviewed in 140). PMA/Io is a pharmacological tool that mimics antigen-T cell receptor ligation to generate the PKC and calcium signals, respectively (141).

IL-2 production is a hallmark of T cell activation. As mentioned briefly above, T cell activation is a series of biochemical changes in the T cell in response to specific antigen recognition (or pharmacological agents such as PMA/Io). The T cell receptor is a complex of several subunits and is sometimes referred to as the TCR/CD3 complex. The ζ chains of the CD3 complex are responsible for part of the initial T cell signaling. In addition, the TCR/CD3 complex associates with a co-receptor, either CD4 or CD8.

These co-receptors dictate whether the T cell will differentiate into T_H or T_C, respectively. Thus, T_H are often referred to as $CD4^+$ cells and T_C are referred to as $CD8^+$ cells. CD45, another membrane bound protein, also gets activated early in the T cell activation process and acts as a phosphatase to, in turn, activate cytosolic tyrosine kinases (fyn and lck). Lck associates with CD4 (in the case of T_H cells) and fyn associates with the ζ chains of the CD3 complex. CD4 and the ζ chains of CD3 are phosphorylated and promote binding of ZAP-70. ZAP-70 and fyn activate PLC-γ to cleave phosphatidylinositol to DAG and IP_3. These signals, in turn, activate PKC and elevate intracellular calcium, respectively, to induce IL-2 as described below (80, 140).

IL-2 is tightly regulated by several transcription factors that bind in the minimal essential promoter of the gene (transcriptional start site to –300 bp) (87). Important transcription factors for IL-2 include AP-1, NF-AT, NF-κB, OCT and proteins which bind to the CD28RE (Figure 4). Several of these transcription factors can be found in many cell types, but it is the cooperative binding between them on a relatively short piece of DNA that regulates IL-2 (142). As the regulation of each of these transcription factors is complex, each will be described individually.

1. AP-1

AP-1 binds the IL-2 promoter in at least two distinct places, the proximal AP-1 site (-150 bp) and the distal AP-1 site (-180 bp) (143). AP-1 sites are also known as TREs because often the proteins are inducible by TPA (144). The proximal (TGACTCT) and distal (TGACTGA) AP-1 sites differ from the AP-1 consensus (TGACTCA) site by only one base pair in mice (87). Both distal and proximal AP-1 sites bind purified AP-1 proteins (145-147); however, in cells that were activated with PMA/Io, proteins bind to the

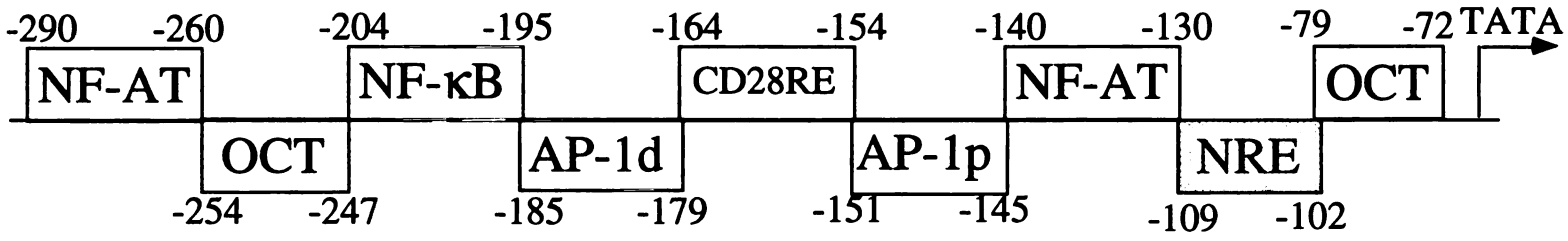


Figure 4. The minimal essential promoter region of IL-2. Several transcription factors bind in the first 300 base pairs upstream of the transcriptional start site in the promoter region of IL-2. Many act cooperatively to induce IL-2; however, the NRE is shaded to indicate that protein binding at this site is often associated with downregulation of the IL-2 promoter.

proximal AP-1 site but not the distal AP-1 site (143). In Jurkat cells, deletion of the distal AP-1 site does not cause inhibition of IL-2 promoter induction (145). The proximal AP-1 site however, confers responsiveness to T cell receptor ligation (148). In addition, in murine T cell clones, mutation of the proximal AP-1 site inhibited IL-2 promoter induction in response to α-CD3 and PMA/Io (143). Interestingly, mutation of the distal site resulted in IL-2 promoter inhibition when cells were stimulated with α-CD3 but not PMA/Io. These results suggest that the proximal AP-1 site is required for IL-2 production whereas the distal AP-1 site might enhance activation *in vivo* (143).

In addition to binding at the proximal and distal AP-1 sites, AP-1 proteins participate in complexes that form at almost every other defined binding site in the promoter. For instance, OCT binds in association with OAP, which has been identified as fos and jun family member proteins (149, 150). Furthermore, fos and jun proteins form complexes with NF-κB proteins and this interaction synergistically activates an NF-κB-responsive gene (151). c-Fos and c-jun participate in binding at the CD28RE and other proteins that bind at the CD28RE function in conjunction with AP-1 proteins at the adjacent proximal AP-1 site (152). Finally, the distal NF-AT site is a composite site comprised of NF-AT proteins from the cytosol and fos and jun proteins from the nucleus (153).

AP-1 has been well characterized as a heterodimer of the c-fos and c-jun nuclear proteins; however, over 50 family members of the BZIP superfamily of proteins might form cross-family homo and heterodimers and bind to AP-1 sites (reviewed in 154, 155). These include members from the jun (c-jun, jun-B, and jun-D), fos (c-fos, fos-B, fra-1, and fra-2), CREB/ATF (CREB, CREM, ATF-1, and ATF-2), and maf (maf-K,

maf-F, maf-B, nrl) families, with the important exception that fos family homodimers will not form. The consensus AP-1 site contains a 7 base pair core sequence TGACTCA. It is not surprising that other BZIP superfamily members can bind to this sequence as the AP-1 consensus site differs from the CRE consensus site by only one base pair, TGACGTCA (154).

Transcriptional and post-translational regulation of the members of the BZIP superfamily varies greatly and this accounts for the differences in inducibility of these proteins. c-Jun is regulated by an AP-1-like site, which binds a heterodimer of c-jun and ATF-2, and acts as a positive autoregulatory loop for the protein (156). In addition, the c-jun promoter also contains a CAAT box and a GC box, which are recognized by the transcription factors CTF and SP1, respectively (156). With the positive feedback loop, c-jun is often expressed in unstimulated cells, and can be upregulated. Interestingly, the c-jun and ATF-2 proteins are both phosphorylated and activated by SAPK, also known as JNK (157-161). The residues, which are phosphorylated on these proteins by SAPK (serine 63 and 73 for c-jun and threonine 63 and 71 for ATF-2), are located in the transactivation domains of the proteins and do not affect DNA binding (162). In addition, c-jun has been shown, at least *in vitro*, to be phosphorylated by several kinases near the C terminus, including casein kinase II (163) and glycogen synthase kinase 3, (164), which negatively regulates its DNA-binding activity. The dephosphorylation necessary for DNA binding is thought to occur via a PKC-dependent phosphatase (164). Thus, c-jun must be dephosphorylated for DNA binding and phosphorylated for transactivation potential. The phosphorylation of c-jun in the transactivation domain (serines 63 and 73) also enhances its ability to interact with a

co-activator CBP, which potentiates activated gene expression by CREB and c-jun and allows for an interaction between transcription factors and TFIIB, part of the complex at the transcriptional start site (165-167). Recently, another transcriptional co-activator for c-jun was discovered, JAB1, which is not affected by phosphorylation state, but confers stability to complexes which interact with AP-1 sites (168). In addition, JNK phosphorylation of c-jun in the transactivation domain stabilizes the protein by suppressing ubiquitination and subsequent degradation by the 26S proteasome (169-171). Therefore, phosphorylation of the c-jun protein affects its ability to dimerize, bind to DNA, transactivate genes, and finally, regulates its degradation.

Cellular stimulation with various agents (serum, calcium, growth factors, cAMP, and PMA/Io) results in a rapid and transient induction of c-fos transcription (172-174). c-Fos is also regulated by several elements in its promoter. Among these are a CRE that is located proximal to the TATA box start site, which confers cAMP-responsiveness to c-fos (154, 173). Another element which regulates c-fos expression is the SIE, originally named for the c-fos promoter response to v-sis-conditioned medium (v-sis is the viral counterpart to the platelet-derived growth factor β-chain) (175). The protein bound to this element was originally described vaguely as SIF, a 91-kDa protein that was induced in response to growth factors (176). Recently, however, it has been established that STAT binds to the SIE in the c-fos promoter conferring cytokine-responsiveness (177-179). A third important regulator of c-fos transcription is the SRE, which is recognized by SRF, a dimer that recruits ternary complex factors, such as elk-1 (180). Similar to c-jun, c-fos is tightly regulated by phosphorylation. One of the most important phosphorylation events in c-fos induction is the ERK phosphorylation of the

ternary response factor, elk-1 (181, 182), which then induces rapid transcription of c-fos in response to growth factors. Additionally, elk-1 is phosphorylated by the SAPK and p38 members of the MAPK family, conferring c-fos responsiveness to UV radiation and inflammatory cytokines, such as IL-1, both of which are not strong inducers of ERK activity (183-185). The c-fos protein itself is also post-transcriptionally phosphorylated. Transcriptional activation is increased with phosphorylation of threonine 232 in the c-fos protein by ras-dependent fos-regulating kinase (FRK), an enzyme which is distinct from the MAPK members ERK and SAPK (186). Furthermore, c-fos is phosphorylated by MAPK and RSK in the transrepression domain of the protein and these two phosphorylation events appear to be cooperative (187). It was later demonstrated that these two phosphorylation events are mediated by mos, a serine/threonine kinase that activates ERK MAPK via phosphorylation of MEK. These phosphorylation events result in increased stabilization of the c-fos protein (188). Thus, similar to c-jun, the c-fos protein is tightly regulated both transcriptionally by regulatory protein binding in the promoter, and post transcriptionally by phosphorylation via several signaling cascades.

CREB has traditionally been described as a nuclear transcription factor that is primarily responsive to agents that elevate intracellular cAMP. It is phosphorylated on serine 133 by PKA (189) and this causes dimerization and binding to DNA at CREs in cAMP-responsive genes, such as c-fos (123, 190). Additionally, serine 133 can be phosphorylated by RSK-2, a CREB kinase. This results in activation of CREB in a cAMP-independent manner as RSK-2 is activated by the MAPK cascade (191). It has also been demonstrated that CREB is phosphorylated by p38 (192, 193) and CaMKIV (194, 195). Although the AP-1 complex is historically thought to be a

heterodimer of c-fos and c-jun proteins, recent data suggest that CREB, via its leucine zipper dimerization region, might form cross-family heterodimers with c-fos and/or c-jun and participate in binding at AP-1 sites (196, 197). Further evidence that the cAMP signaling cascade, presumably via CREB activation, plays a role in AP-1 binding is that treatment of cells with FSK in addition to PMA/Io enhanced binding to proximal AP-1 sites in the murine IL-2 promoter (64, 198). The regulation of AP-1 activity therefore, is very complex, and is subject to modification at several levels, including transcriptional regulation, post-translational phosphorylation, and protein-specific interactions (i.e. c-jun/CREB dimers versus c-jun/c-fos dimers).

In addition to regulation by phosphorylation, AP-1 is also regulated by oxidation/reduction. Both c-fos and c-jun proteins undergo changes in oxidation/reduction status via a conserved cysteine residue (199). It has been demonstrated that anti-oxidants inhibit induction of c-fos and c-jun and AP-1 binding activity (200, 201). The change in oxidation status is attributed to the protein, ref-1 (202, 203). Ref-1 stimulates DNA binding by reducing the cysteine residues in c-fos and c-jun and it also acts as an apurinic/apyramidinic endonuclease (202). It is interesting that a protein responsible for repairing DNA damage also stimulates transcription factor binding to the DNA, perhaps to allow rapid transcription of damaged genes (202). In lymphocytes the effects of oxidation on AP-1 binding activity correlated with IL-2 production in that H_2O_2 treatment induced IL-2 production (204).

2. NF-AT

Despite its name, NF-AT proteins are ubiquitously expressed and there are at least four different NF-AT family members to date (reviewed in 205). NF-AT binds the

IL-2 promoter in two different places, the proximal NF-AT element (approximately –120 bp) and the distal NF-AT element (approximately –280 bp) and they share the core DNA sequence GAGGAAAA (142). As mentioned above, the distal NF-AT site is a composite site at which both NF-AT and AP-1 proteins bind cooperatively (153). Specifically in human PMA/Io-stimulated T cells, the nuclear component was comprised of fra-1 and jun-B (206). Mutation of the distal NF-AT site inhibited IL-2 reporter gene constructs by 70-85% in Jurkat cells (145). Furthermore, cyclosporin A, which has been shown to inhibit the activity of calcineurin, inhibited IL-2 via inhibition of dephosphorylation of the cytosolic component of NF-AT (207, 208).

The regulation and activation of the cytosolic component of NF-AT is mediated via phosphorylation state. NF-AT must be dephosphorylated prior to nuclear translocation and binding to DNA (reviewed in 205). Nuclear translocation following dephosphorylation occurs because of exposure of a nuclear localization signal on NF-AT (209). It has been established that the phosphatase responsible for the dephosphorylation of NF-AT in the cytosol is calcineurin (208). Calcineurin is a calcium- and calmodulin-dependent serine/threonine phosphatase (reviewed in 210). Thus, activation of NF-AT is highly calcium-dependent. Indeed, this was demonstrated using agents that increase intracellular calcium, such as Io, to induce a sustained calcium signal. Under these conditions, NF-AT remained activated and localized to the nucleus for the duration of the sustained calcium signal. Conversely, NF-AT was phosphorylated and returned to the cytosol in the absence of the calcium signal (211-213). Furthermore, it has been demonstrated that PMA enhances the calcium sensitivity of NF-AT transcriptional

activation (214). This might be one reason for the calcium-dependence of the IL-2 response in many lymphoid cell preparations, including SPLC.

As previously mentioned NF-AT binds cooperatively with AP-1 proteins. Therefore, signals that regulate AP-1 will also regulate the activity of NF-AT. For instance, a dominant negative form of c-jun blocked AP-1 and NF-AT transcriptional activity, and IL-2 gene transcription in Jurkat T cells (215). Interestingly, NF-AT cooperativity with AP-1 was determined to be critical for IL-2 production but not for expression of other cytokines such as TNF-α and IL-13 (216). Thus, NF-AT activation in one cell type might or might not induce gene expression depending on the availability of AP-1 proteins (216).

3. NF-κB

NF-κB binds to one site in the IL-2 promoter (approximately –200 bp) (142). The NF-κB site in the IL-2 promoter differs from the consensus NF-κB site (GGGATTTCAC versus GGGACTTTCC, respectively) (142). Mutation of the NF-κB site from the IL-2 promoter partially inhibited transcriptional activity (217, 218). The NF-κB site is different from the other sites discussed thus far because in unstimulated cells, there are proteins that bind the site and act as repressors (p50/p50 homodimers). Upon stimulation, these proteins are replaced by those that act as activators, such as p50/p65 heterodimers. The major difference between the two proteins is that p50 lacks the transactivation domain and therefore, requires a transactivating partner in order to induce transcription (reviewed in 142).

Similar to the other transcription factors which participate in binding to the IL-2 promoter, NF-κB is also regulated by phosphorylation, albeit indirectly. NF-κB

proteins are anchored in the cytoplasm by IκB, which prevents nuclear translocation of NF-κB. Upon phosphorylation, IκB is rapidly degraded via the ubiquitin pathway and NF-κB proteins are now able to translocate to the nucleus to induce transcription of NF-κB-responsive genes. The major regulatory event for NF-κB is the phosphorylation of IκB (reviewed in 219). Several kinases have been demonstrated to phosphorylate NF-κB *in vitro*, including PKA and PKC (220). Recently, however, IκB kinase (IKK) was identified which is now considered the primary kinase responsible for the phosphorylation of IκB (221-223). There are two forms of IκB kinase, IKKα and IKKβ, which receive signals from NF-κB inducing kinase (NIK) and MEKK, respectively (224, 225).

Similar to AP-1, NF-κB is also regulated by oxidation/reduction status. NF-κB induction occurs with agents that induce oxidative stress, including H_2O_2, TNF-α, glutathione depletion, UV, and ionizing radiation (reviewed in 226). The regulation of oxidation/reduction status might be also due to ref-1, as with AP-1 (202). Again, the induction of NF-κB in response to oxidative stress correlated with the demonstration that H_2O_2 treatment induced IL-2 production (204).

4. OCT

OCT binds in two places in the IL-2 promoter region (approximately –60 and –250 bp) (142). One point mutation of the OCT site reduced IL-2 promoter activity by 40% and a double mutation resulted in a stronger suppressive effect (227). These sites are bound by both the ubiquitously expressed OCT-1 and the lymphoid-specific OCT-2 (228-230). Interestingly, in resting cells, OCT contacts all the nucleotides in the OCT element, including the flanking region; whereas, in activated cells, AP-1 proteins bind to the 5' region of the OCT site and prevent OCT binding to those nucleotides (142, 227). The

regulation of OCT is not well characterized in T cells, although one must assume that via its association with OAP (149, 150), the regulation of AP-1 protein members contributes to the activation status of this transcription factor complex.

5. CD28RE

CD28 is a co-stimulatory molecule on T cells that, in addition to antigen-T cell receptor ligation, provides a critical signal for optimal IL-2 production in many cell types, including SPLC (85). Furthermore, *in vivo*, T cells that receive signals from antigen, in the absence of CD28 co-stimulation, become anergic (unresponsive) (231). The CD28RE is located at approximately –160 bp in the IL-2 promoter (142). Mutation of this site causes loss of CD28-responsiveness without affecting signals via T cell receptor ligation, providing evidence that CD28 is a co-stimulatory molecule (232). The protein members of the NF-κB family have been shown to bind to the CD28RE (152, 233, 234). Furthermore, due to its proximity to the AP-1 site at –150, there have been reports of cooperation between AP-1 proteins and those that bind the CD28RE (152).

In addition to the signaling pathways that induce AP-1 and NF-κB, which would also induce binding to the CD28RE, there are other signaling cascades activated by CD28. Signaling cascades activated via CD28 include PLC, ras, PI-3-kinase, and ceramide/sphingomyelinase. All of these pathways have been determined to induce IL-2 production (reviewed in 235).

6. NRE

With the exception of the NF-κB p50/p50 homodimer, most of the transcription factors that bind in the minimal essential promoter region of IL-2 act cooperatively to induce transcription of the gene as described above. It was determined

however, that there is a negative regulatory element of IL-2 in the promoter denoted the NRE located at –102 bp (142). Mutation of the NRE sequence resulted in stimulation of the IL-2 promoter in Jurkat cells (236). The NRE site is bound by ZEB (237), a zinc finger protein that also suppresses immunoglobulin heavy chain transcription in B cells (238). In EL-4 T cells, there was constitutive ZEB binding in unstimulated cells, but PMA/Io further induced ZEB binding (237). This suggests that the NRE acts to silence IL-2 production in unstimulated cells and perhaps to control the activity of the IL-2 promoter in stimulated cells.

In addition to controlling or silencing the IL-2 promoter in stimulated or unstimulated T cells, binding to the NRE is also induced in anergic T cells (239). Anergy is a state of unresponsiveness in T cells as characterized by decreased production of IL-2, AP-1 and NF-κB DNA binding activity (240-243). T cells become anergic if they receive an inappropriate or incomplete activation signals (231). This helps protect from immunological tolerance, that is, the ability of the T cells to recognize self-antigens. Often presentation of self antigens to a T cell occurs in the absence of the appropriate co-stimulatory molecule and therefore, the T cells become unresponsive to the self antigens to prevent autoimmune diseases (80). With the effects of cannabinoid compounds on AP-1, NF-κB and IL-2, it is tempting to speculate that cannabinoids induce an anergic-like state in T cells.

C. ERK MAPK

ERK MAPK refers to two forms of the ERK MAPK protein (p44 and p42), translated from the genes, ERK1 and ERK2. ERK MAPK-1 and –2 are proline-directed protein kinases that phosphorylate targets on serine and threonine residues (reviewed in

244). In addition to ERK MAPK-1 and –2, ERK MAPK-3 has recently been identified and is primarily localized to the nucleus (245). For the purpose of this discussion, ERK MAPK will denote forms 1 and 2, unless noted.

ERK MAPKs are the last kinases in the three-kinase signaling module, MAPKKK-MAPKK-MAPK. The activation of ERK MAPK depends on phosphorylation of two critical residues (threonine and tyrosine) located in the recognition sequence, tyrosine-glutamic acid-threonine (246, 247). This phosphorylation is mediated via MEK (248) and to date, no other MAPKK has been identified that phosphorylates ERK MAPK (249). ERK MAPKs however, also undergo autophosphorylation and therefore, have the capability to target tyrosine residues as well (250, 251). MEK is also regulated by phosphorylation, although, unlike ERK MAPKs, which require dual phosphorylation, MEK can be partially activated with phosphorylation at either one of two critical serine residues (252, 253). MEK is autophosphorylated as well on threonine, tyrosine, and serine residues (253).

There are several kinases that will phosphorylate MEK, which might explain the various cellular processes that ERK MAPKs have been determined to regulate, including proliferation, differentiation, stress responses and apoptosis (reviewed in 254). One of the best-characterized activators of MEK is the MEKK (or MAPKKK), raf (255). Raf activation is also regulated by phosphorylation. Raf is phosphorylated on serine, threonine and tyrosine residues and also undergoes autophosphorylation (256, 257). Phosphorylation of the tyrosine resides seems to be critical for activation and therefore, tyrosine kinases such as src and fyn are possible activators of raf (256, 258-261). This provides one potential link to the ERK MAPK pathway in T cell activation as fyn is a

critical tyrosine kinase in early T cell activation (140). In addition, ras activates raf via interaction with GEFs to promote raf membrane localization (262, 263). The membrane localization of raf might promote interactions between raf and PKC as well (264). Ras activation of raf also occurs in response to activation of receptor tyrosine kinases and G protein-coupled receptors (265-267).

The ERK MAPK cascade is, as described above, activated primarily via phosphorylation; therefore, deactivation of the cascade occurs via dephosphorylation. Indeed, many kinases are also closely associated with phosphatases (reviewed in 268). With respect to ERK MAPK, there is a family of phosphatases, including MKP and PAC, which are responsible for downregulation of kinase activity (269, 270). Often, these phosphatases are induced by similar signals that induce ERK MAPK, thereby providing negative regulation of ERK MAPK (269, 270).

Targets of ERK MAPK include transcription factors such as elk-1 and c-jun, kinases such as RSK (reviewed in 267), transcriptional machinery such as RNA polymerase II (271, 272), and cell cycle regulatory proteins, such as Rb (273, 274). The various targets of ERK MAPKs explain the great diversity of responses that these kinases regulate.

Specifically in T cells, the ERK MAPK signaling cascade has been shown to be critical for IL-2 production in Jurkat and EL-4 T cells (275, 276). In a study by Whitehurst, *et. al.*, NF-AT transcription and IL-2 production was induced by expression of constitutively active raf or MEK (275). On the other hand, Li, *et al.* demonstrated that Jurkat cells transfected with a dominant negative mutant of ERK MAPK-1 suppressed IL-2 production (276). While these data provide evidence for a critical role of the ERK

MAPK signaling cascade in IL-2 production, it has also been shown that IL-2 production in primary human T cells occurred via an ERK MAPK-independent pathway (277). The authors suggested that the ERK MAPK signaling cascade is less critical in primary T cells versus the T cell line. This might be due to signal redundancy or signal compensation in primary T cells.

The demonstration of ERK MAPK-independent mechanisms of IL-2 suggested that other kinases are involved in IL-2 production following T cell activation. In fact, there are two other known MAPK signaling modules, the JNK and p38 cascades. Specifically, JNK is activated in response to the co-stimulatory molecule, CD28 (278), and activation of JNK correlated strongly with IL-2 production (278). An important function of CD28-mediated activation of JNK is stabilization of IL-2 mRNA (279), which might contribute to the JNK-mediated upregulation of the IL-2 gene suggested in several studies. α-CD3- and α-CD3/α-CD28-stimulated-IL-2 production in purified splenic T cells from JNK-2-knockout mice was absent, indicating that JNK was critical for IL-2 production (280). Interestingly; however, Dong, *et. al.* demonstrated potentiation of the Con A-, α-CD3- and α-CD3/α-CD28-stimulated IL-2 response in $CD4^+$ cells from dominant negative JNK-1/JNK-2-knockout mice (281). The major difference between the two studies was the co-expression of the dominant negative JNK-1 in the studies by Dong, *et. al.*; thus, these data suggest that there are distinct roles for each of the members of the JNK family of MAPKs in T cell function. On the other hand, p38 MAPK positively regulates IL-2 production as α-CD3- and α-CD3/α-CD28-stimulated IL-2 production was inhibited in splenic T cells treated with the p38 inhibitor, SB203580 (282, 283). The regulation of MAPK signaling modules are complex,

involving sequential phosphorylation events that are often coupled with phosphatase activity (reviewed in 268). The regulation of the target molecules of these kinases; therefore, is also complex, and likely involves a combination of regulatory events mediated by redundant pathways, as demonstrated with IL-2.

D. Calcium

The T cell activation signals and, subsequently, IL-2 production is highly dependent on intracellular calcium concentration (reviewed in 140, 284). As mentioned above, intracellular calcium is elevated in T cells via activation of PLC-γ, which, in part, generates IP_3 to stimulate release of calcium from intracellular stores. It has been demonstrated that T cell activation requires a sustained calcium increase of at least 30 min (214, 285). The calcium signal has been characterized as biphasic: a rapid spike in intracellular calcium concentration due to release of intracellular stores followed by a sustained plateau due to influx of extracellular calcium via membrane channels (284). The biphasic increase in calcium concentration correlated with activation of NF-AT such that a transient spike in intracellular calcium also caused a transient nuclear localization of NF-AT and subsequently, activation of a NF-AT reporter gene. On the other hand, a sustained calcium signal resulted in both sustained nuclear localization and NF-AT reporter gene activation (286). Oscillatory calcium signals also contribute to T cell activation by enhancing low signaling efficiency and providing specificity. For instance, high frequency calcium oscillation signals (similar to steady state calcium concentration) induced a NF-AT reporter gene, such as IL-2; whereas low frequency calcium oscillation signals induced a NF-κB reporter gene (287).

Influx of extracellular calcium in lymphocytes occurs via, most likely, CRAC channels (284, 288). There is no definitive evidence that T cells express voltage-operated calcium channels (reviewed in 284). CRAC channels are highly selective for calcium (relative to other divalent cations) and are activated in response to depletion of intracellular calcium stores. Thus, CRAC channels are likely activated in response to calcium ionophores, such as Io, stimulators of intracellular calcium stores, such as IP_3, and inhibitors of the endoplasmic reticulum ATPase, such as TG (288). These channels might also be sensitive to mitogenic stimulation (284).

T cells are also regulated by potassium channels. T cells express several potassium channels, including voltage-operated potassium channels and calcium-activated potassium channels (284). Potassium channels have been determined to be critical for T cell activation as assessed by the use of potassium channel blockers (289). Furthermore, expression of calcium-activated potassium channels was increased in response to mitogenic stimulation (290). The primary role of the potassium channels is to maintain the membrane potential, which subsequently provides the gradient for calcium influx (284).

Calcium regulates the function of many proteins, including kinases, phosphatases, transcription factors, adapter proteins and others. In T cells, a primary target of calcium regulation is calcineurin (phosphatase 2B). The best-characterized function of calcineurin is its ability to dephosphorylate NF-AT, thereby allowing nuclear translocation and transcription. Calcineurin is a complex of proteins that includes two subunits and association with calmodulin. Calcium regulates calcineurin activity by binding to both the B subunit of calcineurin and to calmodulin. The cooperative calcium

binding allows calcineurin to become activated over a narrow range of intracellular calcium concentrations (reviewed in 291).

Calcium also enhances the PKC response in T cells by activating calcium-dependent PKC isozymes. In human T cells, PKC isozymes α and θ were upregulated within 10 min of cellular stimulation (292). Interestingly, the α isozyme is calcium-dependent whereas the θ isozyme is not; therefore, both PKC types are induced early in T cell activation (reviewed in 293). Specifically, PKC-θ was shown to synergize with calcineurin at the level of rac (small GTP binding protein) to activate JNK and, subsequently, NF-AT and IL-2 reporter gene constructs (294). Thus, calcium directly activates calcium-dependent PKC isozymes and, via activation of calcineurin, enhances IL-2 production through synergism with other PKC isozymes.

Other important targets of calcium regulation in T cells are the CaMK proteins, II and IV. CaMKs are regulated both by phosphorylation and in response to an elevation in intracellular calcium. CaMK can be phosphorylated by a CaMKK and also undergoes autophosphorylation (reviewed in 295). Interestingly, these two kinases have opposing effects on IL-2 production. Transfection of a constitutively active CaMKII blocked PMA/Io-induced IL-2 reporter gene activity by 90% (296). This was mediated partially via effects on NF-AT, AP-1 and OCT transcriptional activity. The role of calcium in this downregulation was suggested by the fact that pretreatment with Io inhibited PMA/Io-induced IL-2 reporter gene activity by 50%. The expression of active CaMKII also blocked IL-2 promoter activity induced by co-transfection with calcineurin, suggesting perhaps calcineurin is regulated by CaMKII (296). Indeed, there are several CaMK consensus phosphorylation sites in calcineurin (297). As opposed to CaMKII, CaMKIV

was shown to positively regulate IL-2 production. In T cells derived from transgenic mice that express a catalytically-inactive CaMKIV, no IL-2 was produced in response to PMA/Io. In addition, there was no induction of fos-B (298). It was also determined that overexpression of CaMKIV stimulated SRE-dependent transcription (299), which might contribute to calcium-dependent induction of c-fos.

Despite the necessity of calcium for T cell activation and IL-2 production, there have been observations that premature calcium signals, that is, prior to T cell activation, inhibit T cell activation and IL-2 production. One example previously mentioned was the demonstration of inhibition of PMA/Io-induced IL-2 reporter gene activity in response to an 8 hr, 2 μM Io pre-treatment (296). This suggests that calcium induces either T cell activation or anergy depending on the time of generation of the calcium signal and the total concentration of intracellular calcium. Indeed, it was demonstrated that pretreatment of PMA-stimulated Jurkat cells with increasing concentrations of A23187 induced anergy in the cells as measured by an inhibition of IL-2 production (300). Interestingly, the ability of calcium to induce either activation or anergy correlates with its ability to activate either CaMKII or CaMKIV.

VIII. Rationale

Cannabinoid compounds exhibit immunosuppressive effects, particularly with respect to T cells. The signaling that occurs in T cells following cellular activation is complex and thus, there are several potential targets of modulation by these compounds that contribute to their mechanism of immunosuppression. Results of studies demonstrating effects of cannabinoid compounds in primary mouse SPLC are presented

here and suggest that cannabinoids might be developed as potential therapeutics for their immunosuppressive effects. Initially, the studies focused primarily on CBN because it was shown to exhibit ten-fold higher affinity for the CB2 receptor (46); however, it was determined in the course of the studies that other cannabinoid compounds possessed potent immunosuppressive effects as well and these will be discussed. The following hypothesis is tested with the studies presented here: Immune modulation by cannabinoid compounds is mediated via cannabinoid receptors (CB1 and/or CB2), resulting in disruption of AP-1 transcription factor binding in the promoter region of immune system genes, such as IL-2.

MATERIALS AND METHODS

I. Cannabinoid compounds

SR144528, SR141716A, CBD and CBN were provided by the National Institute on Drug Abuse. WIN-2 and WIN-3 were purchased from Sigma (St. Louis, MO). WIN-2, WIN-3, SR144528 and SR141716A were prepared as 10 mM stocks in DMSO, aliquoted, and stored at –80°C until use. CBD and CBN were prepared as 20 mM stocks in EtOH, aliquoted, and stored at –20°C until use. CBD and CBN were diluted 10-fold in buffer and added to culture at a 100-fold dilution.

II. Reagents

PMA, Io, FSK, A23187, TG, and PD098059 were purchased from RBI/Sigma (St. Louis, MO). PMA was prepared as a 10 mM stock and stored at –80°C. Io was prepared as a 10 mM stock and stored at –80°C. Working PMA/Io stocks were subsequently prepared as follows: PMA and Io were prepared together as a 1000x stock in DMSO (80 μM PMA/1 mM Io or 40 μM PMA/0.5 mM Io) and stored at –80°C. FSK was prepared as a 10 mM stock in DMSO and stored at –20°C. A23187 was prepared as a 100 mM stock in DMSO and stored at +4°C. TG was prepared as a 1 mM stock in DMSO and stored at –20°C. PD098059 was prepared as a 25 mM stock in DMSO and stored at –80°C. All reagents were aliquoted prior to storage.

III. Animals

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

IV. Preparation of mouse lymphocyte cultures

Mice were sacrificed and spleens or thymi were aseptically removed. Single cell suspensions were prepared by isolating the lymphocytes from the spleen or thymus. The cells were then washed in RPMI 1640 media followed by centrifugation at 270 x g for 10 min. Cell counting was performed using a Coulter Particle Counter Z1 (lower threshold was set at 4 μm) and cell density was adjusted as appropriate (assays were performed at a density of $5x10^6$ cells/ml unless otherwise specified). Cells were cultured in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 100 units penicillin/ml, 100 units of streptomycin/ml, 50 μM 2-mercaptoethanol, and 2% BCS unless otherwise specified (HyClone, Logan, UT). Select assays were conducted in the absence of the red blood cells. Gey's solution (130 mM NH_4Cl, 5 mM KCl, 0.84 mM Na_2HPO_4, 5.6 mM dextrose, 0.01% phenol red, 1 mM $MgCl_2$, 0.28 mM $MgSO_4$, 1.15 mM $CaCl_2$, 13.4 mM $NaHCO_3$) was utilized for red blood cell lysis. SPLC were incubated for 5 min on ice in the presence of Gey's solution (5ml/spleen), washed twice, and resuspended in appropriate medium.

CH12.LX B cells for cAMP determinations were harvested from culture flasks at cell density of approximately 2-5x10^5 cells/ml. Cells were pelleted by centrifugation at 270 x g for 10 min followed by resuspension in FAP-BSA. Cell counting was performed using a hemacytometer (total cell count X 2 dilution factor for dye X 1x10^4 = cells/ml). Cells were adjusted to 1x10^6 cells/ml for use in the cAMP determination assay.

V. Preparation of nuclear and cytosolic proteins

SPLC were cultured in 60 mm^2 tissue culture plates at a density of 5x10^6 cells/ml (5 ml/plate). THMC were cultured in 100 mm^2 tissue culture plates at a density of 1x10^6 cells/ml (10 ml/plate). SPLC or THMC were lysed with HB buffer (10 mM HEPES, 1.5 mM $MgCl_2$), and nuclei were pelleted by centrifugation at 6700 x g for 5 min. The supernatant was retained for cytosolic protein whereas the pellet was used for nuclear lysis. The supernatant fraction was centrifuged for 1 h at 100000 x g and the resulting supernatant following ultracentrifugation, which contained cytosolic protein, was stored in 10% glycerol. During the 1 h ultracentrifugation for the cytosolic fraction, the original pellet was used for nuclear lysis. Nuclear lysis of the pellet was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA and 10% glycerol) which contained 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and leupeptin, and 1 mM sodium orthovanadate for 15 min on ice. Samples were then centrifuged at 17500 g for 15 min and the supernatant was retained. Samples were aliquoted and stored at –80°C in the presence of protease inhibitors.

VI. Protein determination

Protein determination was performed using a BCA protein assay kit (Sigma, St. Louis, MO). Protein determination reagent was prepared by adding 1 part 4% copper (II) sulfate solution to 50 parts BCA. This reagent was then combined with nuclear or cytosolic protein extracts and incubated for 30 min at 37°C. Alternatively, samples were incubated overnight at 4°C. Standard curves were generated using a 1 mg/ml BSA protein standard (0-25 μg/μl standard curve). Absorbance at 562 nm was determined using a Beckman DU640 Spectrophotometer.

VII. EMSA

A. Sequences of DNA probes

DNA oligomers were synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University and are as follows: proximal AP-1 from the IL-2 promoter, 5' GATCTCTGATGACTCTCTGGAATT (adapted from 143); AP-1 consensus, 5' GATCCGGCTGACTCATCAGTA (adapted from 198); distal NF-AT from the IL-2 promoter: 5' GATCTGGAGGAAAAACTGTTTCATACAGA AGGCGTA (adapted from 206).

B. Preparation of acrylamide gel

Glass plates were assembled with 1.5 mm spacers. Gel solution (89 mM tris, 89 mM boric acid, 2 mM EDTA, 4% acrylamide, 1% APS, 0.05% TEMED) was placed between the glass plates with the 1.5 mm comb in place. The gel was allowed to polymerize for at least 2 h at RT.

C. Sample preparation and running of gel

Nuclear protein was isolated and 5 μg was incubated with 1.2 μg of poly (dI-dC) and binding buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 75 mM NaCl (for AP-1) or 100 mM NaCl (for NF-AT), 0.05% Nonidet p-40, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate and 1 μg/ml aprotinin and leupeptin) for 20 min on ice. A ^{32}P-labeled DNA probe was added and incubated with the samples for an additional 30 min at RT. DNA binding activity was resolved from free probe on a 4% acrylamide gel in TBE buffer (89 mM tris, 89 mM boric acid, 2 mM EDTA) at 110V for 2.5 h at RT. The gel was then dried for 1 h and autoradiographed overnight at –80°C. For cold competitor studies, 1 pM of unlabeled double-stranded DNA was added prior to the ^{32}P-labeled probe using an appropriate sample as noted in the figure legends. Bands were quantified using a densitometer visual imaging system (BioRad, Hercules, CA).

D. Supershift analysis

For supershift analysis, 0.75 μg/ml of antibody was incubated at RT for 30 min in addition to the above incubations required for binding to the probe. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CREB-1 antibody was added prior to the ^{32}P-labeled probe, whereas the anti-c-jun antibody was added after the probe. The anti-fos antibody recognizes all fos family members (c-fos, fos-B, fra-1 and fra-2) and was added either before or after the probe as described in the figure legends.

VIII. Western blotting

A. Preparation of acrylamide gels

Glass plates were assembled with 1.5 mm spacers. Separating gel solution (375 mM tris, 10% acrylamide, 0.1% SDS, 0.03% APS, 0.0005% TEMED) was placed between the glass plates, leaving approximately 30 mm for stacking gel. A thin layer of water was placed on top of gel solution. The gel was allowed to polymerize for at least 2 h at RT. Stacking gel solution (125 mM tris, 4% acrylamide, 0.1% SDS, 0.02% APS, 0.001% TEMED) was prepared and placed on top of the separating gel between the glass plates with the 1.5 mm comb in place. The gel was allowed to polymerize for at least 1 h at RT.

B. Sample preparation and running of gel

Nuclear or cytosolic protein (20 or 25 μg) was prepared in buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 225 mM NaCl, 0.05% Nonidet p-40, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate and 1 μg/ml aprotinin and leupeptin), incubated with 4x loading buffer (0.0625M tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 1% 2-mercaptoethanol), and heated at 95°C for 10 min. The samples were then loaded in each lane and resolved on a 10% SDS-PAGE gel at 100V for 2 h at RT. The proteins were then transferred to nitrocellulose at 20V overnight at 4°C.

C. Protein detection

1. c-Fos and c-jun

Nitrocellulose membranes were blocked with 5% milk in TBST (10 mM tris, 150 mM NaCl, 0.1% tween) for 2 h at RT. The membranes were then incubated

with 0.1 μg/ml of a rabbit polyclonal antibody for c-fos or c-jun (Santa Cruz Biotechnology, Santa Cruz, CA) prepared in 1% BSA in TBST for 2 h at RT, followed by a series of washes in TBST. Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin followed by exposure to ECL Western blotting detection reagents (Amersham, Arlington Heights, IL). Phospho- and non-phospho-c-jun cell extracts (New England Biolabs, Beverly, MA) were used as positive controls for Western detection of c-fos and c-jun. Bands were quantified using a densitometer visual imaging system (BioRad, Hercules, CA).

2. Phosphorylated ERK MAPK

Nitrocellulose membranes were blocked with 1% BSA in TBST for 2 h at RT. The membranes were then incubated with 25 ng/ml of rabbit polyclonal antibody for phosphorylated ERK MAP kinase (Promega, Madison, WI) prepared in 0.1% BSA in TBST for 2 h at RT, followed by a series of washes in TBST. Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin followed by exposure to ECL Western blotting detection reagents (Amersham, Arlington Heights, IL). Bands were quantified using a densitometer visual imaging system (BioRad, Hercules, CA).

3. Phosphatase digestion

Phosphatase digestion was performed using a lambda protein phosphatase (New England Biolabs, Beverly, MA). This is a non-specific phosphatase that dephosphorylates serine, threonine and tyrosine residues. Nuclear protein was prepared in buffer and incubated with 1000 units of phosphatase for 30 min at 30°C. Samples

were then incubated with 4x loading buffer and Western blotting for c-fos was performed as stated above.

IX. RT-PCR

A. Sequences of DNA primers

Primers for mouse genes were synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University and are as follows: c-jun forward primer, 5' AGAGGACCGGTAACAAGTGG; c-jun reverse primer, 5' AGTCGTCACGGAATTCATCG; c-fos forward primer, 5' TGCCTGCAT TCTTCTCTCG; c-fos reverse primer, 5' GAGTCTCCAGAATGAACTCGC; IL-2 forward primer, 5' TGCTCCTTGTCAACAGCG; IL-2 reverse primer, 5' TCATCA TCGAATTGCCACTC; CB1 forward primer, ACCTGATGTTCTGGATCGGA; CB1 reverse primer, TGTTATCTAGAGGCTGCGCA; CB2 forward primer, TTCTT ACCTGCCGCTCATG; CB2 reverse primer, CGGATCTCTCCACTCCGTAG. The internal standard primers are as follows: c-jun forward IS primer, T7 (TAATACGACTCACTATAGG) + c-jun forward (as above) + rat β-globin forward (CCTGCAGTGTCTGATATTGTTG); c-jun reverse IS primer, $(dT)_{18}$ + c-jun reverse (as above) + rat β-globin reverse (AACACACCATTGCGATGAA); c-fos forward IS primer, T7 (as above) + c-fos forward (as above) + rat β-globin forward (as above); c-fos IS reverse primer, (dT)18 + c-fos reverse (as above) + rat β-globin reverse (as above); IL-2 forward IS primer, T7 (as above) + IL-2 forward (as above) + rat β-globin forward (GGTGCTTGGAGACAGAGGTC); IL-2 reverse IS primer, (dT)18 + IL-2 reverse (as above) + rat β-globin reverse (TCCTGTCAACAATCCACAGG). No internal standards

for CB1 and CB2 were used for these studies (qualitative RT-PCR only for these genes). The sizes of the PCR products are as follows: c-jun, 251 bp; c-jun IS, 370 bp; c-fos, 219 bp; c-fos IS, 370 bp; IL-2, 390 bp; IL-2 IS, 474 bp; CB1, 450 bp; CB2, 429 bp.

B. Preparation of IS

An RNA IS was synthesized from rat genomic DNA using rat β-globin for a spacer gene based on the method by Vanden Heuvel (301, 302). Using the above primers for IS, rat genomic DNA was amplified. The resulting products were purified with the Wizard PCR Prep purification system (Promega, Madison, WI) and transcribed into RNA with the Gemini II *In Vitro* Transcription System (Promega, Madison, WI). The IS was then treated with DNase to remove the DNA template and contamination. Following quantitation with a Beckman DU640 Spectrophotometer, the number of molecules of IS RNA per µl was calculated with this equation: ((µg/µl RNA)/330 µg/µmol/bp X bp IS) X 6.022 x 10^{17} molecules/µmol.

C. Isolation of total RNA

Total RNA was isolated using TRI reagent (Sigma, St. Louis, MO). Total RNA was separated from other cellular components with BCP, precipitated with iospropyl alcohol, and washed with ethanol. Samples were then treated with DNase for 30 min at 37°C to prevent genomic DNA contamination and amplification. Subsequently, the samples were phenol/chloroform-extracted (in order to remove the DNase protein), precipitated in isopropyl alcohol and washed with ethanol. RNA samples were quantitated at 260 nm with a Beckman DU640 Spectrophotometer. Nuclease-free water containing RNasin (Promega, Madison, WI) was used for sample dilution to 50 ng/µl. Samples were stored at –80°C.

D. Reverse transcription and amplification

RT-PCR was performed with 100 ng total RNA and IS RNA of known amounts were reverse transcribed into cDNA using oligo(dT) as primers. Various concentrations of IS were used in order to generate a standard curve from which numbers of molecules in RNA samples could subsequently be determined. A PCR master mix (PCR buffer, 4 mM $MgCl_2$, 6 pmol forward primer, 6 pmol reverse primer, 2.5 units Taq polymerase) was added to the cDNA samples for amplification of the gene of interest. RT-PCR was performed in a Perkin Elmer Gene Amp PCR System 9600.

E. Running of gel and quantitation

Following reverse transcription and amplification, RT-PCR products for the IS and gene of interest were separated by electrophoresis on a 3% Nusieve 3:1 (FMC Bioproducts, Rockland, ME) agarose gel and visualized by ethidium bromide staining. PCR products were combined with PCR loading dye (0.01% xylene-cyanol in 30% Ficoll) prior to gel loading. cDNA bands were quantitated using the Gel Doc 1000 (BioRad, Hercules, CA), and standard curves were generated by calculating the log of the ratio of intensity of the IS RT-PCR product to that of the gene of interest RT-PCR product versus the log of the number of known molecules of IS. The number of molecules of transcripts per 100 ng of total RNA can be calculated from the standard curve.

X. ELISA

SPLC were cultured in triplicate in 48-well culture plates at 0.5 ml/well. THMC were cultured in triplicate in 12-well culture plates at 1 ml/well. Drugs, VH, antagonists,

or other compounds were added to the culture wells as described in the figure legends. Following the appropriate incubation period at 37°C (usually 24 h), cells were harvested by centrifugation at 270 x g for 10 min. Supernatants were collected, aliquoted and stored at –80°C until assayed. IL-2 was quantified using the sandwich ELISA method. Immulon IV strip wells (Dynatech Laboratories, Chantilly, VA) were coated for 1 h at 37°C with 1 μg/ml purified rat anti-mouse IL-2 antibody (Pharmingen, San Diego, CA). Wells were washed with PBST (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, 0.02% tween) after each incubation period. Wells were blocked for 30 min at 37°C with 3% BSA in PBST. Standard curves were generated using mouse recombinant IL-2 (0-512 units/mL; Pharmingen, San Diego, CA) and samples were diluted, where necessary, in 2% BCS complete medium. Samples and standards were placed, in triplicate, into the wells and incubated for 1 h at 37°C. Samples were then incubated for 1 h at RT with 1 μg/ml biotinylated anti-mouse IL-2 antibody (Pharmingen, San Diego, CA) followed by a 1 h incubation at RT with 1.5 μg/ml streptavidin peroxidase (Sigma, St. Louis, MO). IL-2 was detected with TMB (Fluka, and the reaction was terminated with 6N H_2SO_4. 96-well plates were read with a Bio-Tek Instruments EL-808 plate reader at 450 nm.

XI. cAMP determination

SPLC were treated with Gey's solution in order to lyse the red blood cells. SPLC or CH12.LX cells were resuspended in RPMI 1640 supplemented with 1 mg/ml fatty-acid poor bovine serum albumin (Calbiochem, La Jolla, CA). 1-ml aliquots of cells were placed in 12 x 75 mm borosilicate glass tubes and treated in triplicate as described in the figure legends. The cells were treated with acidic ethanol (0.01 N HCl in 95% ethanol) to

inactivate adenylate cyclase and were subsequently sonicated to facilitate release of cAMP into the extraction buffer. The samples were then centrifuged at 1600 x g for 15 min to remove cell fragments. The supernatants, containing the intracellular cAMP, were incubated at –80°C for 2-3 h followed by overnight lyophilization. The quantitation of cAMP from cells were performed using cAMP assay kits (Diagnostic Products Inc., Los Angeles, CA). The method is based on competition for binding to a cAMP binding protein between intracellular cAMP and known quantities of ^{3}H-cAMP, thus resulting in quantitation of the bound ^{3}H-cAMP. The amount of bound ^{3}H-cAMP therefore, is inversely proportional to the amount of cAMP in the samples, which can be determined using standard curves generated with cAMP calibrator solution. The excess free ^{3}H-cAMP was removed by incubation for 30 min at 0°C with dextran-coated charcoal. 1-ml samples were then placed into scintillation counting cocktail and quantitated with a Packard Tri-Carb 2100 TR liquid scintillation analyzer.

XII. Calcium determination

SPLC were treated with Gey's solution in order to lyse the red blood cells. Cells were then washed twice in calcium-KREB buffer (129 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 2.8 mM glucose, 0.2% BSA). Intracellular calcium concentration was determined by measuring the fluorescence of fura-2 dye, which is dually excited at 340 nm and 380 nm. Fura-2 AM ester dye (1 μM, Molecular Research Products, Eugene, OR) was added to the cells and incubated for 30 min at RT in the dark. Cells were harvested, washed three times with calcium KREB buffer to remove extracellular fura-2 dye, and readjusted to $5x10^{6}$

cells/ml in calcium-KREB buffer. Cells were stored at RT in the dark until used. Cells were placed in a 3-ml quartz cuvette with constant stirring. Calcium determinations were performed at RT with a Spex 1681 0.22 Spectrometer with dual excitation at 340 and 380 nm and emission at 510 nm (all slit widths were 1 mm). Intracellular calcium concentration calculations were based on maximum and minimum calcium values, as assessed with use of 0.1% Triton-X and 250 mM EGTA, respectively. The dissociation constant for the fura-2-calcium complex was 1.45×10^{-7}. For studies conducted in the absence of extracellular calcium, the KREB buffer was prepared as above without $CaCl_2$ and supplemented with 20 μM EGTA.

XIII. Viability determination

Single cell suspension lymphocytes were combined in equal volume with 0.4% trypan blue solution (Sigma, St. Louis, MO). Samples were enumerated with a hemacytometer. Viable cells were characterized by the ability of the cell to exclude the trypan blue solution. Percent viability was calculated as the ratio of live cells over the total number of cells in the sample.

XIV. Statistical analysis

The mean ± S.E. was determined by a parametric analysis of variance for each treatment group. When significant differences were detected, treatment groups were compared with the appropriate control with the Dunnett's two-tailed *t* test. IC_{50} values were determined from plots of log M concentration of cannabinoid versus % VH control.

EXPERIMENTAL RESULTS

I. Effect of cannabinoids on proximal AP-1 binding from the IL-2 promoter.

It is well established that PMA/Io mimics signaling via the T cell receptor (141) and induces AP-1 binding to the TRE (144). In EL-4 cells and Jurkat cells, a mouse and human T cell line, respectively, proximal AP-1 binding was further induced with FSK, suggesting a role for the cAMP pathway in activation of AP-1 (64, 198). In addition, in double positive THMC, CREB participated in the binding complex that formed at the proximal AP-1 site from the IL-2 promoter (303). Thus, cAMP was determined to play a critical role in induction of AP-1 binding to the proximal TRE, suggesting that TRE binding activity is a potential target for cannabinoid compounds. Indeed, the proximal TRE binding was inhibited by CBN in EL-4 T cells (64). Therefore, the first objective of these studies was to examine cannabinoid compounds effects on proximal AP-1 binding in primary mouse SPLC. Proximal AP-1 binding was induced with PMA/Io within 30 min and remains induced through 240 min, with peak induction occurring at the 240-min timepoint (Figure 5). This result was consistent with studies conducted in AR-5 cells, a murine T cell line, in which proximal AP-1 binding was maximally detected 2-4 h post α-CD3 or PMA/Io treatment (143). PMA/Io-induced binding was inhibited with 20 μM CBN at all times of cellular activation (Figure 6). It is important to note that the concentrations of cannabinoids used in these studies were not cytotoxic as determined by trypan blue exclusion assays. Interestingly, CBN-induced inhibition of binding was serum concentration-dependent such that inhibition with CBN was more robust in cells that had been cultured in 2% BCS as opposed to 5% BCS (Figure 7). The use of

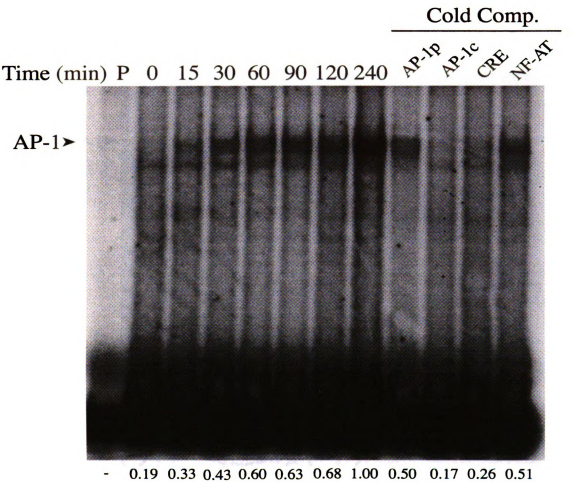


Figure 5. Induction of proximal AP-1 binding in response to PMA/Io. SPLC were treated with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the proximal AP-1 site by EMSA. P represents probe alone. Various cold competitors (1 pmol) were used to determine whether proteins that bind to proximal AP-1 can also bind to consensus AP-1, CRE or NF-AT. The 240-min stimulated sample was used for the cold competitor studies.

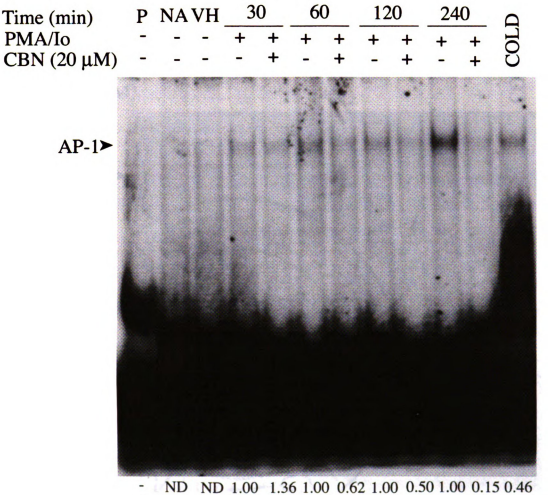


Figure 6. Inhibition of PMA/Io-stimulated proximal AP-1 binding by CBN. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the proximal AP-1 site by EMSA. P represents probe alone. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold proximal AP-1 DNA.

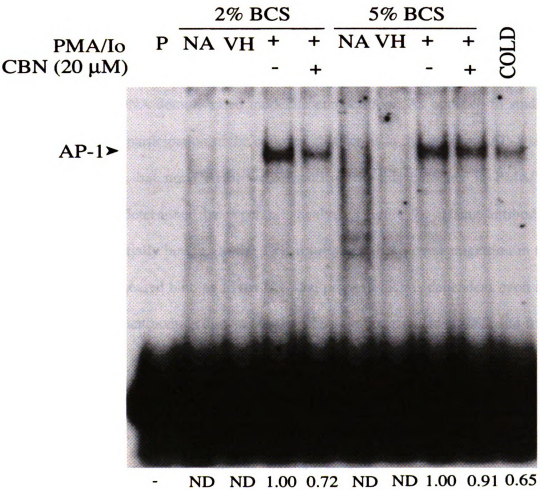


Figure 7. Effects of serum concentration on CBN-induced inhibition of PMA/Io-stimulated proximal AP-1 binding. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for 240 min in either 2% or 5% BCS. Nuclear proteins were assayed for binding ability to the proximal AP-1 site by EMSA. P represents probe alone. The 240-min stimulated sample (2% BCS) was used for the cold competitor studies with 1 pmol cold proximal AP-1 DNA.

unlabeled (cold) AP-1 proximal DNA provided some competition for ^{32}P-labeled (hot) binding (Figures 5-8). It was determined that proteins that bind to the proximal AP-1 site do so with high affinity as shown in Figure 8. The IC_{50} value of inhibition of binding to the proximal AP-1 site with cold proximal AP-1 DNA was between 2-7.5 pmol DNA. In addition, as shown in Figure 5, cold consensus AP-1 DNA and cold CRE DNA provided effective competition suggesting that similar proteins bind to these DNA elements. The use of cold NF-AT DNA demonstrated moderate competition. As opposed to studies demonstrating CREB participation in the complex that forms at the proximal AP-1 site, fos and jun proteins, but not CREB, were identified in this complex in PMA/Io-stimulated SPLC as determined by supershift analysis (Figure 9). Using antibodies against proteins potentially binding to the TRE results in either slower migration in the acrylamide gel or decreased binding to the hot TRE probe, which is dependent upon the antibody used. If the antibody is directed to the DNA binding domain (CREB and fos), the antibody must be added prior to the hot DNA probe and antibody binding to the protein results in decreased binding. If the antibody recognizes any other part of the protein (fos and c-jun), the antibody must be added after the hot DNA probe and antibody binding to the protein results in slower migration ("supershift") on the acrylamide gel. The fos antibody recognizes residues in the DNA-binding domain and outside of this domain and therefore could be used to identify fos by either method (here it was added after the probe). It was readily apparent that the fos family members were present in the complex, as this antibody recognizes the fos family members c-fos, fos-B, fra-1 and fra-2. In addition, there was a contribution by c-jun (this antibody is specific for c-jun) in this complex as demonstrated by a supershift by the c-jun antibody. There was a modest

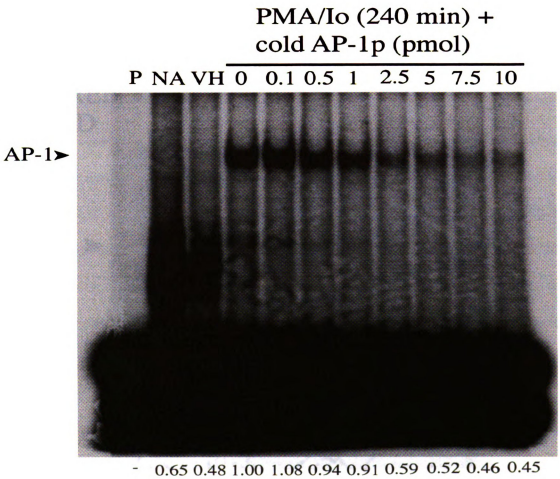


Figure 8. Concentration-response of inhibition of proximal AP-1 binding by cold proximal AP-1 DNA. SPLC were treated with PMA/Io (80 nM/1 μM) for 240 min. Nuclear proteins were assayed for binding ability to the proximal AP-1 site by EMSA. P represents probe alone. Increasing concentrations of cold proximal AP-1 DNA were added to the reaction mixture.

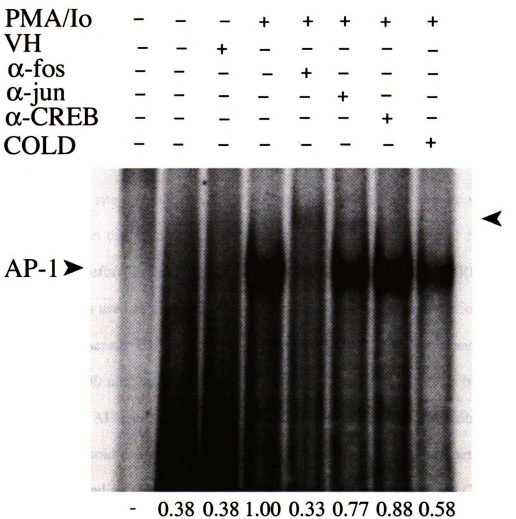


Figure 9. Identification of proteins in the complex that forms at the proximal AP-1 site in PMA/Io-stimulated SPLC. SPLC were treated with PMA/Io (80 nM/1 μM) for 240 min. Nuclear proteins were assayed for binding ability to the proximal AP-1 site by EMSA. The first lane is probe alone. Antibodies against fos and jun family members and CREB-1 were added to the reaction mixture. Retardation or disappearance of the band indicates presence of the protein(s).

decrease in the band intensity in the presence of the CREB antibody, indicating modest involvement of CREB in this complex.

II. Effect of cannabinoids on consensus AP-1 binding.

The TRE site, found in many gene promoters, was originally identified in the human metallothionein promoter. Upon comparison of several genes which were AP-1-responsive, a consensus TRE site, TGACTCA, was identified (144). This differs from the IL-2 proximal site by only one base pair and yet, results in a drastic increase in binding intensity to the TRE site (304). A consensus TRE is not found in the IL-2 promoter and, therefore, in combination with studies using the proximal TRE from the IL-2 promoter, was used as a tool to study specific effects on immune function. Protein binding to the consensus TRE was readily induced by PMA/Io by 15 min and remained induced through 240 min, with peak induction at 240 min (Figure 10). This binding was specific as a cold AP-1 consensus probe effectively competed for hot probe binding. Interestingly, the cold proximal TRE or NF-AT did not effectively compete with hot consensus TRE binding (Figure 10). There was a modest effect of CBN (20 μM) on consensus TRE binding at several different stimulation times as demonstrated in Figure 11. This modest effect, however, was serum dependent as a similar nuclear protein preparation conducted in 2% BCS (as opposed to 5% BCS in Figure 11) resulted in more dramatic inhibition by CBN (Figure 12). This serum dependence is not surprising in light of the fact that cannabinoid compounds are highly lipophilic and might be sequestered by the higher protein and lipid content in 5% BCS. Additionally, the AP-1 complex, c-fos in particular, is very serum-responsive (174, 305). In comparison with the proximal AP-1

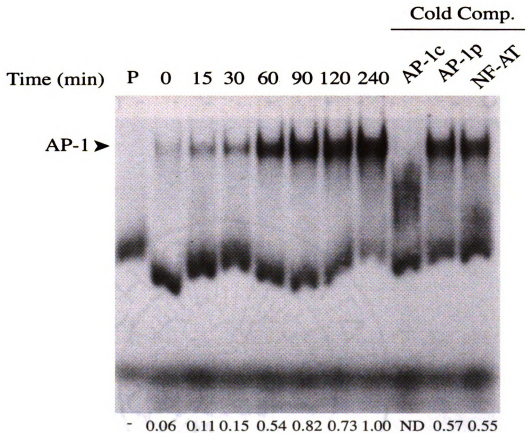


Figure 10. Induction of consensus AP-1 binding in response to PMA/Io. SPLC were treated with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the consensus AP-1 site by EMSA. P represents probe alone. Various cold competitors (1 pmol) were used to determine whether proteins that bind to consensus AP-1 can also bind to proximal AP-1 or NF-AT. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold consensus AP-1 DNA.

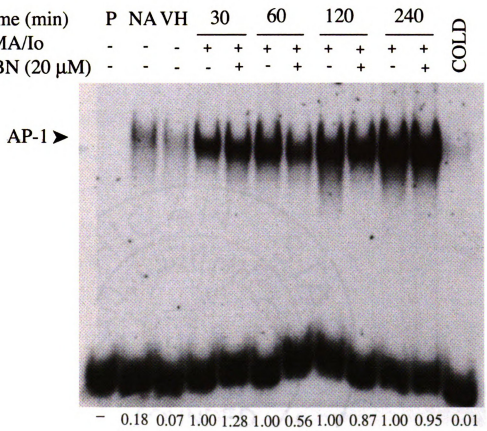


Figure 11. Inhibition of PMA/Io-stimulated consensus AP-1 binding by CBN in 5% BCS. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the consensus AP-1 site by EMSA. P represents probe alone. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold consensus AP-1 DNA. Densitometric values for NA and VH were determined as compared to the 240-min stimulated sample.

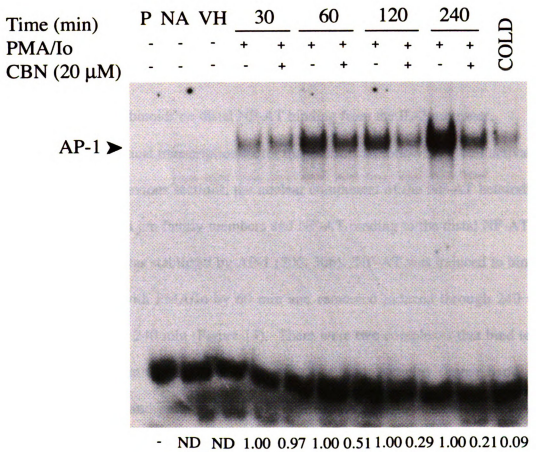


Figure 12. Inhibition of PMA/Io-stimulated consensus AP-1 binding by CBN in 2% BCS. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the consensus AP-1 site by EMSA. P represents probe alone. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold consensus AP-1 DNA.

binding studies, the consensus AP-1 binding was more intense and appeared to be one complex. It is possible, however, that several complexes were represented but could not be resolved due to the intensity of binding. There was evidence for multiple complexesas determined by supershift analysis as fos, jun and CREB proteins were all identified as participants in the complex that forms at the consensus AP-1 site (Figure 13).

III. Effect of cannabinoids on distal NF-AT binding from the IL-2 promoter.

NF-AT is a critical transcription factor that is induced following T cell activation. As discussed in the previous sections, the nuclear component of the NF-AT heterodimer is composed of fos and jun family members and NF-AT binding to the distal NF-AT site in the IL-2 promoter was stabilized by AP-1 (206, 306). NF-AT was induced to bind its consensus sequence with PMA/Io by 60 min and remained induced through 240 min, with peak induction at 240 min (Figure 14). There were two complexes that bind to the distal NF-AT site from the IL-2 promoter and the slower complex (upper band) was shown to contain fos and jun proteins (153). NF-AT binding was inhibited following treatment with 20 μM CBN at all activation times (Figure 15).

IV. Effect of cannabinoids on c-fos and c-jun expression.

Cannabinoid compounds inhibited binding to the proximal AP-1 and distal NF-AT sites in the IL-2 promoter. Therefore, the next major objective of these studies was to analyze the effects of these compounds on protein expression of the two major components of the AP-1 complex, c-fos and c-jun, using Western analysis. c-Fos protein expression was readily induced by PMA/Io by 30 min and remained induced through 240

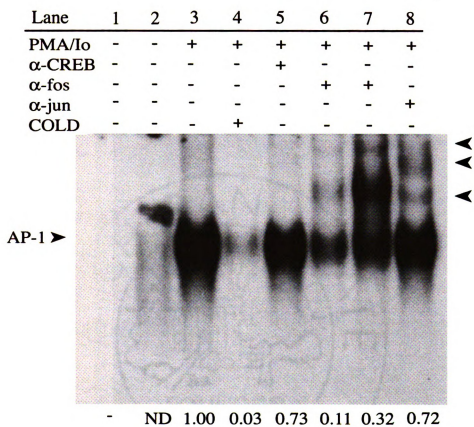


Figure 13. Identification of proteins in the complex that forms at the consensus AP-1 site in PMA/Io-stimulated SPLC. SPLC were treated with PMA/Io (80 nM/1 μM) for 240 min. Nuclear proteins were assayed for binding ability to the consensus AP-1 site by EMSA. Lane 1 is probe alone. Antibodies against fos and jun family members and CREB-1 were added to the reaction mixture. Fos antibody was added both before and after the probe (lanes 6 and 7, respectively). Jun antibody was added before the probe; CREB antibody was added after the probe. Retardation or disappearance of the band indicates presence of the protein(s).

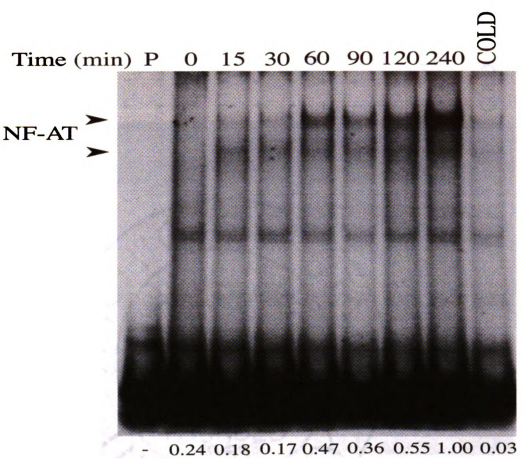


Figure 14. Induction of distal NF-AT binding in response to PMA/Io. SPLC were treated with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the distal NF-AT site by EMSA. P represents probe alone. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold distal NF-AT DNA. The lower complex represents NF-AT binding and the upper complex represents NF-AT in combination with AP-1 proteins (composite NF-AT/AP-1 site).

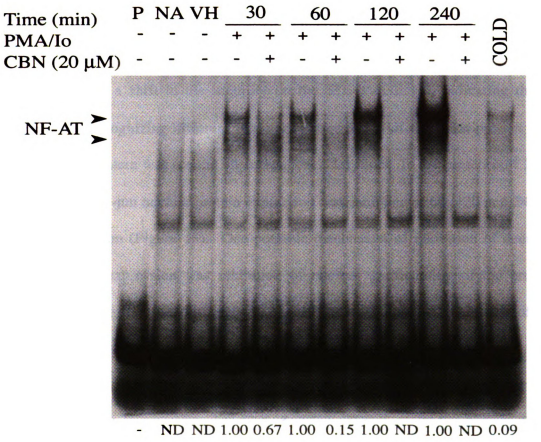
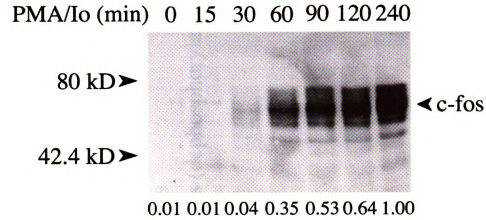


Figure 15. Inhibition of PMA/Io-stimulated distal NF-AT binding by CBN. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for binding ability to the distal NF-AT site by EMSA. P represents probe alone. The 240-min stimulated sample was used for the cold competitor studies with 1 pmol cold distal NF-AT DNA. The lower complex represents NF-AT binding and the upper complex represents NF-AT in combination with AP-1 proteins (composite NF-AT/AP-1 site).

min (Figure 16). Similarly, c-jun protein expression was induced by 60 min and peaked at 240 min (Figure 16). One striking difference between the two Western analyses for c-fos and c-jun was the broad banding pattern on the c-fos blots. In order to verify that thispattern represented various phosphorylation states of c-fos recognized by the antibody, a phosphatase digestion of the nuclear extract was performed (188, 307). As shown in Figure 17, treating the nuclear extract from the 240 min-stimulated sample with λ-ppase induced a shift in the mobility on the SDS-PAGE gel, indicating that the antibody was recognizing different phosphorylation states of the c-fos protein. This broad banding pattern was seen in all subsequent c-fos blots. In the presence of 20 μM CBN, c-fos and c-jun nuclear protein expression was inhibited at 60, 120 and 240 min post cell activation (Figure 18). One possible mechanism of inhibition of decreased nuclear protein expression was inhibition of nuclear translocation of the proteins. However, there was no cytosolic retention of either c-fos or c-jun in the presence of CBN (20 μM) in SPLC that had been stimulated for 240 min (Figures 19 and 20).

Inhibition of the nuclear protein expression of both c-fos and c-jun was not due to an inhibition of nuclear translocation of the proteins. Therefore, studies were performed in order to determine whether the decrease in c-fos and c-jun protein expression by CBN was due to inhibition of steady state mRNA expression using quantitative RT-PCR. Examples of standard curve gels for c-fos and c-jun are shown in Figure 21. These gels were used to quantify the number of molecules of c-fos or c-jun using the respective RNA internal standards. Determination of numbers of molecules of both c-fos and c-jun from two separate preparations are presented in Table I. c-Fos gene expression was readily induced within 15 min following cellular activation with PMA/Io to levels of



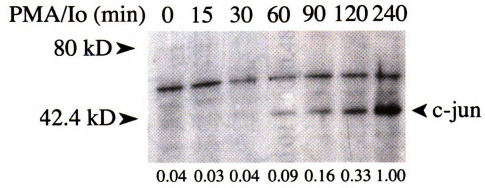


Figure 16. Induction of c-fos and c-jun nuclear protein expression in response to PMA/Io. SPLC were treated with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for c-fos and c-jun protein expression by Western analysis. c-Fos is a 62-kD protein; c-jun is a 39-kD protein.

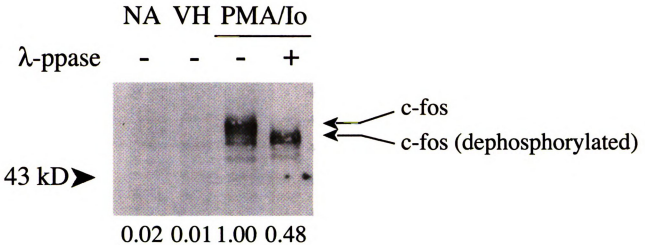
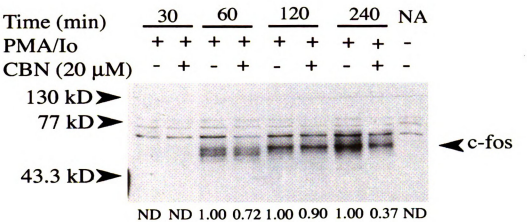


Figure 17. The broad banding pattern of c-fos represents different phosphorylation states of the protein. SPLC were treated with PMA/Io (80 nM/1 μM) for 240 min. Nuclear proteins were treated with 1000 units of λ-ppase for 30 min at 30°C. Samples were then assayed for c-fos expression by Western analysis.



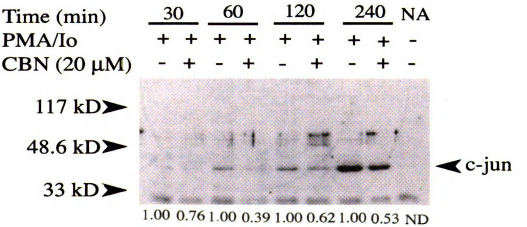
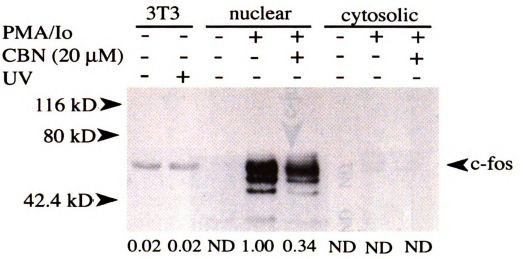


Figure 18. Inhibition of PMA/Io-stimulated c-fos and c-jun nuclear protein expression by CBN. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for c-fos and c-jun protein expression by Western analysis.

A.



B.

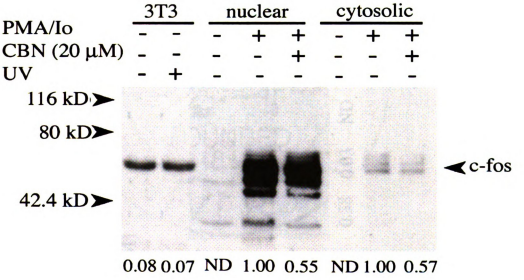


Figure 19. Effect of CBN on nuclear and cytosolic protein expression of c-fos. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for various times. UV-treated cellular extracts from NIH3T3 cells were used as positive controls for identification of the protein. Nuclear and cytosolic proteins were assayed for c-fos protein expression by Western analysis. A.) blot exposed for 10 min; B.) blot exposed for 30 min.

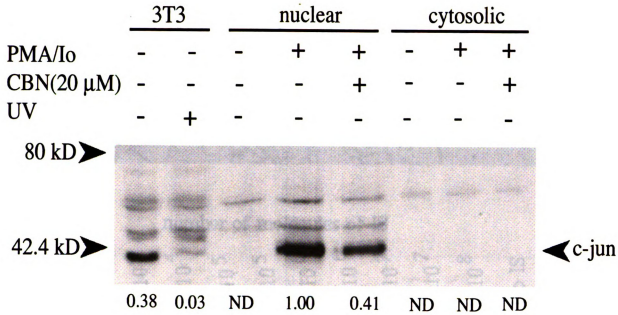


Figure 20. Effect of CBN on nuclear and cytosolic protein expression of c-jun. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (80 nM/1 μM) for 240 min. UV-treated cellular extracts from NIH3T3 cells were used as positive controls for identification of the protein. Nuclear and cytosolic proteins were assayed for c-jun protein expression by Western analysis.

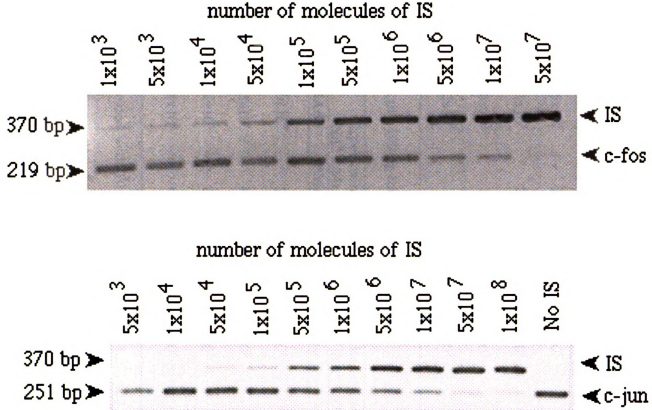


Figure 21. Representative quantitative RT-PCR standard curves for c-fos and c-jun. Primers for c-fos and c-jun were designed for specific recognition of the genes and their respective internal standards. Unknown RNA samples and known internal RNA standards were reverse transcribed and amplified in one reaction tube. Standard curves were generated using densitometry.

Table 1. Effects of CBN on c-fos and c-jun steady state mRNA expression as analyzed by quantitative RT-PCR.

Molecules c-fos per 100 ng total RNA				
Time (min)	**Experiment #1** NA[b] 4.80×10^4		**Experiment #2** NA[b] 9.19×10^4	
	PMA/Io	**PMA/Io + CBN**	**PMA/Io**	**PMA/Io + CBN**
15	2.65×10^5	2.61×10^5	2.84×10^5	2.56×10^5
30	2.01×10^5	2.51×10^5	3.08×10^5	3.66×10^5
45	1.28×10^5	2.43×10^5	3.03×10^5	3.53×10^5
60	7.62×10^4	8.2×10^4	1.76×10^5	1.59×10^5
Molecules c-jun per 100 ng total RNA				
Time (min)	**Experiment #1** NA[b] 2.24×10^6		**Experiment #2** NA[b] 4.97×10^6	
	PMA/Io	**PMA/Io + CBN**	**PMA/Io**	**PMA/Io + CBN**
15	2.29×10^6	2.24×10^6	3.98×10^6	4.80×10^6
30	2.22×10^6	4.63×10^6	8.62×10^6	1.06×10^7
45	3.21×10^6	3.56×10^6	1.04×10^7	1.11×10^7
60	3.48×10^6	4.57×10^6	9.49×10^6	1.06×10^7

[a] SPLC were pretreated with 20 μM CBN for 15 min followed by PMA/Io. RNA isolation and quantitative PCR was performed as described using internal standards and primers for mouse c-fos or c-jun. Numbers of molecules (per 100 ng total RNA) determined from two separate experiments are shown.

[b] NA represents unstimulated splenocytes.

about $1x10^5$ to $5x10^5$ transcripts of c-fos per 100 ng total RNA. The amount of c-fos transcripts began to return to basal levels almost immediately (by 30 min). This was consistent with previous reports demonstrating that the transcript half-life for c-fos is short (174). Treatment with 20 μM CBN had no inhibitory effect on c-fos gene expression within 60 min. Similar to c-fos, the c-jun transcript was induced within 30 min following cellular activation and there were no inhibitory effects by 20 μM CBN on c-jun gene expression at any times examined following cellular activation. The levels of c-jun transcripts were $5x10^6$ to $1x10^7$ c-jun transcripts per 100 ng total RNA and this level was consistent for as long as the cells were activated.

The effects of CBN on c-fos steady state mRNA expression were unexpected in that the c-fos promoter region contains a CRE (reviewed in 154), a DNA element that is sensitive to inhibition by CBN (65, 82). Hence, gene expression for both c-fos and c-jun was also examined at later times of cellular activation (at 6 h) and there was no inhibition of c-jun mRNA expression. The effects on c-fos; however, were difficult to determine as the levels of c-fos transcripts had returned to near basal by 120 min ($< 5 \times 10^4$ transcripts per 100 ng total RNA) and were, therefore, often too close to the detection limit of the technique. Furthermore, the steady state expression of both c-fos and c-jun was highly variable between experiments and might be the result of the lack of stability of the immediate early gene message. The general trends shown in Table I, however, were consistent throughout the studies. Thus, there was no inhibitory effect on c-fos or c-jun steady state gene expression early during cellular activation. In order to verify CBN drug activity, quantitative RT-PCR for steady state IL-2 expression was performed, although longer treatment times with PMA/Io were required as IL-2 is not an immediate early gene

(Figure 22). As previously demonstrated (64), 20 μM CBN inhibited PMA/Io-stimulated IL-2 steady state mRNA expression.

V. Effect of cannabinoids on phosphorylated ERK MAPK expression.

As c-fos and c-jun steady state mRNA expression was not inhibited in the presence of CBN, this suggested that the decreased nuclear protein expression could be the result of a perturbation of the post-translational modifications of c-fos and c-jun. c-Fos and c-jun are tightly regulated by phosphorylation state; thus, inhibition of an upstream kinase seemed likely. Therefore, the role of ERK MAPKs in PMA/Io-stimulated SPLC, and subsequently, effects of CBN on the kinase, were studied.

In order to assess the role of ERK MAPK in PMA/Io-stimulated SPLC, the MEK inhibitor, PD098059, was utilized. Inhibition of MEK by PD098059 results in inhibition of ERK MAPK activation (308). Indeed, treatment of SPLC with increasing concentrations of PD098059 inhibited expression of phosphorylated ERK MAPK in PMA/Io-stimulated SPLC with an IC_{50} value of approximately 100 nM (Figure 23). The expression of phosphorylated forms of ERK MAPK is an indication of the activity of the kinase (246). The PD098059-induced inhibition of ERK MAPK phosphorylation demonstrated both activity of the inhibitor and the ability of the antibody to specifically recognize phosphorylated forms of ERK MAPK.

It has been recently demonstrated that ERK MAPKs are required for the production of IL-2 in Jurkat T cells as demonstrated using cells transfected with a dominant negative form of ERK (276). Using PD098059, it was determined that ERK MAPK also played a critical role in IL-2 production in PMA/Io-stimulated SPLC with an

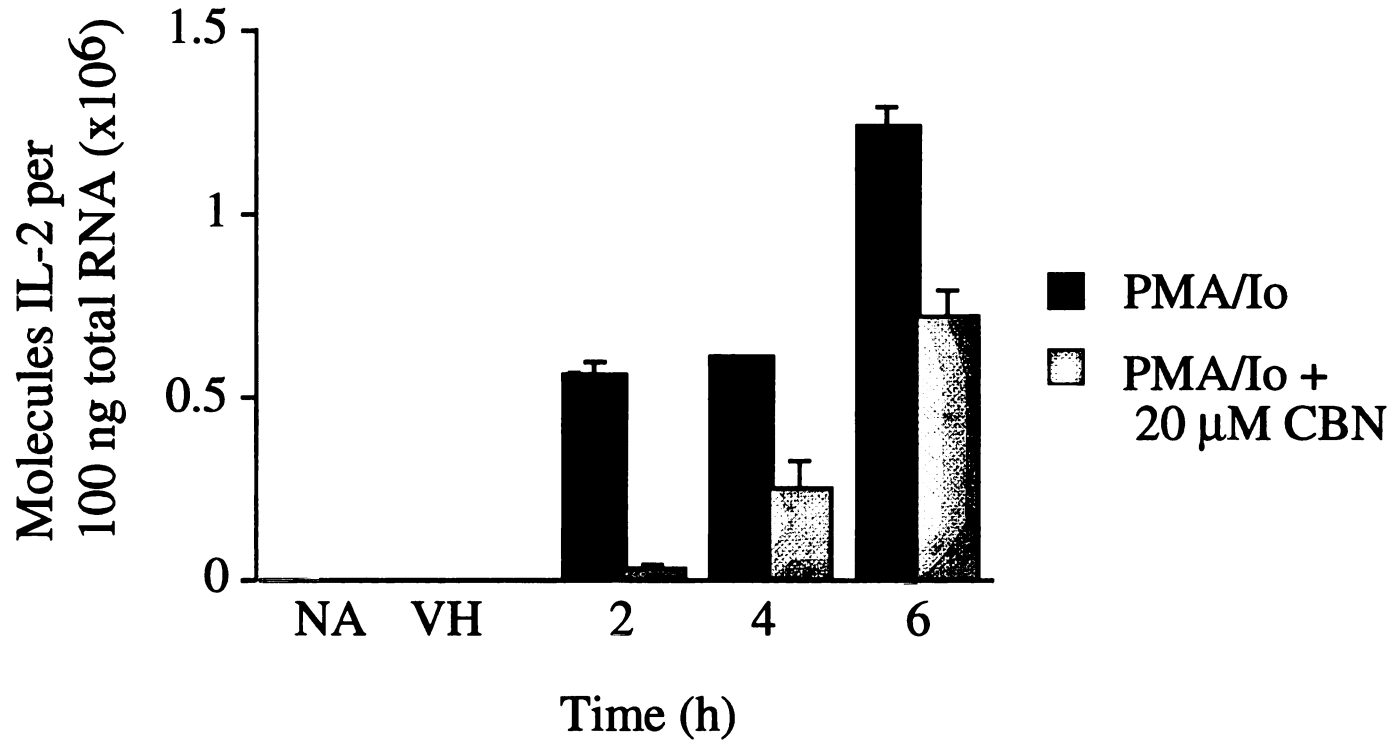


Figure 22. Inhibition of PMA/Io-stimulated IL-2 steady state mRNA expression. SPLC were treated for 15 min with 20 μM CBN followed by activation with PMA/Io (80 nM/1 μM) for various times. Total RNA was isolated and IL-2 steady state gene expression was assessed by Q RT-PCR. Bars represent one RNA isolate performed in duplicate.

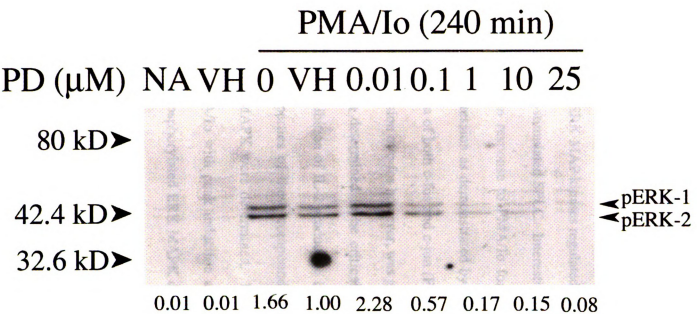


Figure 23. Inhibition of PMA/Io-stimulated phosphorylated ERK MAPK nuclear protein expression by PD098059. SPLC were pretreated for 30 min with PD098059, followed by activation with PMA/Io (40 nM/0.5 μM) for various times. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis. ERK-1 is a 44 kD protein; ERK-2 is a 42 kD protein.

IC_{50} value of approximately 5 μM (Figure 24). This demonstrates that T cell signaling in SPLC mimics signaling in purified T cell preparations and, in fact, ERK MAPK also played a critical role in IL-2 production in PMA/Io-stimulated THMC (Figure 25). As mentioned above, both c-fos and c-jun participate in complexes that bind in the IL-2 promoter and both are regulated by ERK MAPK in several cell types. Thus, PD098059 was used to determine if ERK MAP kinases regulated the nuclear protein expression of c-fos and c-jun in PMA/Io-stimulated SPLC. Interestingly, although both c-fos and c-jun were robustly induced in response to PMA/Io for 240 min, ERK MAPK played a minimal role in this expression as demonstrated by a minimal effect of PD098059 on nuclear protein expression of both c-fos and c-jun (Figure 26). The effect of PD098059 on nuclear protein expression of c-fos however, was greater than the effect on c-jun.

ERK MAPK was determined to be critical for IL-2 production in PMA/Io-stimulated SPLC and inhibition of IL-2 production by cannabinoids is indicative of the immunosuppressive properties of these compounds (64). Therefore, the effects of cannabinoids on ERK MAPK were determined. ERK MAPK phosphorylation was rapidly induced by PMA/Io with peak induction at 20 min (Figure 27). While the expression of nuclear phosphorylated ERK MAPK decreased over time, the expression level of phosphorylated ERK MAPK at 240 min post cellular stimulation was above that found in naive cells. This indicates that ERK MAPK was rapidly activated, followed by a downregulation to a sustained level (above basal) for several hours. As seen in Figure 28, CBN (20 μM) inhibited the expression of phosphorylated ERK MAPK at 30, 60, 120 and 240 min post cellular activation. Furthermore, treatment of activated SPLC with various concentrations of CBN for 5 or 240 min demonstrated that activation of ERK

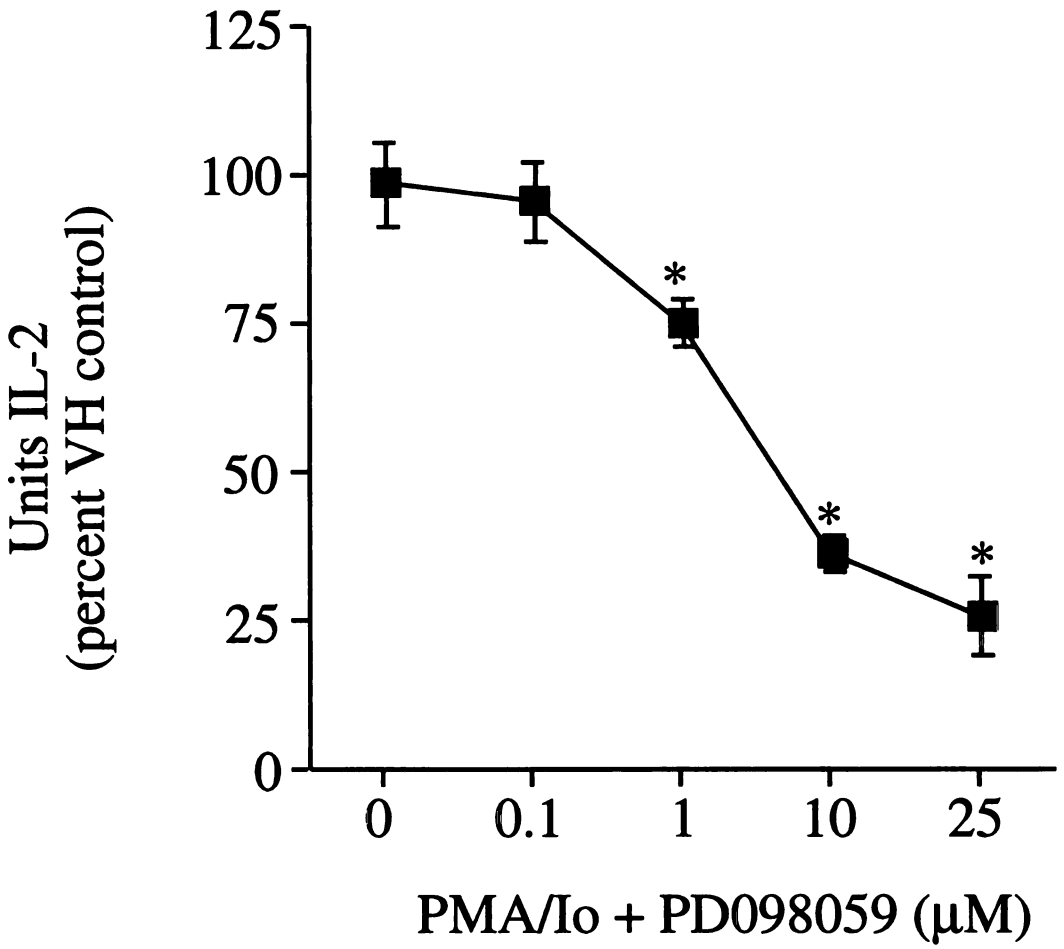


Figure 24. Effect of PD098059 on PMA/Io-stimulated IL-2 in SPLC. SPLC were treated with PD098059 for 30 min followed by activation with PMA/Io (40 nM/0.5 μM) for 24 h. IL-2 production was determined with an ELISA with a standard curve in units IL-2/ml. No IL-2 production was detected in unstimulated cells. Average IL-2 production in PMA/Io-stimulated cells was 2348 ± 446 units IL-2/ml. Zero represents stimulation with PMA/Io in the absence of 0.2% DMSO VH. Asterisks denote statistical significance ($p < 0.05$) compared to VH control. Results are pooled from at least three separate experiments.

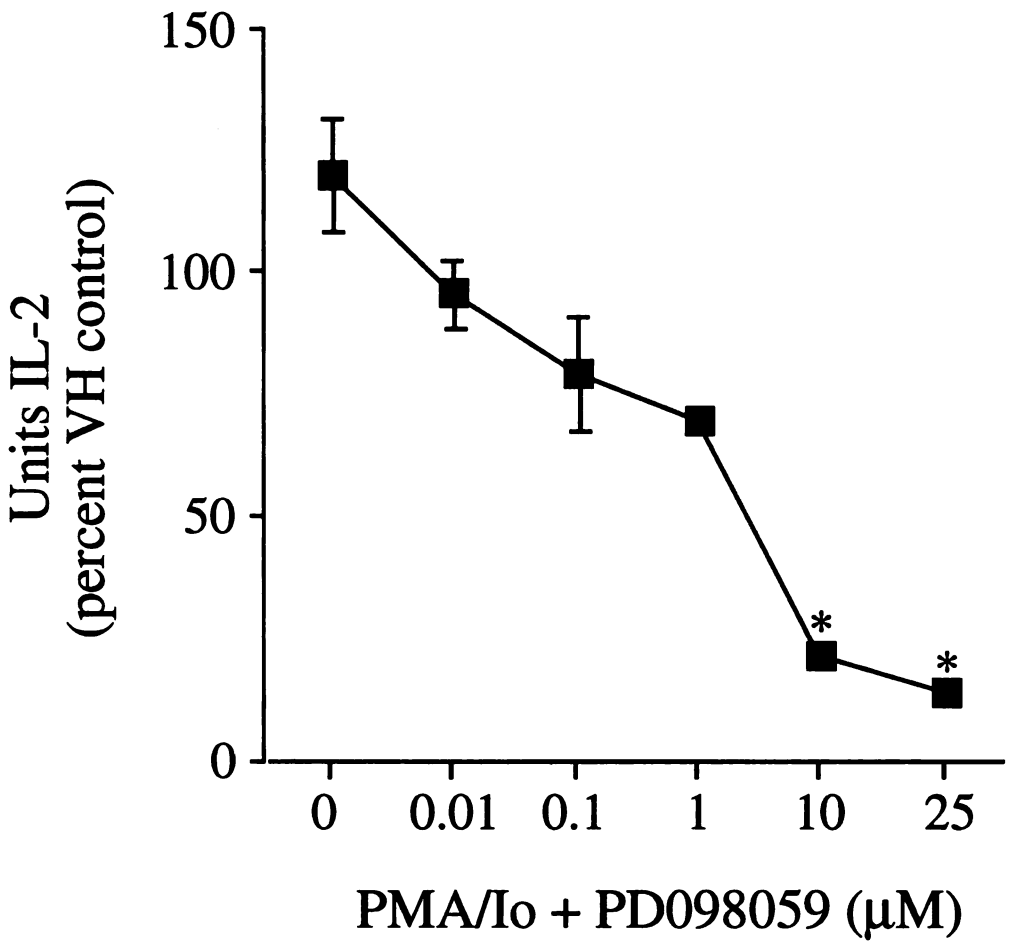


Figure 25. Effect of PD098059 on PMA/Io-stimulated IL-2 in THMC. THMC were treated with PD098059 for 30 min followed by activation with PMA/Io (80 nM/1 μM) for 24 h. IL-2 production was determined with an ELISA with a standard curve in units IL-2/ml. No IL-2 production was detected in unstimulated cells. Average IL-2 production in PMA/Io-stimulated cells was 18 ± 6 units IL-2/ml. Zero represents stimulation with PMA/Io in the absence of 0.2% DMSO VH. Asterisks denote statistical significance ($p < 0.05$) compared to VH control. Results are pooled from at least three separate experiments.

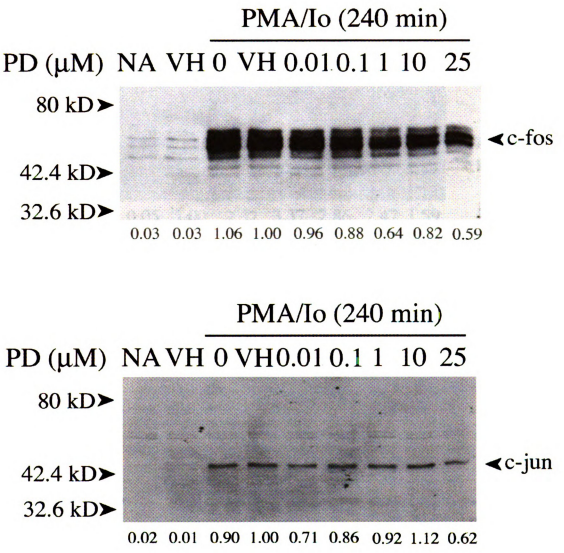


Figure 26. Effect of PD098059 on PMA/Io-stimulated c-fos and c-jun nuclear protein expression. SPLC were pretreated for 30 min with PD098059, followed by activation with PMA/Io (40 nM/0.5 μM) for various times. Nuclear proteins were assayed for c-fos and c-jun nuclear protein expression by Western analysis.

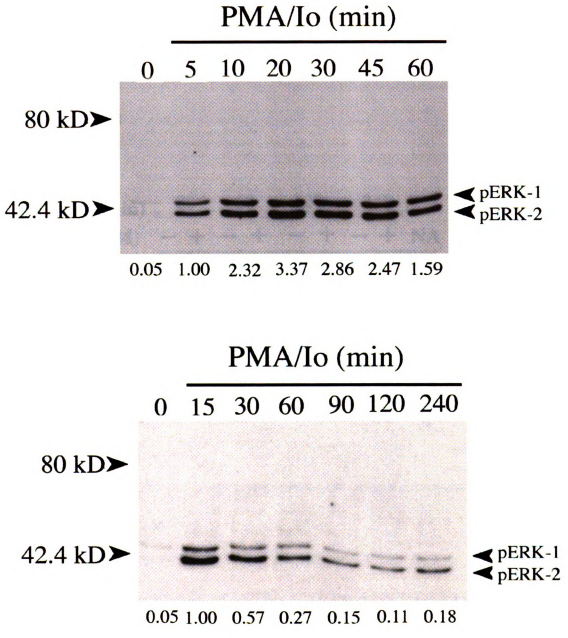


Figure 27. Induction of nuclear phosphorylated ERK MAPK protein expression in response to PMA/Io. SPLC were treated with PMA/Io (40 nM/0.5 μM) for various times. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.

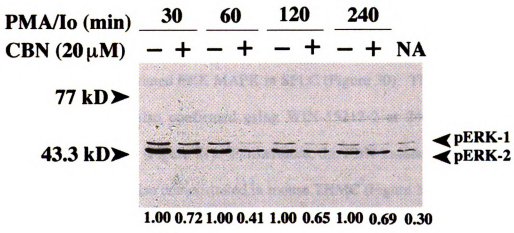


Figure 28. Inhibition of PMA/Io-stimulated nuclear phosphorylated ERK MAPK protein expression by CBN. SPLC were pretreated for 15 min with CBN (20 μM), followed by activation with PMA/Io (40 nM/0.5 μM) for various times. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.

MAPK was inhibited at concentrations as low as 1 μM CBN at 240 min post cellular activation (Figure 29). Although the effects of CBN varied at concentrations below 1 μM, there was consistency in the ability of CBN to inhibit the activation of ERK at concentrations above 1 μM throughout several replicates of the experiment. Thus, activation of ERK MAPK was a particularly sensitive target of CBN treatment in activated mouse SPLC. This observation is in contrast to several studies demonstrating that cannabinoids activate ERK MAPK in cell systems in which no activation was required (73, 74). However, CBN, in the absence of PMA/Io, did not modulate expression of phosphorylated ERK MAPK in SPLC (Figure 30). The inhibition of ERK MAPK by CBN was also confirmed using WIN-55212-2 at 240 min post cellular activation with PMA/Io (Figure 31). Furthermore, the CBN-induced inhibition of ERK MAPK activation was also demonstrated in mouse THMC (Figure 32), confirming again that effects in SPLC mimic effects in T cells.

VI. Effect of cannabinoid receptor antagonists on cannabinoid-induced inhibition of PMA/Io-stimulated IL-2.

The mechanism of inhibition of PMA/Io-stimulated IL-2 in SPLC involves decreased AP-1 transcription factor activity, which might ultimately be due to inhibition of activation of ERK MAPK. Thus, the objective of the next set of studies was to determine whether one of both of the cannabinoid receptors was involved in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production. As previously reported, mRNA expression of CB2 is approximately ten-fold higher than CB1 in lymphoid tissues. In addition, cannabinoid receptor mRNA expression for both CB1 and CB2 is

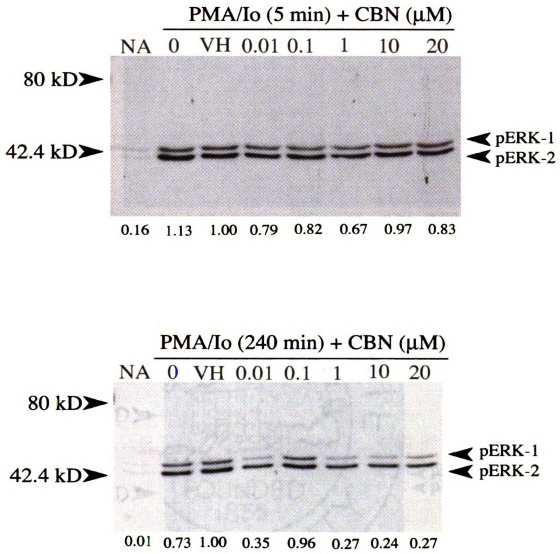
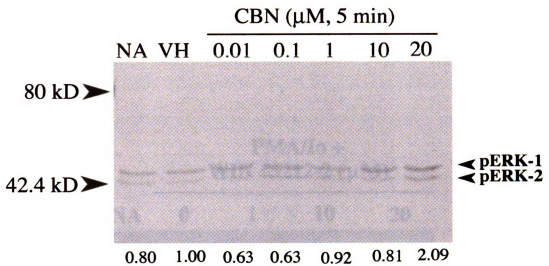


Figure 29. Effect of CBN on PMA/Io-stimulated phosphorylated ERK MAPK at 5- and 240-min post cellular stimulation. SPLC were pretreated for 15 min with CBN followed by activation with PMA/Io (40 nM/0.5 μM) for either 5 or 240 min. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.



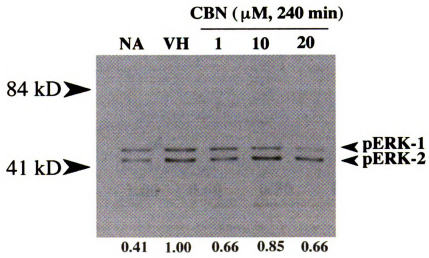


Figure 30. Effect of CBN on phosphorylated ERK MAPK in resting SPLC for 5 and 240 min. SPLC were treated with CBN for either 5 or 240 min. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.

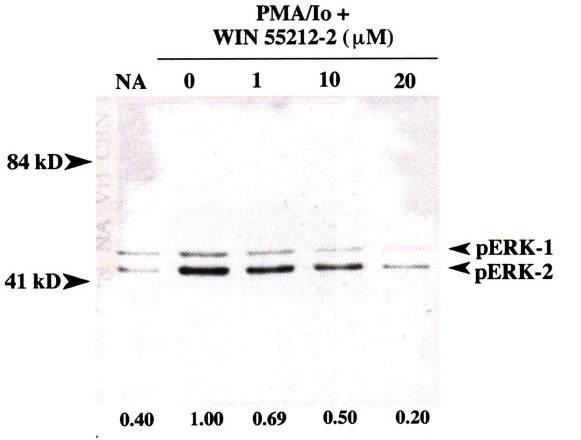


Figure 31. Inhibition of PMA/Io-stimulated nuclear phosphorylated ERK MAPK protein expression by WIN-55212-2. SPLC were pretreated for 15 min with WIN-2 followed by activation with PMA/Io (40 nM/0.5 μM) for 240 min. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.

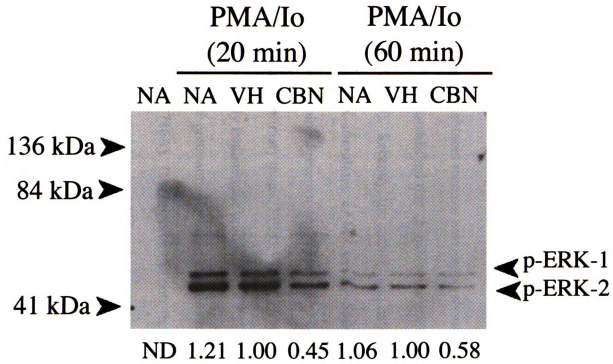


Figure 32. Inhibition of PMA/Io-stimulated nuclear phosphorylated ERK MAPK protein expression by CBN in THMC. THMC were pretreated for 15 min with CBN (15 μM) followed by activation with PMA/Io (80 nM/1 μM) for various times. Nuclear proteins were assayed for phosphorylated ERK MAPK protein expression by Western analysis.

highest in B cells and lowest in T cells (51). Qualitative RT-PCR was performed in order to confirm cannabinoid receptor expression in SPLC, EL-4 T cells and CH12.LX B cells (Figure 33). CH12.LX B cells expressed the highest amount of CB2 mRNA, which was slightly higher than SPLC and approximately double that found in EL-4 T cells. CB1 however was expressed exclusively in SPLC. The expression pattern of cannabinoid receptors in EL-4 T cells was similar to that found in THMC (52). Expression patterns of cannabinoid receptors in lymphocytes were confirmed in order to determine whether one or both of the cannabinoid receptor antagonists should be used for the next set of studies.

It is well established that cannabinoids inhibit FSK-stimulated cAMP in several cell types (93, 309). Recently, the cannabinoid antagonists were used to determine that inhibition of FSK-stimulated cAMP by CP-55940 was mediated via cannabinoid receptors in cannabinoid receptor-transfected CHO cells (38, 39). In order to characterize the antagonists in primary mouse SPLC, cAMP assays were performed with CBN (Figure 34). CBN modestly inhibited the production of FSK-stimulated cAMP, which could not be attenuated with pretreatment with a combination of both cannabinoid antagonists (SR144528/SR141716A). It is important to note that the antagonists alone did not affect the levels of FSK-stimulated cAMP (average percent inhibition by the antagonists was 90.8%). In addition, inhibition of FSK-stimulated cAMP by WIN-2 could not be attenuated by this antagonist combination (Figure 35). Due to the relatively lower levels of receptor expressed by SPLC, the ability of the antagonists to reverse cannabinoid-mediated inhibition in CH12.LX cells, a B cell line, was performed. As seen in Figure 33, the level of CB2 receptor mRNA in CH12.LX cells as analyzed by qualitative RT-PCR was higher than that detected in either SPLC or EL-4 T cells and CB1 mRNA was

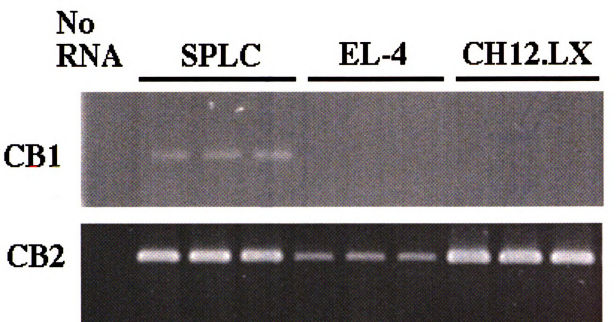


Figure 33. Expression of CB1 and CB2 in lymphocytes. Total RNA from SPLC, EL-4 T cells and CH12.LX B cells was isolated and qualitative RT-PCR was performed for CB1 and CB2 steady state gene expression.

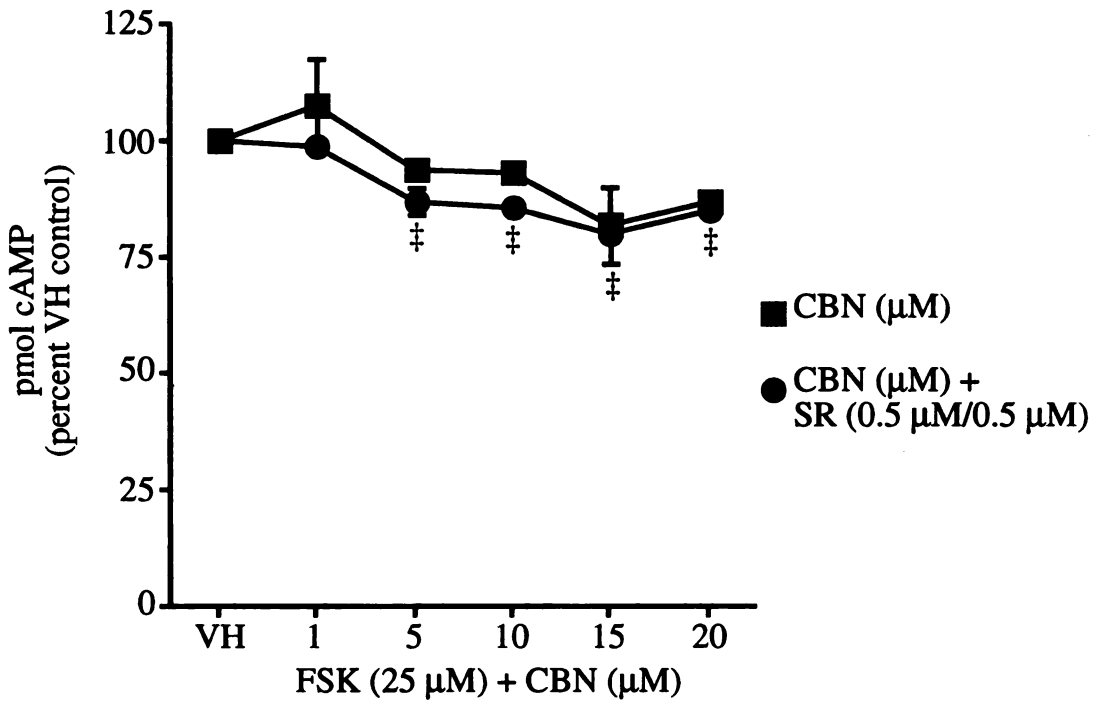


Figure 34. Effect of cannabinoid receptor antagonists on CBN-induced inhibition of FSK-stimulated cAMP in SPLC. SPLC were pretreated with SR144528 and SR141716A (0.5 μM each) for 30 min followed by CBN treatment for 30 min. Cells were stimulated with 25 μM FSK for 15 min then assayed for cAMP. Average stimulation with FSK was 6.3 ± 0.6 pmol cAMP/5x10^6 SPLC. * $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the SR + VH group. Results represent three separate experiments with three replicates per treatment group.

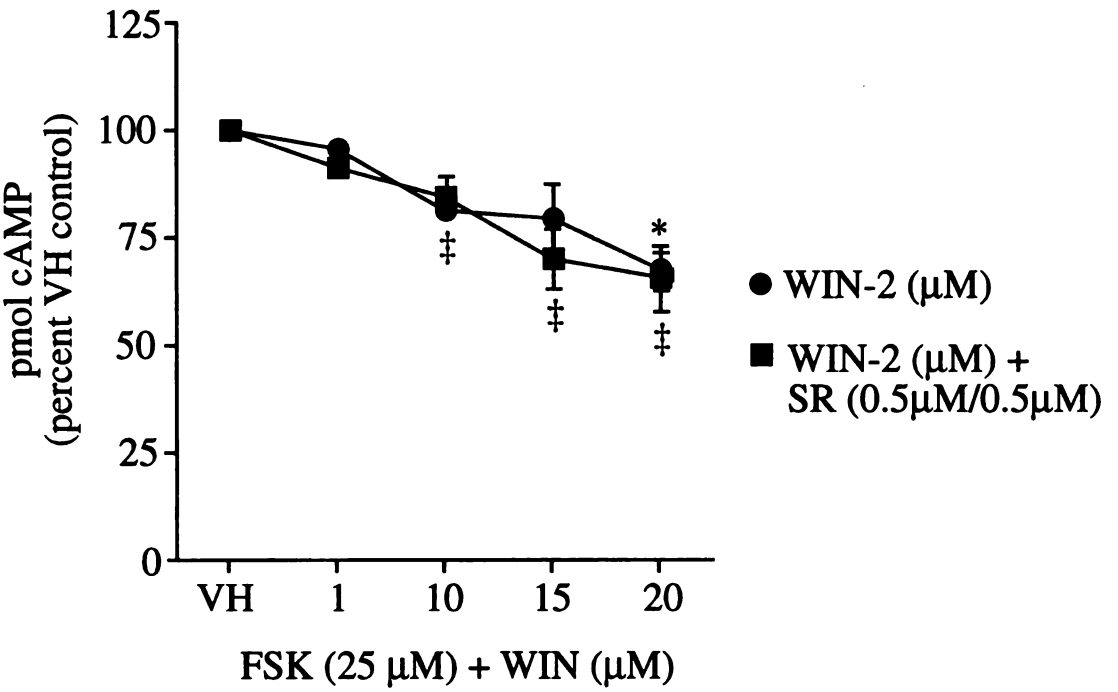


Figure 35. Effect of cannabinoid receptor antagonists on WIN-2-induced inhibition of FSK-stimulated cAMP in SPLC. SPLC were pretreated with SR144528 and SR141716A (0.5 μM each) for 30 min followed by WIN-2 treatment for 30 min. Cells were stimulated with 25 μM FSK for 15 min then assayed for cAMP. Average stimulation with FSK was 5.4 ± 0.4 pmol cAMP/5x10^6 SPLC. * $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the SR + VH group. Results represent three separate experiments with three replicates per treatment group.

not detected. Therefore, only the CB2 receptor antagonist (SR144528) was utilized in the CH12.LX studies. Despite the higher level of CB2 expression, SR144528 failed to attenuate cannabinoid-induced inhibition of FSK-stimulated cAMP by either CBN or WIN-2 in the CH12.LX cells (Figures 36 and 37). It is important to note also that the antagonists did not demonstrate any inverse agonist activity as has been previously reported (Figures 34-37) (310-312). These results suggest that the inhibition of FSK-stimulated cAMP production in these lymphoid preparations were not mediated via CB1 or CB2 as assessed by use of the cannabinoid receptor antagonists, SR141716A and SR144528. Although an initially surprising result, other reports have confirmed non-cannabinoid receptor-mediated effects of cannabinoids on cAMP (313).

Previous results from our laboratory have established a positive role of cAMP on IL-2 production (135), specifically depending on low concentrations of cAMP in the early stages of T cell activation (93). Often, inhibition of FSK-stimulated cAMP by cannabinoids was detected in the same cell preparations as those in which inhibition of PMA/Io-stimulated IL-2 occurred (64, 65, 82). Thus, characterization of the cannabinoid receptors involved in inhibition of PMA/Io-stimulated IL-2 production in SPLC was conducted. As presented in Figure 38, IL-2 was readily induced by PMA/Io treatment and was inhibited in the presence of CBN (1-20 μM) in a concentration-dependent manner. Similar to the effect of the antagonists in the cAMP assay, SR144528/SR141716A in combination did not attenuate the CBN-induced inhibition of PMA/Io-stimulated IL-2 production. In addition, there was no inverse agonist activity detected by either antagonist. The estimated IC_{50} value for CBN was 11.6 ± 1.2 μM in

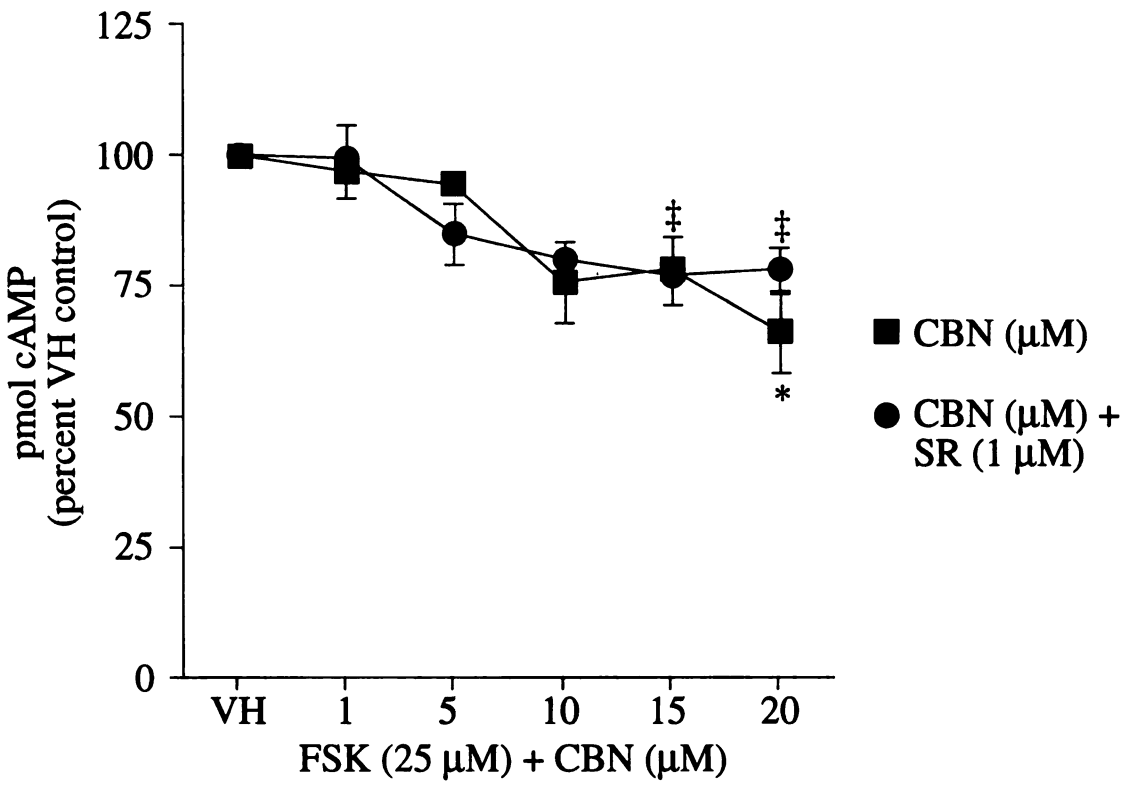


Figure 36. Effect of cannabinoid receptor antagonists on CBN-induced inhibition of FSK-stimulated cAMP in CH12.LX cells. CH12.LX cells were pretreated with SR144528 (1 μM) for 30 min followed by CBN treatment for 30 min. Cells were stimulated with 25 μM FSK for 15 min then assayed for cAMP. Average stimulation with FSK was 4.5 ± 1.4 pmol cAMP/1x10^6 CH12.LX cells. * $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the SR + VH group. Results represent three separate experiments with three replicates per treatment group.

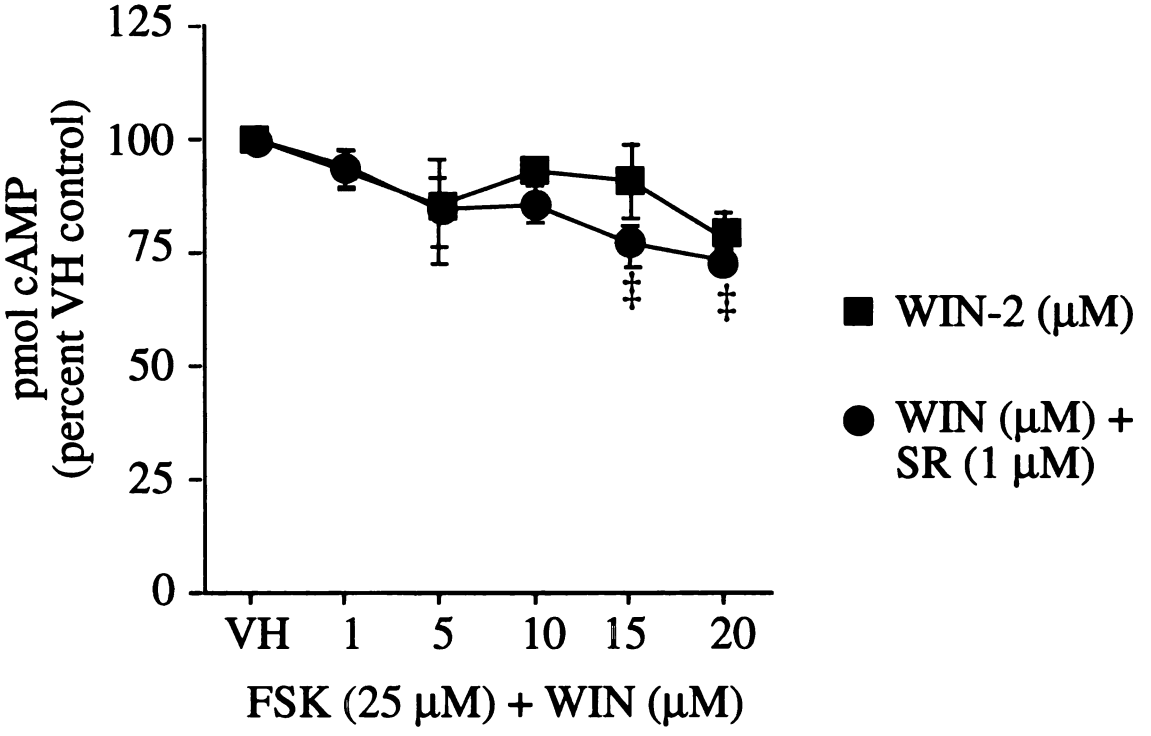


Figure 37. Effect of cannabinoid receptor antagonists on WIN-2-induced inhibition of FSK-stimulated cAMP in CH12.LX cells. CH12.LX cells were pretreated with SR144528 (1 μM) for 30 min followed by WIN-2 treatment for 30 min. Cells were stimulated with 25 μM FSK for 15 min then assayed for cAMP. Average stimulation with FSK was 5.6 ± 1.9 pmol cAMP/1x10^6 CH12.LX cells. * $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the SR + VH group. Results represent three separate experiments with three replicates per treatment group.

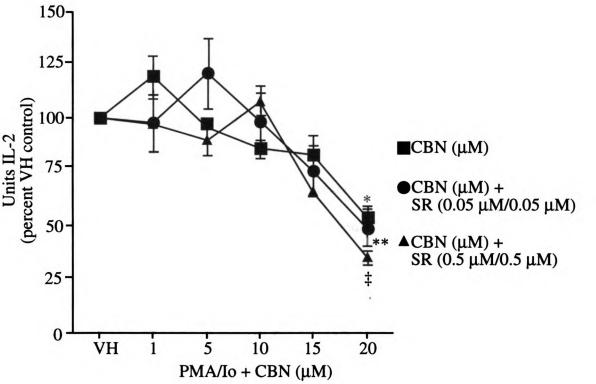


Figure 38. Effect of cannabinoid antagonists on CBN-induced inhibition of PMA/Io-stimulated IL-2 production. SPLC were treated with both SR141716A and SR144528 for 30 min followed by CBN treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. Average stimulation with PMA/Io was 1636 ± 432 units IL-2/ml. The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group; ** $p < 0.05$ as compared to the SR 0.0.5 μM/0.0.5 μM + VH group; ‡ $p < 0.05$ as compared to the SR 0.5 μM/0.5μM + VH group. Results represent four separate experiments with three replicates per treatment group.

the absence of antagonists and 11.6 ± 1.1 μM and 12.8 ± 0.8 μM in the presence of 0.05 μM/0.05 μM and 0.5 μM/0.5 μM SR144528/SR141716A, respectively.

Due to the inability of the cannabinoid antagonists to reverse the cannabinoid-induced inhibition of either cAMP or IL-2 production, PTX was utilized to examine the potential involvement of $G\alpha_i/G\alpha_o$ proteins in these effects. PTX (100 ng/ml) pretreatment for 24 h increased (29.0%) FSK-stimulated cAMP production in SPLC, which was statistically different from FSK-stimulated SPLC in the absence of PTX. However, CBN treatment (1-20 μM), in the presence or absence of PTX, modestly inhibited FSK-stimulated cAMP production in a concentration-dependent manner (Figure 39). Furthermore, PTX pretreatment did not attenuate the CBN-induced inhibition of PMA/Io-stimulated IL-2 production (Figure 40). The estimated IC_{50} value for IL-2 inhibition by CBN in PMA/Io-stimulated SPLC was 7.7 ± 2.2 μM in the absence of PTX and 8.6 ± 3.5 μM and 9.3 ± 2.1 μM in the presence of 10 ng/ml and 100 ng/ml PTX, respectively. PTX did not significantly modulate IL-2, with the exception that there was a modest but consistent increase in basal IL-2 secreted from the cells that were treated with 100 ng/ml PTX (approximately 10-15 units/ml). While these data demonstrated that PTX increased cAMP production as expected, they also suggested that CBN-induced inhibition of FSK-stimulated cAMP and PMA/Io-stimulated IL-2 was not mediated via a PTX-sensitive G protein-coupled receptor.

The results with PTX supported the theory that the inhibition of PMA/Io-stimulated IL-2 by CBN was not mediated via CB1 or CB2. Further evidence for this theory was provided with use of the WIN stereoisomer pair. One of the characteristics of receptor-mediated effects is stereospecific activity (92). Both WIN-2 (high affinity

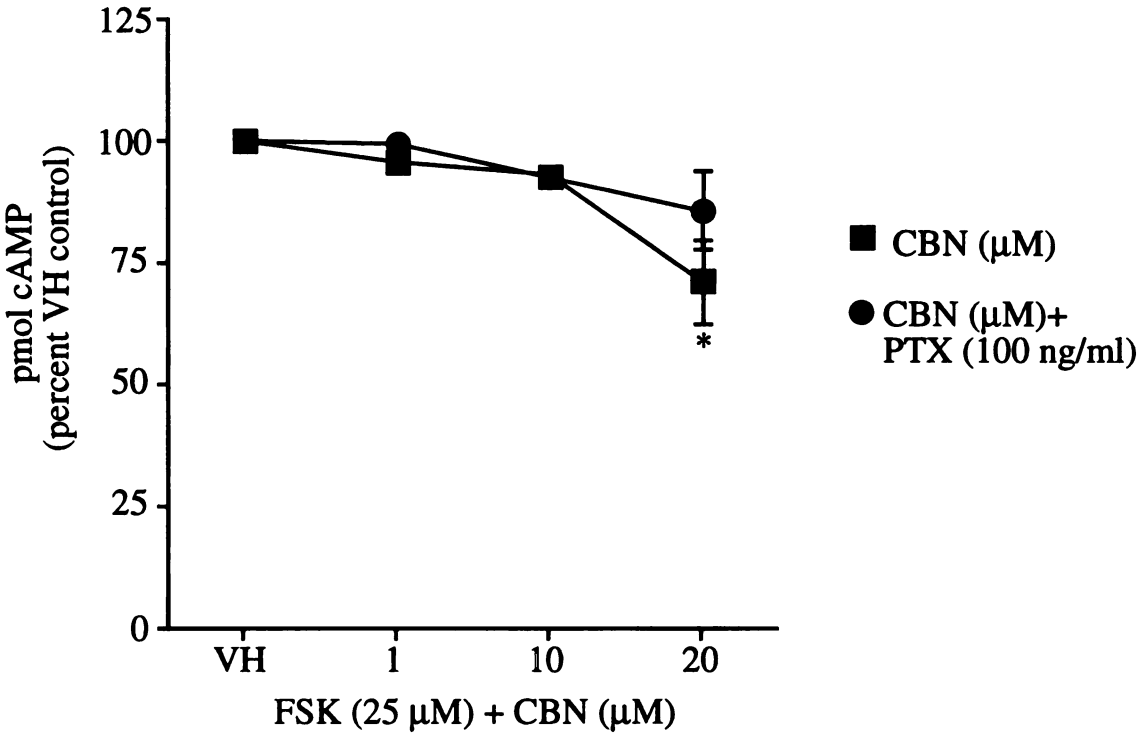


Figure 39. Effect of PTX on CBN-induced inhibition of FSK-stimulated cAMP production. SPLC were treated with 100 ng/ml PTX for 24 h. The cells were harvested, washed, and resuspended in 1 mg/ml FAP-BSA. Cells were treated with CBN for 30 min followed by 25 μM FSK for 15 min then assayed for cAMP. Average stimulation with FSK was 4.6 ± 0.5 pmol cAMP/5x10^6 SPLC in the absence of PTX. Average increase in cAMP production in the presence of 100 ng/ml PTX was 29.0%, which was significantly different from average stimulation in the absence of PTX ($p < 0.05$). * $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the PTX + VH group. Results represent five separate experiments with three replicates per treatment group.

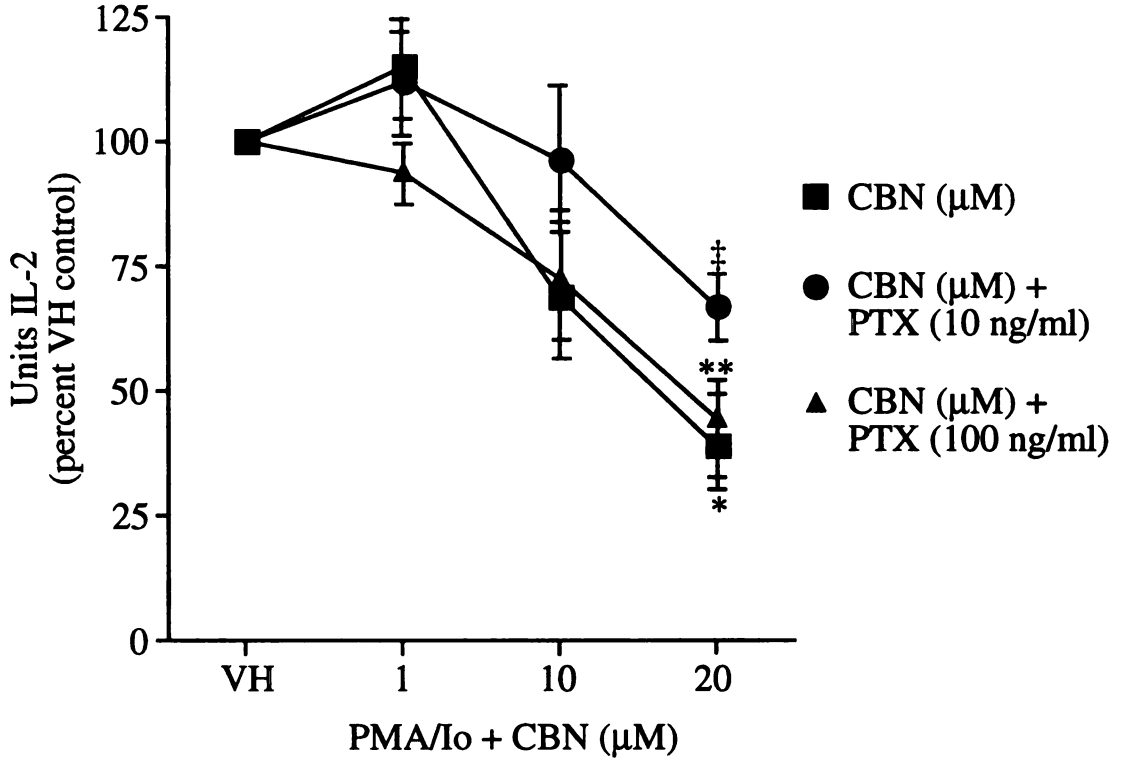


Figure 40. Effect of PTX on CBN-induced inhibition of PMA/Io-stimulated IL-2 production. SPLC were treated with 10 or 100 ng/ml PTX for 24 hours. The cells were harvested, washed, and resuspended in 2% BCS. Cells were treated with CBN for 30 min followed by PMA/Io (40 nM/0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Average stimulation with PMA/Io was 948 ± 184 units IL-2/ml.* $p < 0.05$ as compared to the VH group; ** $p < 0.05$ as compared to the PTX 10 ng/ml + VH group; ‡ $p < 0.05$ as compared to the PTX 100 ng/ml + VH group. Results represent five separate experiments with three replicates per treatment group.

isomer) and WIN-3 (low affinity isomer) inhibited PMA/Io-induced IL-2 production in a concentration-dependent manner; however, WIN-2 demonstrated robust, and consistently greater, inhibition than that of WIN-3 (Figure 41). The IC_{50} values for WIN-induced inhibition of IL-2 were 3.5 ± 0.8 μM for WIN-2 and 5.3 ± 0.4 μM for WIN-3. While the difference in potency between the two isomers was not evident by the IC_{50} values, there was a greater degree of separation in the potency at higher concentrations. For instance, at 10 μM, the mean percent VH control was 6.7 ± 3.1% for WIN-2 versus 38.1 ± 3.7% for WIN-3. Interestingly, WIN-3 treatment of PMA/Io-stimulated SPLC inhibited IL-2 more robustly than CBN, a ligand that exhibits selectivity for the CB2 receptor. Furthermore, the antagonists (0.5 μM/0.5 μM SR144528/SR141716A) did not attenuate the inhibition of PMA/Io-stimulated IL-2 by either WIN isomer (Figure 42). In the presence of SR141716A/SR144528, the IC_{50} values did not significantly change from those calculated in the absence of the antagonists (2.8 ± 0.3 μM for WIN-2; 6.1 ± 2.4 μM for WIN-3).

In light of the above results, CBD, another plant-derived cannabinoid was utilized to assess the role of cannabinoid receptors in cannabinoid-induced IL-2 inhibition. CBD was previously determined to exhibit low-affinity for the CB2 receptor in HL-60 cells (46) and did not inhibit FSK-stimulated cAMP production in CB1-transfected CHO cells (45). In SPLC, however, CBD (1-20 μM) robustly inhibited PMA/Io-stimulated IL-2 production with an IC_{50} value of 2.9 ± 0.3 μM and this inhibition was not attenuated by 0.5 μM/0.5μM SR144528/SR141716A (Figure 43). Higher concentrations of antagonists (5 μM/5 μM SR144528/SR141716A) were utilized in these studies due to the low binding affinity of CBD for the cannabinoid receptors; yet, there was no attenuation of

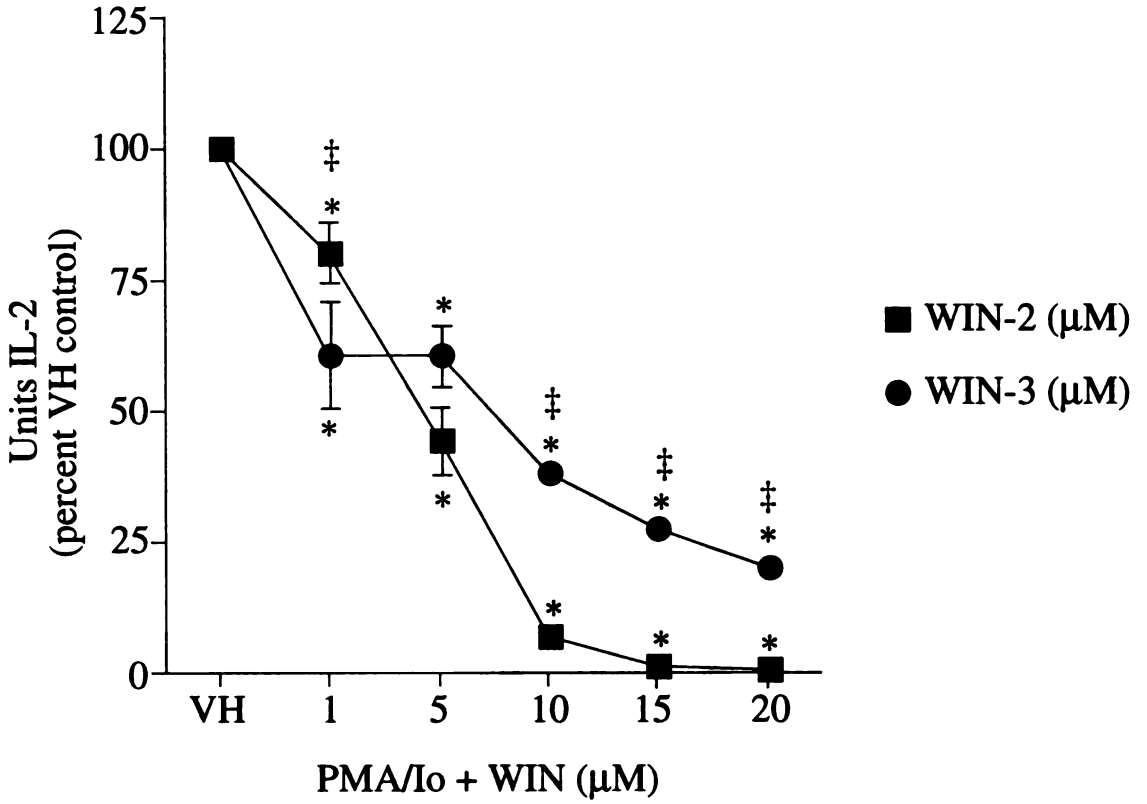


Figure 41. Effect of WIN stereoisomers on PMA/Io-stimulated IL-2 production. SPLC were treated with WIN for 30 min followed by activation with PMA/Io (40 nM/0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Average stimulation with PMA/Io was 1889 ± 251 units IL-2/ml.* $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the WIN-2 group for each concentration. Results represent three separate experiments with three replicates per treatment group.

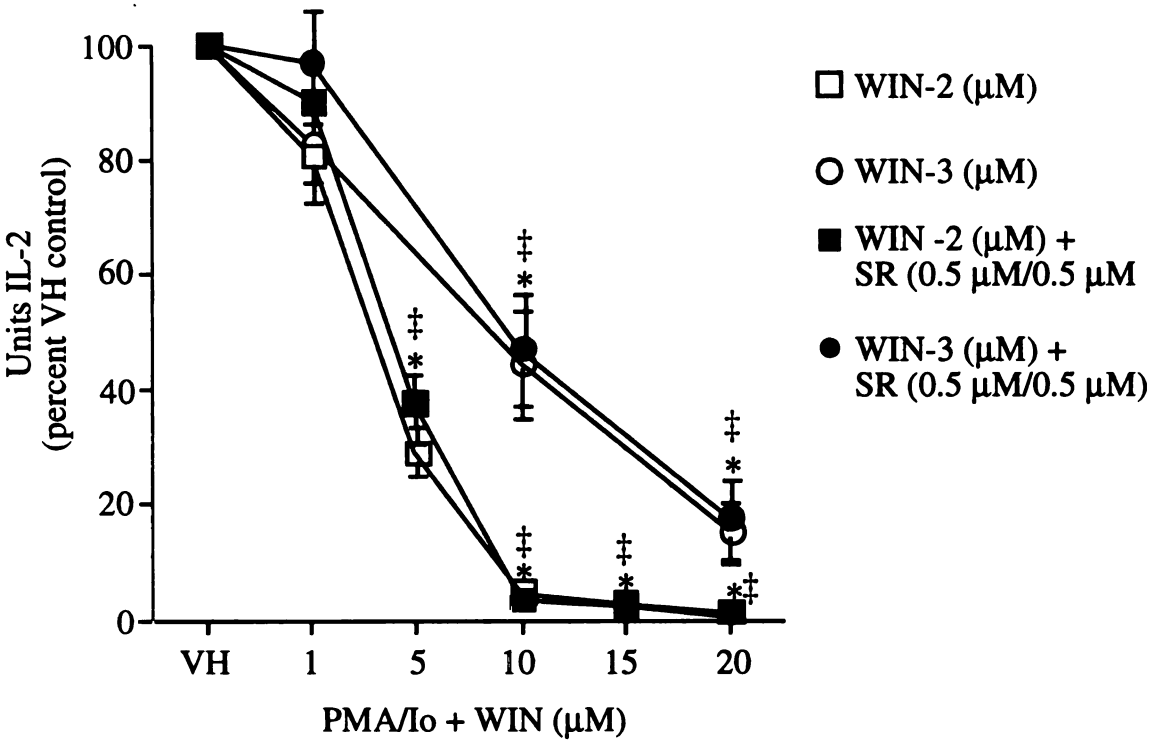


Figure 42. Effect of cannabinoid antagonists on WIN-induced inhibition of PMA/Io-stimulated IL-2 production. SPLC were treated with both SR141716A and SR144528 (0.5 μM/0.5 μM) for 30 min followed by WIN treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Average stimulation with PMA/Io was 1690 ± 191 units IL-2/ml.* $p < 0.05$ as compared to the VH group; ‡ $p < 0.05$ as compared to the SR + VH group. Results represent four separate experiments with three replicates per treatment group.

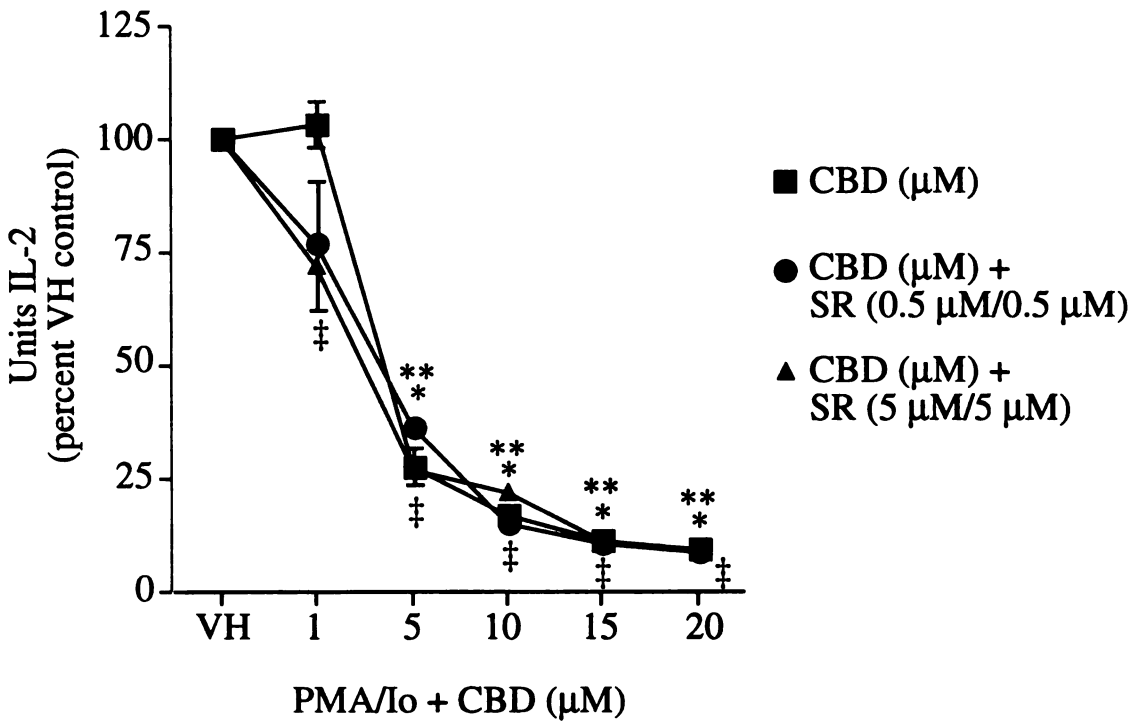


Figure 43. Effect of cannabinoid antagonists on CBD-induced inhibition of PMA/Io-stimulated IL-2 production. SPLC were treated with both SR141716A and SR144528 (0.5 μM/0.5 μM or 5 μM/5 μM) for 30 min followed by CBD treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. Cells were stimulated with 40 nM PMA/0.5 μM Io for 24 h. Average stimulation with PMA/Io was 1639 ± 342 units IL-2/ml. The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group. ** $p < 0.05$ as compared to the SR (0.5 μM/0.5 μM) + VH group; ‡ $p < 0.05$ as compared to the SR (5 μM/5 μM) + VH group. Results represent three separate experiments with three replicates per treatment group.

CBD-induced inhibition of PMA/Io-stimulated IL-2. The IC_{50} values for CBD-induced inhibition in the presence of the antagonists was 2.4 ± 0.8 μM and 2.0 ± 0.2 μM for 0.5 μM/0.5 μM and 5 μM/5 μM concentrations of SR144582/SR141716A, respectively. It was notable that the magnitude of inhibition of IL-2 by CBD was greater than that of CBN, in spite of the fact that CBN possesses greater CB1 and CB2 affinity.

It is well established that PMA/Io treatment of lymphocytes in culture causes a morphological change that is best described as "cellular aggregation" (314). Indeed, in the SPLC cultures, it was noted that cellular aggregation occurred within 24 h following PMA/Io treatment (Figure 44). Interestingly, however, WIN-2 pretreatment of SPLC resulted in a remarkable abrogation of the ability of the cells to aggregate, which corresponded with the inhibition of IL-2 production. Treatment of the SPLC cultures with the combination of SR144528/SR141716A at 0.5 μM each did not reverse the WIN-2-induced abrogation of cellular aggregation (Figure 44). Moreover, the antagonists alone did not alter cellular aggregation induced by PMA/Io.

VII. Role of oxidation/reduction status in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2.

There is much evidence supporting the notion that cannabinoid receptors were likely not involved in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2. It was possible then, that the mechanism was receptor-independent. For example, both Δ^9-THC and CBD were determined to be anti-oxidants (315). Furthermore, H_2O_2 induced T cell activation and IL-2 production (204). Thus, studies were conducted in order to determine whether oxidant treatment could attenuate cannabinoid-induced inhibition of PMA/Io-

Figure 44. Effect of cannabinoid antagonists on WIN-2-induced inhibition of PMA/Io-stimulated cellular aggregation. SPLC were treated with both SR141716A and SR144528 (0.5 μM/0.5 μM) for 30 min followed by WIN treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. Cells were observed at 40x with a Nikon epifluorescence phase contrast microscope equipped with a 35mm camera. Images were captured by a COHU video camera and digitized and processed with NucleoTech Gel Expert (NucleoTech Corp, San Mateo, CA). Results are representative of four separate experiments.

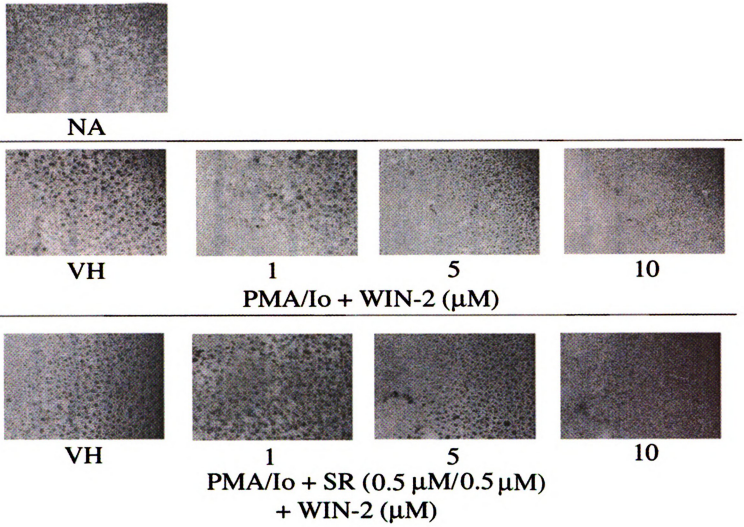


Figure 44. Effect of cannabinoid antagonists on WIN-2-induced inhibition of PMA/Io-stimulated cellular aggregation.

stimulated IL-2 production. However, pre-treatment with H_2O_2 did not prevent CBN- or CBD-induced inhibition of PMA/Io-stimulated IL-2 production (Figure 45).

VIII. Effect of cannabinoid compounds on regulation of intracellular calcium concentration.

With the demonstration that cannabinoid receptors were likely not involved in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2, other potential T cell targets were examined. As previously stated, the two critical signals for IL-2 production in SPLC are those generated via PKC and an elevation in intracellular calcium. Furthermore, the PKC response can be enhanced by calcium activation of calcium-dependent PKC isozymes. A major target of calcium regulation in T cells is NF-AT and interestingly, NF-AT binding activity was inhibited by CBN (Figure 15). In addition, NF-AT was determined to be one of the most critical targets of inhibition by cannabinoids as demonstrated by an inhibition of NF-AT binding activity in PMA/Io-stimulated EL-4 T cells that correlated with inhibition of an NF-AT reporter gene construct (88). Therefore, alteration in calcium regulation and signaling seemed like a plausible mechanism for cannabinoid-mediated dysregulation of IL-2. However, CBN and CBD (1-20 μM) elevated intracellular calcium for at least 30 min in resting SPLC in a concentration-dependent manner (Figures 46 and 47). Interestingly, a sustained elevation (at least 30 min) in intracellular calcium concentration is required for optimal T cell activation (214, 285). The shapes of the curves and magnitude of intracellular calcium elevation were different for the two cannabinoids. Notably, the time to onset of intracellular calcium elevation by CBD was similar to that of Io (0.5 μM, Figure 48).

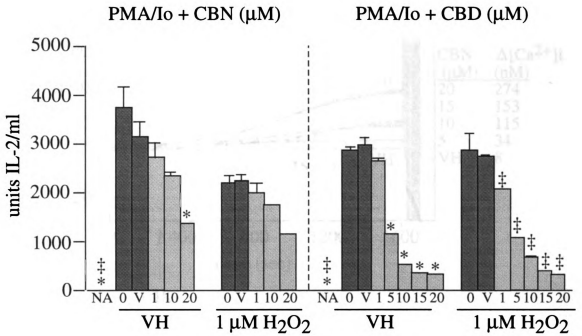


Figure 45. Effect of H_2O_2 on cannabinoid-induced inhibition of PMA/Io-stimulated IL-2. SPLC were treated with H_2O_2 (1 μM) for 30 min followed by CBN or CBD treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the CBN VH group; ‡ $p < 0.05$ as compared to the CBD VH group. Results represent two separate experiments with three replicates per treatment group.

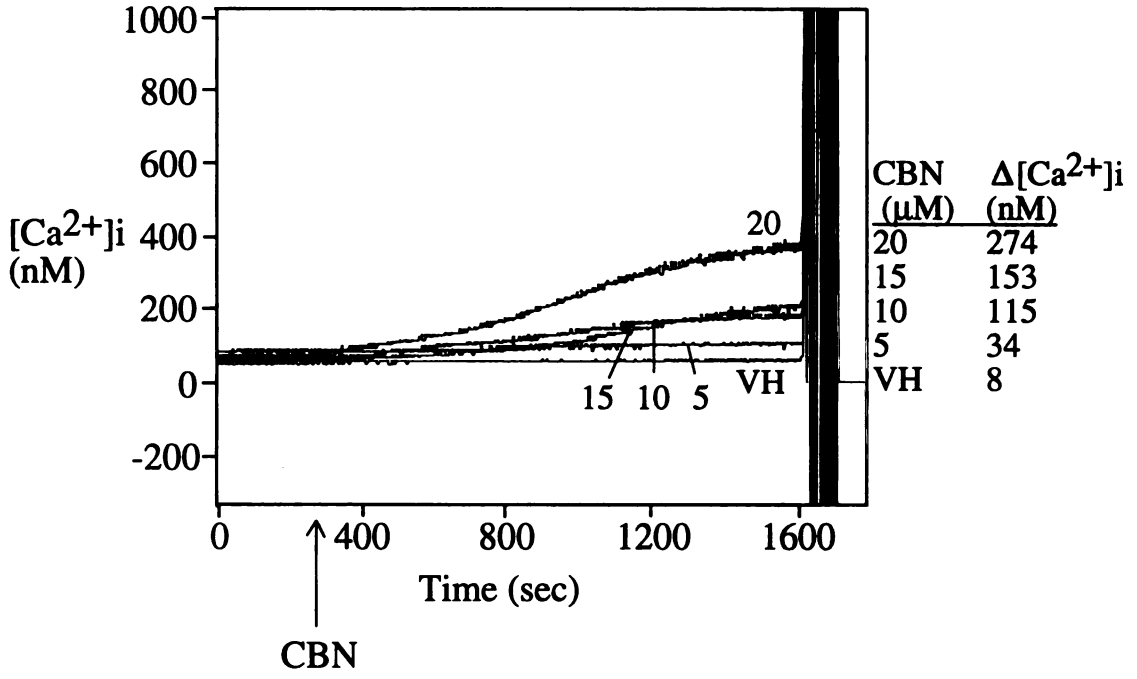


Figure 46. CBN elevates intracellular calcium in resting SPLC. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. CBN was added by glass syringe to the cuvette at 300 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of two separate experiments.

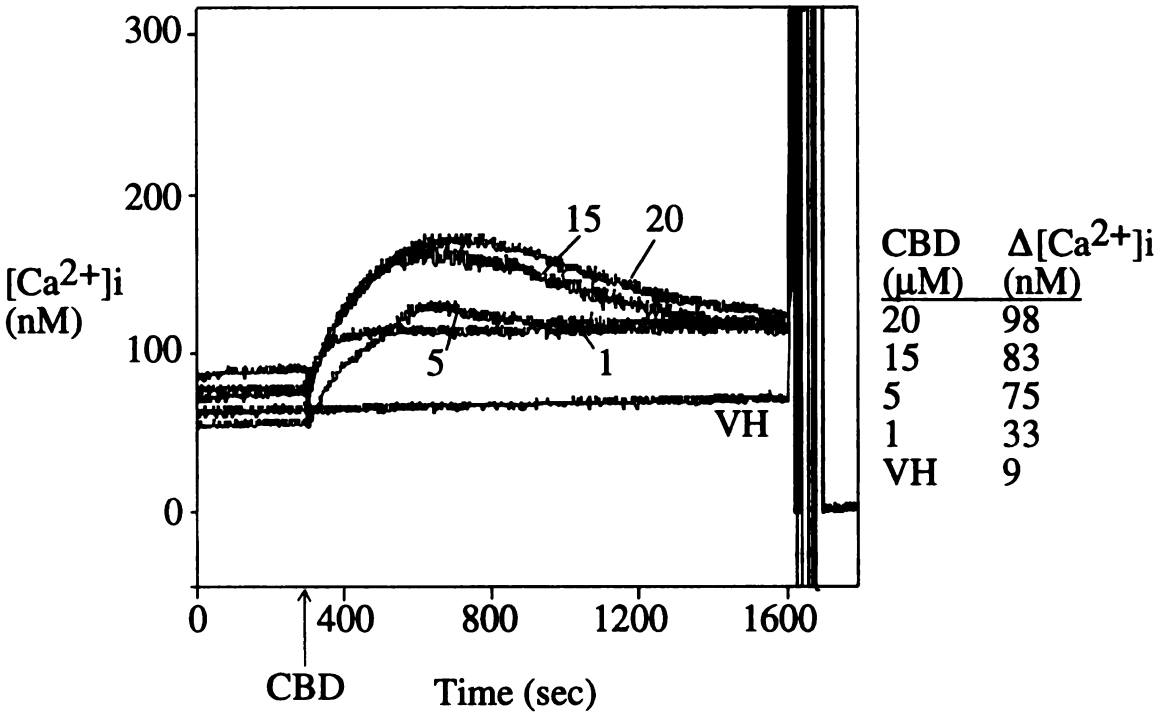


Figure 47. CBD elevates intracellular calcium in resting SPLC. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. CBD was added by glass syringe to the cuvette at 300 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of two separate experiments.

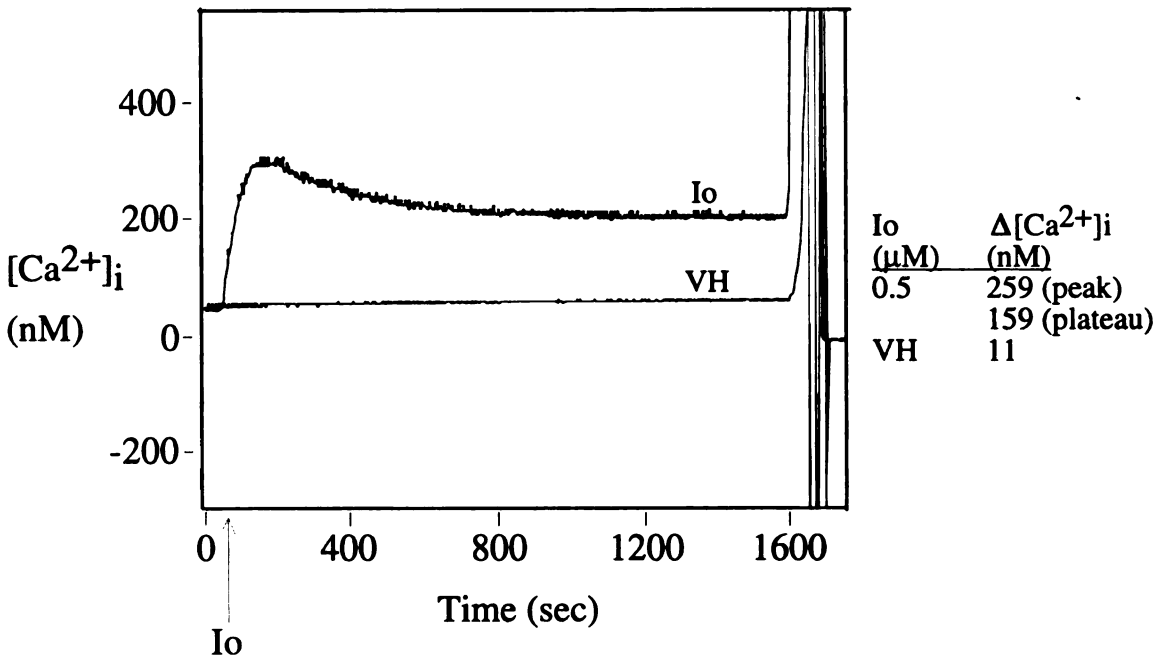


Figure 48. Io elevates intracellular calcium in resting SPLC. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. Io was added by glass syringe to the cuvette at 50 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of four separate experiments.

Moreover, the time to onset of intracellular calcium elevation by TG was also rapid (Figure 49). Unlike other cell populations in which TG induced a transient elevation in intracellular calcium (50), in resting SPLC, the calcium elevation in response to TG was sustained for at least 30 min. This is likely due to stimulation of influx of extracellular calcium via the CRAC channels (316) and in fact, TG can also be utilized to activate T cells (317).

CBN and CBD elevated intracellular calcium in resting SPLC, yet the mechanism of this elevation was not evident. In several cell types, the transient nature of TG permits its use as a powerful tool to determine whether the elevation in intracellular calcium is due to an intracellular store release. However, as demonstrated in Figure 49, TG treatment of resting SPLC induced a sustained elevation in intracellular calcium. Thus, studies were performed in the presence and absence of extracellular calcium in order to determine whether the CBN-induced elevation in intracellular calcium was due to influx of extracellular calcium. The calcium response induced by 15 μM CBN was drastically reduced when the studies were conducted in the absence of extracellular calcium, indicating the elevation of intracellular calcium by CBN occurred primarily via influx of extracellular calcium (Figure 50). Io (0.5 μM)-stimulated elevation in intracellular calcium was used to confirm the absence of extracellular calcium in the buffer (Figure 51). As expected for Io, the first phase was primarily due to release of intracellular stores of calcium which was not affected by the zero-extracellular calcium conditions, and the second phase was due to influx of extracellular calcium which was abolished under these conditions (140).

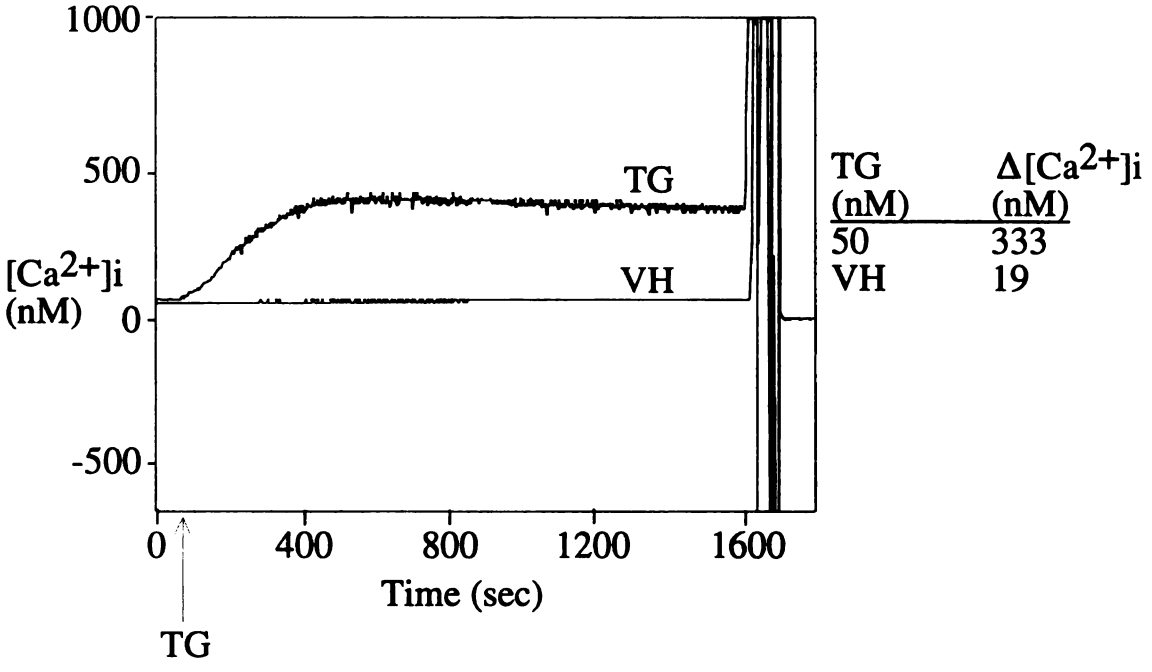


Figure 49. TG elevates intracellular calcium in resting SPLC. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. TG was added by glass syringe to the cuvette at 50 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of two separate experiments.

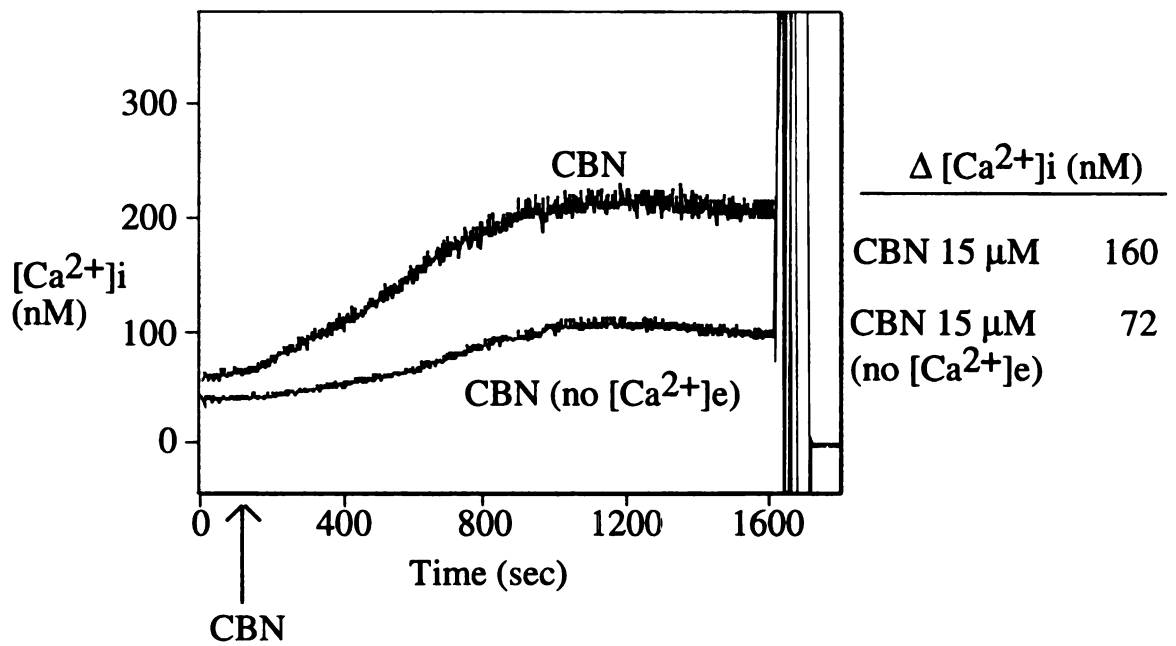


Figure 50. Effect of zero extracellular calcium on CBN-induced elevation in intracellular calcium. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. CBN was added by glass syringe to the cuvette at 50 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of two separate experiments.

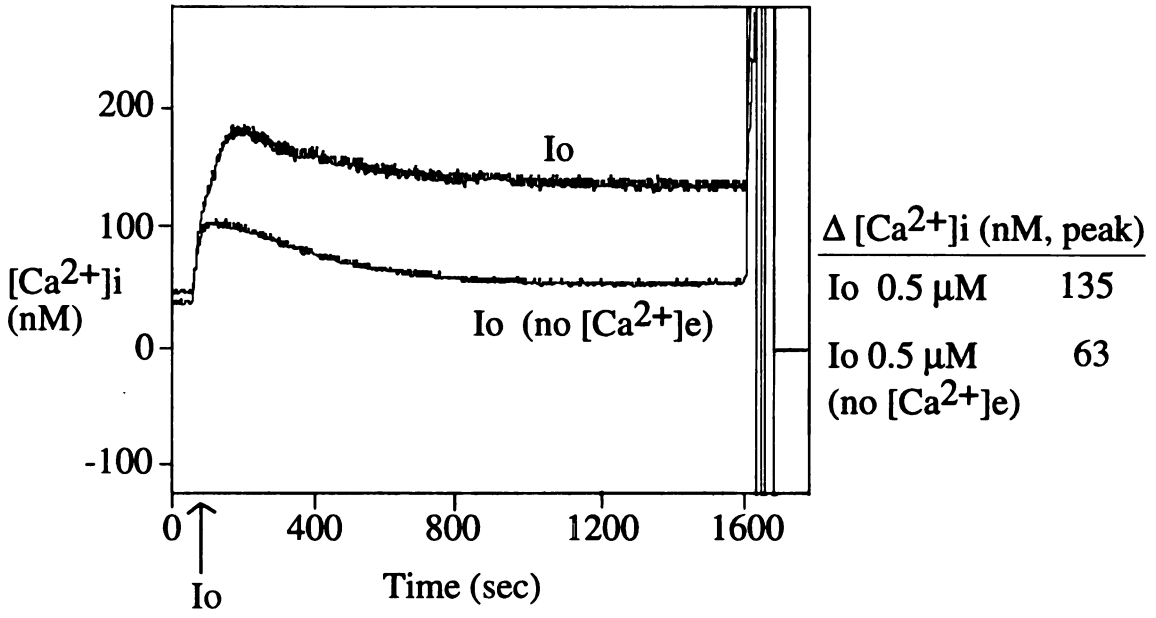


Figure 51. Effect of zero extracellular calcium on Io-induced elevation in intracellular calcium. SPLC were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in Ca^{2+}-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. Io was added by glass syringe to the cuvette at 50 s and the elevation in intracellular calcium was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Calcium concentration was calculated from the change in the ratio of bound to free calcium. Results are representative of two separate experiments.

CBN-induced elevation in intracellular calcium in resting SPLC was mediated primarily via an influx of extracellular calcium, suggesting a putative role for membrane-bound receptors. Induction of elevation in intracellular calcium with cannabinoid treatment has been demonstrated in a number of other cell types in a cannabinoid receptor-dependent manner (71, 72). In light of these previous findings, SR144528 and SR141716A were utilized to examine the involvement of CB1 and CB2 in CBN-induced elevation of intracellular calcium in resting SPLC and THMC. Studies were conducted in THMC in order to verify that the elevation in intracellular calcium also occurred in T cells. CBN (15 μM)-induced elevation in intracellular calcium was markedly attenuated by both cannabinoid receptor antagonists (5, 10 μM alone or in combination) in resting SPLC (Table 2). Similar to SPLC, CBN (5-15 μM) treatment elevated intracellular calcium in resting THMC. As THMC do not express CB1 mRNA (52), only the CB2 antagonist, SR144528, was utilized in order to address the putative role of the CB2 receptor in CBN-induced elevation in intracellular calcium in THMC. As presented in Table 3, SR144528 (10 μM) partially attenuated the CBN-induced elevation in intracellular calcium. Furthermore, SR144528 in combination with CBN modestly elevated intracellular calcium in THMC.

IX. Effect of intracellular calcium elevation on PMA/Io-stimulated IL-2 production.

CBN elevated intracellular calcium in resting SPLC and THMC in a cannabinoid receptor-dependent manner. Previous studies have demonstrated that a premature elevation in intracellular calcium results in inhibition of T cell activation (296, 300). Indeed, pretreatment of SPLC with Io for 30 min prior to activation resulted in a drastic

Table 2. Summary of effects of cannabinoid receptor antagonists on CBN-induced elevation in intracellular calcium in resting SPLC*.

		CBN 15 μM + SR144528 (μM)		
VH	CBN 15 μM	1	5	10
17	222	207	124	124

		CBN 15 μM + SR141716A (μM)		
VH	CBN 15 μM	1	5	10
17	190	184	112	60

		CBN 15 μM + SR144528 (μM)/SR141716A (μM)		
VH	CBN 15 μM	0.5/0.5	2.5/2.5	5/5
20	206	197	147	117

* values are $\Delta [Ca^{2+}]_i$ as measured from base to peak

Results are representative of two separate experiments.

Table 3. Summary of effects of cannabinoid receptor antagonists on CBN-induced elevation in intracellular calcium in restingTHMC*.

		CBN (μM)		
	VH	5	10	15
CBN (μM)	8	27	81	108
CBN (μM) + SR144528 10 μM	23	49	45	39

* values are $\Delta\ [Ca^{2+}]_i$ as measured from base to peak

Results are representative of two separate experiments.

inhibition of IL-2 production. This result was confirmed with two other agents that increase intracellular calcium, A23187 and TG (Figure 52). The concentrations used for both Io and TG are in the same range as those used in the intracellular calcium determination studies, providing a correlation between the magnitude of intracellular calcium elevation and the magnitude of IL-2 inhibition.

X. Cannabinoid induction of IL-2 in PMA-stimulated SPLC.

Cannabinoids, similar to Io and TG, elevated intracellular calcium in resting SPLC. Therefore it was determined whether cannabinoids would provide the necessary calcium signal required for IL-2 production in the presence of PMA. CBN (up to 20 μM), in the presence of 40 nM PMA, did not induce IL-2 production. CBD (1-20 μM) however, induced IL-2 production in a concentration-dependent manner in PMA-stimulated SPLC (Figures 53 and 54). The magnitude of IL-2 production with PMA/CBD was much lower than that produced with PMA/Io; yet this was the first demonstration that cannabinoids, in the absence of any other calcium signal, induced IL-2 production. The cannabinoid receptor antagonists were used to determine if the stimulation of IL-2 by CBD was cannabinoid receptor-mediated; however, 1 μM of cannabinoid receptor antagonist individually or in combination did not attenuate CBD-induced increase in PMA-stimulated IL-2 (Figure 53). Again, due to the relatively low binding affinity that CBD exhibits for the cannabinoid receptors, higher concentrations of antagonists were used. In fact, 10 μM of cannabinoid receptor antagonists individually or in combination attenuated the CBD-induced IL-2 production in PMA-stimulated SPLC

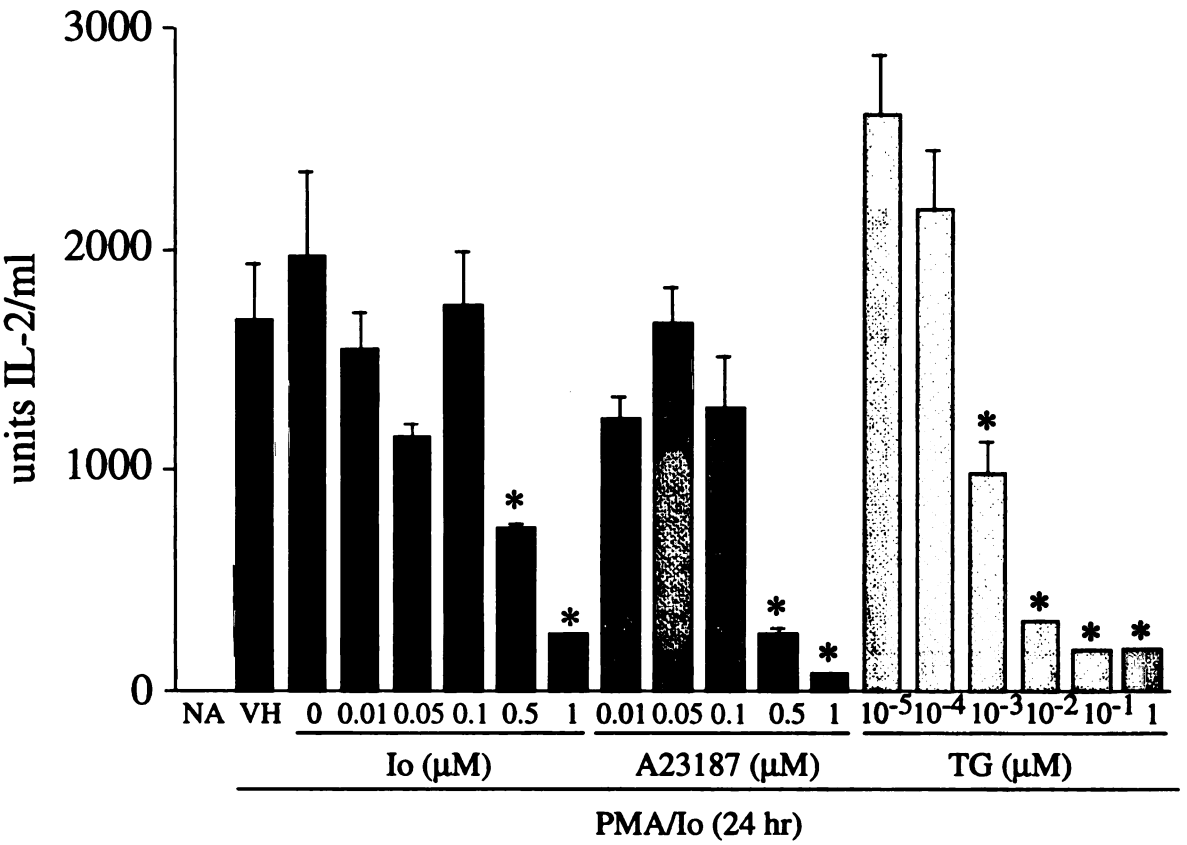


Figure 52. Inhibition of PMA/Io-stimulated IL-2 production by Io, A23187 and TG. SPLC were treated with Io, A23187, or TG for 30 min followed by activation with PMA/Io (40 nM/0.5 μM) for 24 h. For the Io-treated samples, the concentrations of Io are in addition to that given in combination with PMA (i.e., 0.5 μM plus the indicated concentrations). The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group. Results represent three separate experiments with three replicates per treatment group.

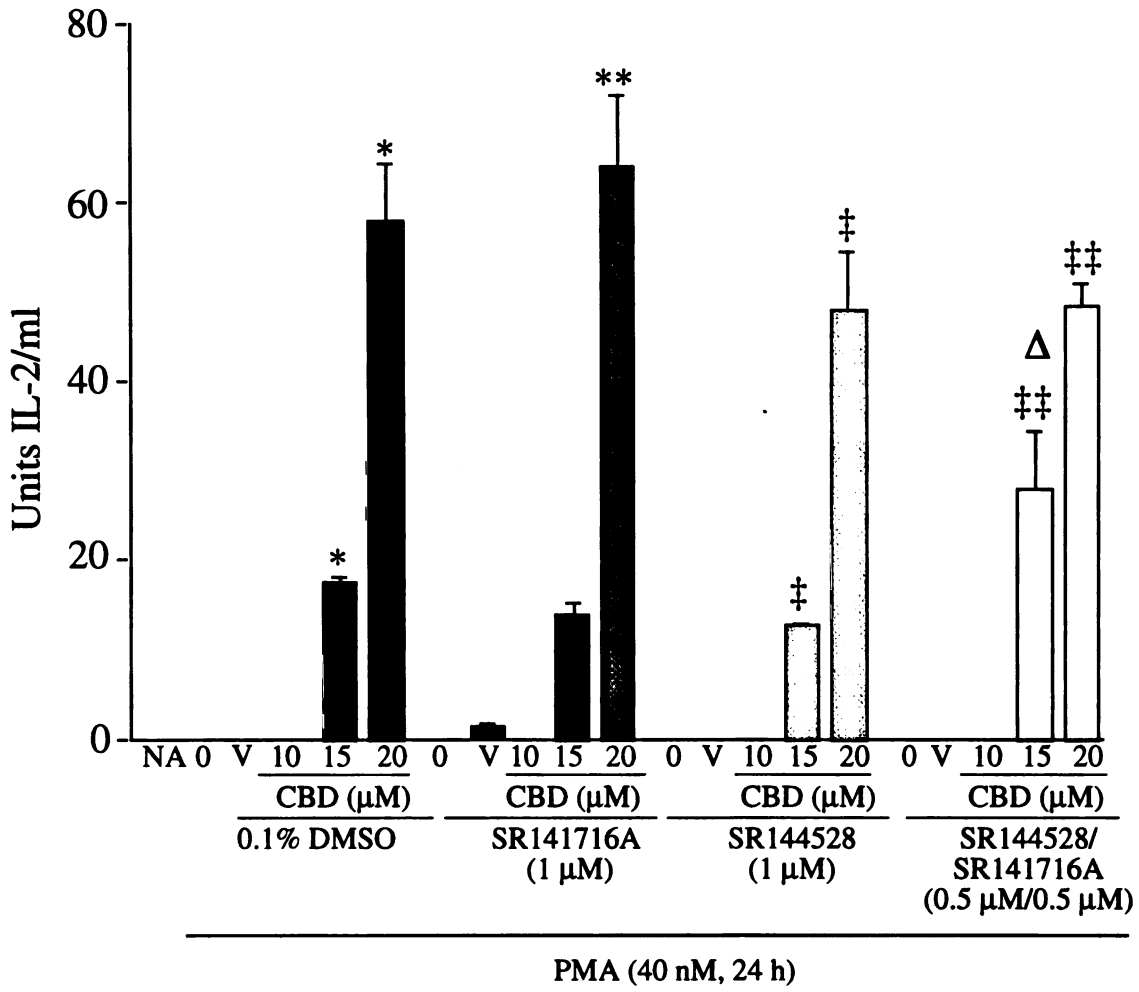


Figure 53. Effect of cannabinoid receptor antagonists (1 μM each or in combination) on CBD-induced production of IL-2 in PMA-stimulated SPLC. SPLC were treated with SR141716A and SR144528 (1 μM each or in combination) for 30 min followed by CBD treatment for 30 min. Cells were stimulated with PMA (40 nM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group; ** $p < 0.05$ as compared to the SR144528 + VH group; ‡ $p < 0.05$ as compared to the SR141716A + VH group; ‡‡ $p < 0.05$ as compared to the combined + VH group; Δ $p < 0.05$ as compared to CBD 15 μM group. V, VH. Results represent three separate experiments with three replicates per treatment group.

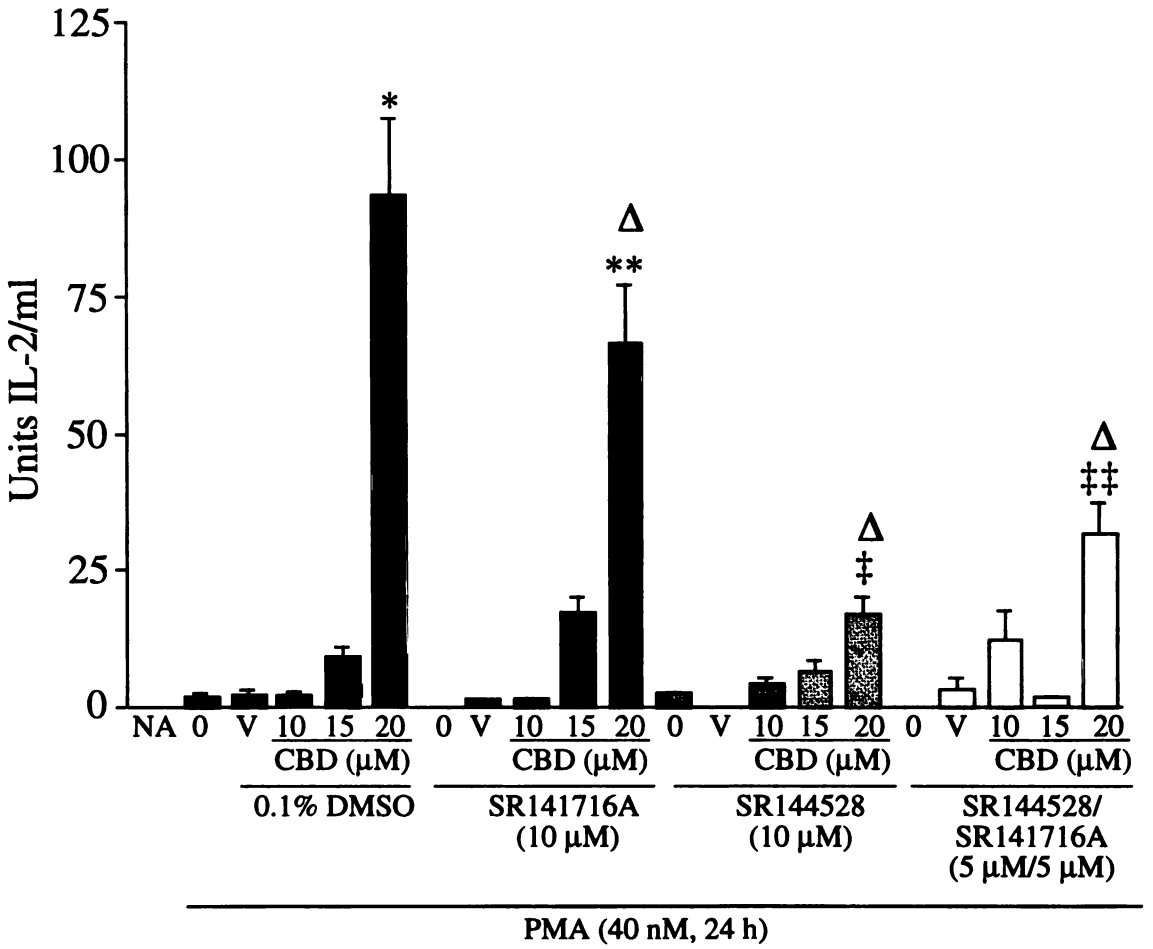


Figure 54. Effect of cannabinoid receptor antagonists (10 μM each or in combination) on CBD-induced production of IL-2 in PMA-stimulated SPLC. SPLC were treated with SR141716A and SR144528 (5 μM each or in combination) for 30 min followed by CBD treatment for 30 min. Cells were stimulated with PMA (40 nM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group; ** $p < 0.05$ as compared to the SR144528 + VH group; ‡ $p < 0.05$ as compared to the SR141716A + VH group; ‡‡ $p < 0.05$ as compared to the combined + VH group; Δ $p < 0.05$ as compared to CBD 20 μM group. V, VH. Results represent three separate experiments with three replicates per treatment group.

(Figure 54), with more effective antagonist by the CB2 receptor antagonist versus the CB1 receptor antagonist.

DISCUSSION

Cannabinoid compounds exhibit immunosuppressive effects in T lymphocytes. One such effect is the suppression of IL-2 production (64) in lymphocytes treated with PMA/Io, a stimulus that mimics antigen receptor activation (141). While the mechanism of this inhibition involves inhibition of transcription of IL-2 (64, 88), there are many signals that contribute to IL-2 transcription and the signal(s) that are specifically targeted by these compounds in T cells is not yet known.

I. Effect of cannabinoid compounds on AP-1 transcription factor activity in PMA/Io-stimulated SPLC.

Cannabinoid compounds have been shown to inhibit both the cAMP signaling cascade (65, 82) and PMA/Io-stimulated IL-2 production in lymphocytes (64). Therefore, it was tempting to speculate that the inhibition of the cAMP signaling cascade contributed to the inhibition of PMA/Io-stimulated IL-2 production. While the role of cAMP in T cell function has been disputed, there is evidence to suggest that low level cAMP production early in T cell activation is critical for T cell function (93, 135). Furthermore, it was demonstrated that CREB proteins participate in the binding at the proximal AP-1 site from the IL-2 promoter (303) and this suggested that cannabinoids inhibited IL-2 production at the transcriptional level via inhibition of transcription factor binding activity.

AP-1 proteins participate in the complexes that form at almost every site in the IL-2 promoter. Therefore, initially these studies focused on c-fos and c-jun regulation by

cannabinoid compounds based on the observation by Barton and coworkers (134) that T cells expressing a dominant negative form of CREB, which was no longer PKA-responsive, resulted in decreased IL-2 production, inhibition of PMA/Io-induced proliferation, G1 cell cycle arrest, and decreased mRNA expression of c-fos, c-jun, fra-2 and fos-B (134). Consistent with these studies, we have demonstrated that CBN treatment of mouse SPLC inhibited PMA/Io-induced transcription factor binding to proximal AP-1, consensus AP-1 and distal NF-AT elements, all complexes which contain fos, jun and CREB family proteins (153, 154, 206). This inhibition almost certainly contributes to the decreased IL-2 production in SPLC following treatment with cannabinoid compounds (64). In fact, in EL-4 T cells, Yea, *et. al.* demonstrated that CBN could inhibit the transiently transfected p(IL-2)-CAT expression plasmid in activated EL-4 cells in a concentration-dependent manner (88). This was partially due to an inhibition of NF-AT-mediated transcriptional regulation as demonstrated by a p(NF-AT)3-CAT expression vector. Interestingly, there was no effect on transcriptional regulation using a p(AP-1)3-CAT expression plasmid, which contains the AP-1 consensus motif. The authors noted that there was a marked inhibition of AP-1 binding in the EL-4 cells at 120 min post cellular activation, which was less apparent at 240 min, indicating partial recovery of binding in the presence of CBN at later times following cellular activation. Hence, the lack of inhibition by CBN of the AP-1 expression plasmid (stimulated for 18 h) represents this transient effect of CBN on protein binding to AP-1 elements in EL-4 cells.

Using supershift analysis, it was determined that fos and jun proteins participate in the complex that forms at the proximal AP-1 sites in PMA/Io-stimulated SPLC. This

was in contrast to studies demonstrating a critical role for CREB in AP-1 binding to the proximal site in T cells (303). These data suggest that: 1.) other CREB proteins were binding to the site but were not recognized by the antibody; 2.) the participation of CREB in the complex at the proximal TRE was cell-type specific; or 3.) CREB proteins participate only if appropriately stimulated (i.e., with FSK). On the other hand, there was one complex induced by PMA/Io at the AP-1 consensus site that included fos, jun and CREB proteins. It was possible that this represented two complexes composed of fos-CREB and fos-jun heterodimers which could not be resolved (fos/CREB is a 62/43 kD heterodimer and fos/jun is a 62/39 kD heterodimer).

The fact that CBN was capable of preventing AP-1 binding to both the proximal and consensus elements not only suggests that these effects were not specific for IL-2, but were the result of a depletion of the protein pool necessary for binding to these sites. Indeed, Western analysis demonstrated that c-fos and c-jun nuclear protein expression was inhibited by CBN treatment. Thus, the inhibition of nuclear protein expression of c-fos and c-jun likely contributes to the inhibition of transcription factor complex formation at AP-1-containing sites. It is important to emphasize that there might be other fos, jun or CREB family members whose protein expression was also inhibited by these compounds, thereby contributing to the decreased transcription factor binding. The mechanism(s) of cannabinoid-induced inhibition of nuclear protein expression of c-fos and c-jun could involve one or more of the following: inhibition of transcription, increased degradation of either the transcript or the protein, or inhibition of nuclear translocation. In fact, it has been demonstrated that retention of c-fos in the cytosol is controlled by a liable transport inhibitor, which itself can be inhibited by cAMP (318). However, the inhibition of c-fos

and c-jun nuclear protein expression was not due to retention of these proteins in the cytosol.

An alternative mechanism to explain the inhibition of c-fos and c-jun nuclear protein expression by cannabinoids is cannabinoid-induced inhibition of steady state mRNA levels. However, there was no inhibitory effect on steady state mRNA expression of c-fos and c-jun in the presence of CBN. This effect of CBN on c-fos and c-jun mRNA expression was different than anticipated based on the studies by Barton, *et al.*, who demonstrated an decrease in mRNA expression of c-fos, c-jun, fra-2 and fos-B in T cells expressing the dominant negative CREB protein (134). One possible explanation for this difference is that the inhibition of the cAMP signaling cascade by CBN is modest relative to overexpression of a mutant CREB protein as used in the Barton studies. Furthermore, effects of CBN on other CREB/ATF family members were not examined and it is possible that CBN-induced inhibition of the cAMP signaling cascade causes compensatory pathways to be activated to prevent the inhibition of c-fos and c-jun mRNA levels. Other studies have shown an increase in immediate early gene expression following treatment with cannabinoid agonists, which was attributed to cannabinoid-induced activation of ERK MAPK activity (73, 74). This result however, is in direct opposition to our results in which we demonstrated an inhibition of ERK MAPK activation following cellular activation for 240 min. There are several possibilities for this discrepancy. First, the cells utilized in the studies conducted by Bouaboula, *et al.* were serum-starved cannabinoid receptor-transfected CHO cells. This resulted in cells that expressed approximately 152,000 CB2 receptors/cell (74); whereas determination of number of total receptors on spleen cells was approximately 1,000 receptors/cell (92).

Second, ERK MAPK activity was measured following treatment with CP-55940, a cannabinoid agonist, at various intervals up to 30 min in CHO cells transfected with either CB1 (73) or CB2 (74). ERK MAPK activity in cells transfected with either CB1 or CB2 peaked at 10 min, with indications of the activity decreasing with further agonist treatment. This suggests that there was a temporal effect of ERK MAPK activation with cannabinoid agonist activation; that is, ERK MAPK was activated early after ligand binding, whereas longer treatment resulted in decreased activity, which might be due to receptor desensitization (319). Indeed, ERK MAP kinase activation was inhibited at 240 min post cellular activation. Third, the SPLC were activated with PMA/Io, indicating that cannabinoids were capable of blocking the signal necessary for activation of ERK in stimulated lymphocytes. Collectively, these studies suggest that cannabinoid compounds inhibited c-fos and c-jun nuclear protein expression and subsequently, AP-1 and NF-AT transcription factor activity, in part via inhibition of ERK MAPK activation.

II. Effects of cannabinoid compounds on ERK MAPK activation in SPLC.

ERK MAPK activation was induced as early as 5 min post cellular activation with peak activation at 20 min. The activation of ERK MAPK was sustained for 240 min, the point at which the most robust inhibition of AP-1 binding activity, NF-AT binding activity, and c-fos and c-jun nuclear protein expression by cannabinoids was demonstrated. The early peak of activation followed by downregulation suggests there is a mechanism in T cell activation to suppress the strong ERK MAPK activity. In fact, there are phosphatases that are induced in response to similar signals that activate

MAPKs (269, 270). The possibility that cannabinoid compounds might be activating a phosphatase to inhibit kinase activity cannot be excluded at this time.

ERK MAPK activation was inhibited by CBN following a 240 min cellular activation with PMA/Io. This might contribute to the inhibition of c-fos and c-jun by cannabinoids as ERK MAPKs have been shown to participate in the post-translational modifications of both c-fos and c-jun. Therefore, the role of ERK MAPK was assessed in PMA/Io-stimulated SPLC using PD098059, the MEK inhibitor (308, 320). Treatment of PMA/Io-stimulated SPLC resulted in a more robust inhibition of PMA/Io-induced c-fos nuclear protein expression than that of c-jun, although PD098059-induced inhibition of either c-fos or c-jun was modest. This result was somewhat surprising for c-fos because ERK MAPKs induce c-fos transcription via elk-1 (182, 321). However, it was demonstrated that 50 μM PD098059 did not inhibit basal or okadaic acid-induced c-fos and c-jun protein expression in mouse keratinocytes, but that inhibition of ERK MAPK prevented transactivation of AP-1 reporter gene constructs (322). Thus, the effect of inhibition of ERK MAPK was the inability of c-fos and c-jun to transactivate AP-1-responsive genes. This suggests that inhibition of ERK MAPK by cannabinoids might inhibit transcription of AP-1-dependent genes, such as IL-2, via an ERK MAPK-dependent inhibition of AP-1 transactivation, without a drastic effect on the individual protein levels. An alternative explanation for these data is involvement of other signaling pathways for c-fos and c-jun nuclear protein expression. This is a likely possibility because elk-1, the critical transcription factor for c-fos, is induced via phosphorylation by JNK and p38 in addition to ERK MAPK (183, 185, 323, 324). The modest effect on c-jun might be due to the fact that c-jun is tightly regulated by JNK rather than ERK

MAPK (162). It is likely that the modest effects of PD098059 on c-fos and c-jun nuclear protein expression were due to a combination of the aforementioned mechanisms.

PD098059-induced inhibition of PMA/Io-stimulated IL-2 in SPLC and THMC demonstrated that ERK MAPKs were critical for IL-2 production. The demonstration of the critical role of ERK MAPKs in PMA/Io-stimulated IL-2 was important because there is conflicting evidence that ERK MAPKs regulate IL-2 production (276, 277). These studies also confirmed that pathways involved in T cell activation are similar in SPLC and THMC, despite the differences in cell population composition and homogeneity. Thus, in primary mouse lymphocytes, ERK MAPKs regulate IL-2 production and thus, inhibition of ERK MAPK activation could contribute to inhibition of IL-2 production in PMA/Io-stimulated lymphocytes. Indeed, it was demonstrated that CBN (in addition to effects on ERK MAPK at 240 min) inhibited activation of ERK MAPK at several different time points following cellular activation with PMA/Io. These results suggest that CBN might be preventing the phosphorylation of several targets of ERK MAPK in the first several hours following cellular activation. For instance, an important target of ERK MAPK is RNA polymerase II, which aids in the elongation phase of transcription (271, 272, 325). Thus, inhibition of activation of ERK MAPK by cannabinoids might prevent phosphorylation of RNA polymerase II, and subsequently inhibit transcription. In fact, inhibition of phosphorylation of RNA polymerase II inhibited the induction of c-fos (326). This effect could contribute to the cannabinoid-induced inhibition of IL-2. Another important target for phosphorylation by ERK MAPK is Rb, which, when phosphorylated, prevents association of Rb with E2F and allows the G1 to M transition in the cell cycle (273, 274). Therefore, inhibition of ERK MAPK by cannabinoids might

inhibit cell cycle progression via decreased phosphorylation of Rb, and this might explain cannabinoid-induced inhibition of proliferation in response to mitogens or PMA/Io. These studies demonstrate that cannabinoid-induced inhibition of ERK MAPK mediates, in part, the pleiotropic effects of these compounds. This observation is in contrast to others demonstrating activation of ERK MAPK by cannabinoids in a cannabinoid receptor-dependent manner (73, 74). However, the inhibition of ERK MAPK was consistent with previous reports of immune suppression by these compounds in PMA/Io-stimulated SPLC as characterized by inhibition of IL-2 production, inhibition of T cell proliferation, and inhibition of the T cell-dependent sheep erythrocyte antibody forming cell response (64, 81, 82). Moreover, the observation that cannabinoids inhibited activation of ERK MAPK suggests a cannabinoid receptor-independent mechanism, although the role of the cannabinoid receptors in cannabinoid-induced inhibition of ERK MAPK activation in PMA/Io-stimulated SPLC has not yet been determined.

III. The role of the cannabinoid receptors in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2.

Cannabinoid-mediated inhibition of PMA/Io-stimulated IL-2 production in mouse SPLC was not dependent on the presence of CB1 or CB2, as determined with the use of the specific CB1 and CB2 receptor antagonists. The rank order of cannabinoid-induced IL-2 inhibition for both potency and efficacy did not correlate with the published K_i values for receptor binding. The rank order potency for cannabinoid agonist-induced inhibition of IL-2 in PMA/Io-stimulated SPLC as assessed by IC_{50} values was: WIN-2 $\approx$ CBD > WIN-3 > CBN. The rank order for efficacy as assessed by the ability of the

agonist to produce 100% inhibition of PMA/Io-induced IL-2 at the maximum concentration tested was: WIN-2 > CBD > WIN-3 > CBN. In SPLC, the K_i for WIN-2 was lower than that for CBN (52). In HL-60 cells, rank order binding activity to the CB2 receptor has been reported to be: Δ^9-THC ≈ CBN >> CBD (46). In addition, there was a 1000 fold difference in affinity between the WIN stereoisomers in CB2-transfected COS cells (327). It has also been reported that CBN exhibited a 10-fold higher affinity for the CB2 receptor, the predominant cannabinoid receptor expressed in the immune system (51, 52). Indeed, CBN exhibits immunomodulating effects (64, 65, 82, 88), but, as demonstrated here, so did CBD and WIN-3, both of which exhibit low binding affinity for CB1 and CB2 (reviewed in 56).

In contrast to the data presented here, Buckley, *et al.* determined that Δ^9-THC–induced inhibition of IL-2 in α-CD3 plus peritoneal macrophage-stimulated T cells was lost in CB2 receptor knockout mice (112). However, the experimental design was very different from the studies described here. In the present studies, cannabinoid-induced inhibition of IL-2 from splenic T cells was directly measured. In the study by Buckley, *et al.*, activated peritoneal macrophages, derived from either wild-type or CB2 receptor knockout mice, were treated with VH or Δ^9-THC for 4 h. Subsequently, the macrophages were co-cultured with a helper T cell hybridoma cell line, which produced IL-2 in response to the macrophages plus α-CD3. In these studies, the macrophages were utilized as co-stimulators for optimal IL-2 production as they presumably express the B7 molecule required to bind the co-stimulatory T cell molecule, CD28 (reviewed in 328). However, the expression level of B7 on the macrophages was not measured in either wild-type or CB2 receptor knockout mice, nor in response to Δ^9-THC treatment.

Furthermore, the helper T cell hybridoma cell line that produced IL-2 in response to α-CD3 plus peritoneal macrophages was not assessed for cannabinoid receptor expression. These critical issues greatly influence the interpretation of the results. Nevertheless, the authors concluded that the loss of cannabinoid-induced inhibition of IL-2 was due to the loss of CB2 in the stimulator macrophages, and not through direct effects on the helper T cell hybridoma cell line (112). Concordant with the aforementioned studies, similar studies conducted by McCoy, *et al.* demonstrated that the CB2 antagonist was involved in Δ^9-THC-induced inhibition of antigen processing and presentation (113). Again, although IL-2 production from T cells was used as an endpoint, the effect by Δ^9-THC was exerted on the macrophages used to stimulate the T cells (not the T cells themselves) (111, 113). Thus, the CB2 receptor seems to be important for antigen processing and/or presentation in macrophages (112, 113) but neither CB1 nor CB2 were critical for cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production from splenic T cells.

Similar to the effects of the antagonists on cannabinoid-induced inhibition of PMA/Io-stimulated IL-2, the antagonists in combination were not able to attenuate CBN-induced inhibition of FSK-stimulated cAMP production, suggesting that the cannabinoid mechanism was not dependent on a PTX-sensitive G protein-coupled receptor. These results were unexpected since inhibition of cAMP production has traditionally been the hallmark response for cannabinoid receptor activity. This conclusion was further substantiated by the inability of PTX to attenuate CBN-induced inhibition of FSK-stimulated cAMP production. In fact, the determination of PTX on CBN-induced inhibition of FSK-stimulated cAMP production was primarily performed in order to

assess the activity of PTX because it did not attenuate CBN-induced inhibition of IL-2. The current interpretation of the cAMP data was in contrast to the conclusion reached by Kaminski, *et al.* who demonstrated that PTX treatment could reverse the Δ^9-THC-induced inhibition of cAMP production in SPLC (93). The difference in interpretations between these two studies could be due to the fact that the enhancing effect of PTX on cAMP was not accounted for in the previous study. Conversely, the inability of PTX to attenuate CBN-mediated inhibition of either cAMP or IL-2 inhibition suggests that the cannabinoid receptors might couple to other G proteins. In fact, the ability of cannabinoid receptors to couple to $G\alpha_s$ has been suggested, particularly for CB1 (61-63).

With the demonstration that cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production was not attenuated by either cannabinoid receptor antagonist, it was possible that the mechanism of IL-2 inhibition involved a non-receptor mediated mechanism. Several of the transcription factors that control IL-2 transcription are regulated by oxidation/reduction status, including AP-1 and NF-κB (reviewed in 226). In fact, it was demonstrated that oxidants, such as H_2O_2, induced T cell activation and IL-2 production (204). Furthermore, it was demonstrated that anti-oxidants inhibit induction of c-fos and c-jun and AP-1 binding activity (200, 201). Interestingly, cannabinoids were shown to be anti-oxidants (315). Thus, it was possible that cannabinoids were inhibiting IL-2 as a result of their anti-oxidant properties. However, cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production was not reversed by treatment with H_2O_2. This suggests that the reported anti-oxidant properties of these compounds did not contribute to inhibition of PMA/Io-stimulated IL-2. Alternatively, perhaps the transient nature of H_2O_2 was not able to reverse the sustained inhibition of IL-2 that was demonstrated by

cannabinoid compounds at 24 h post-cellular stimulation. Finally, SPLC require the presence of 2-ME, a reducing agent, in the culture media; thus, the presence of 2-ME could have counteracted the presence of H_2O_2 in these studies. None of these possibilities can be excluded at this time.

It was interesting to note that despite the fact that both WIN isomers inhibited IL-2, there were consistent differences in potency. Although the IC_{50} values of WIN-2 and WIN-3 were not significantly different, there were significant differences in the effects at concentrations above 10 μM. Therefore, comparing merely IC_{50} values for these compounds might not best reflect the differences in potency between the stereoisomers, especially in light of the extremely steep concentration response curve produced by the WIN compounds. This difference in efficacy suggests that the two isomers differentially affected a specific cellular target. Thus, while the experiments in which the antagonists, PTX and CBD were employed collectively suggest that cannabinoid receptors were not involved in inhibition of PMA/Io-induced IL-2 production, the stereoselectivity with the WIN isomers suggests a specific cellular target of these compounds for IL-2 inhibition. The WIN-2-induced inhibition of cellular aggregation suggests there might be an extracellular target as well. Potential extracellular targets of cannabinoid inhibition include leukocyte integrins, adhesion molecules that direct the interaction between T cells and other cells, such as APCs or endothelial cells (reviewed in 329). The inhibition of cellular aggregation might also contribute to the inhibition of IL-2 because a correlation exists between the ability of the lymphocytes to aggregate and their ability to secrete IL-2 (330).

IV. Effect of CBN on intracellular calcium concentration.

The observation that cannabinoids elevated intracellular calcium concentration in a cannabinoid receptor-dependent manner is consistent with several other studies (50, 55, 71, 72, 331, 332). Although a mechanism for the elevation in intracellular calcium by cannabinoids has not been definitively established, it is tempting to speculate that cannabinoids modulate the calcium channel directly. Alternatively, perhaps cannabinoid receptors couple to $G\alpha_s$ in lymphocytes to mediate the cannabinoid-induced elevation in intracellular calcium. Again, it has been suggested that CB1 might couple to $G\alpha_s$ (61-63).

While the cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 was not attenuated by either the CB1 or CB2 antagonists, the cannabinoid receptor antagonists attenuated (both alone and in combination) the CBN-induced elevation in intracellular calcium in resting SPLC. This differential role of the cannabinoid receptors in these two cannabinoid effects suggests that the elevation in intracellular calcium concentration did not contribute to the cannabinoid-induced inhibition of PMA/Io-stimulated IL-2. However, both cannabinoids and agents that increase intracellular calcium (such as Io, A23187 and TG) inhibited PMA/Io-stimulated IL-2 production in SPLC. In concordance with these observations, T cells become unresponsive (or anergic) if they receive an inappropriate or incomplete activation signal (231). Anergy is a state of unresponsiveness in T cells as characterized by decreased production of IL-2, and AP-1 and NF-κB DNA binding activity (240-243). In fact, it has been demonstrated that T cells become anergic, as characterized by > 90% block in the ability to produce IL-2, if they were prematurely stimulated with the calcium ionophore A23187 (300). In addition,

PMA/Io-induced IL-2 reporter gene activity was inhibited in response to an 8 h, 2 μM Io pre-treatment (296). This suggests that a premature elevation in intracellular calcium might induce anergy depending on the time of generation of the calcium signal and/or the total concentration of intracellular calcium. It is not yet known whether the time of generation or magnitude of the calcium signal (or both) contributes to an unresponsive state. Nevertheless, this might explain how the intracellular calcium elevation by cannabinoids contributes to the inhibition of IL-2 production in PMA/Io-stimulated SPLC.

The differential role of the cannabinoid receptors in cannabinoid-induced calcium elevation and cannabinoid-induced IL-2 inhibition in PMA/Io-stimulated SPLC might be due to the requirement for cellular activation in the studies in which IL-2 production was measured. Perhaps the discrepancy was due to the observation that cellular activation modulates cannabinoid receptor expression levels in T cells and B cells (97, 98, 333). The ability of cellular stimulation to positively or negatively modulate cannabinoid receptor expression depends on cell type, cell maturity, and activation stimulus. Specifically, in T cells, CB1 receptor mRNA was decreased in response to PMA/Io or α-CD3, indicating that T cell activation inhibits expression of cannabinoid receptors (98). Thus, assuming a minimal role of cannabinoid receptors in cannabinoid-induced inhibition of PMA/Io-stimulated IL-2, the part of the mechanism attributed to the receptors might not be detected in stimulated cell systems. Another possibility for the discrepancy is that these two responses occurred in distinct cell populations in the SPLC cell preparation. IL-2 production is limited to T cells whereas intracellular calcium elevation likely occurred in all three cell types (T cells, B cells and macrophages),

suggesting that cannabinoid receptor-dependent increases in intracellular calcium by CBN was due to the higher expression level of cannabinoid receptors on B cells and macrophages (51). However, this latter scenario seemed unlikely based on the demonstration that CBN induced intracellular calcium elevation in a CB2-dependent manner in THMC, a predominantly T cell preparation.

V. Effect of CBD on intracellular calcium concentration.

CBD also elevated intracellular calcium, although the time to onset, shape of the response, and magnitude of intracellular calcium elevation was different from that of CBN. The initial rise in intracellular calcium in response to CBD was similar to Io. In fact, CBD provided the necessary calcium signal for IL-2 production in PMA-stimulated SPLC whereas CBN could not. It is tempting to speculate that the rapid onset of intracellular calcium elevation in response to Io or CBD was the result of an initial release of intracellular stores, and that this initial release was required for IL-2 production. This was supported by the observations with CBN showing that the increase in intracellular calcium was not only gradual, but that the first phase of the response was primarily due to influx of extracellular calcium. In concordance with the above speculation, CBN did not induce IL-2 in PMA-stimulated SPLC.

Interestingly, although both CBD and Io elevated intracellular calcium in a similar fashion, the difference in magnitude of IL-2 produced in response to these two agents in the presence of PMA was striking. Moreover, CBD either stimulated or inhibited IL-2 production, relative to the PMA or PMA/Io control, respectively. These observations support the notion that cannabinoids act through multiple, parallel mechanisms. The

differences in the magnitude of IL-2 production might be due to CBD-mediated anergy induction. Perhaps the low level of IL-2 produced in these cells was the result of receiving the premature calcium signal via CBD followed by PMA, consistent with the aforementioned study in which Jurkat cells became anergic in response to calcium ionophore pre-treatment followed by PMA stimulation (300). Alternatively, the overall increase in intracellular calcium might dictate the magnitude of cellular activation as assessed by IL-2 production. This was supported by the demonstration that high concentrations of CBD robustly inhibited PMA/Io-stimulated IL-2 production; whereas high concentrations of CBD enhanced PMA-stimulated IL-2 production, albeit modestly. Under the aforementioned conditions, intracellular calcium concentrations in CBD plus PMA/Io-treated cells would be higher than in CBD plus PMA-treated cells. These studies might also explain some of the differential effects of cannabinoids on IL-2 production in response to various stimuli (85, 86). Again, it has not definitively established whether the time of generation of the calcium signal, magnitude of the calcium signal, or both, contributes to the unresponsive state.

Notably, the cannabinoid receptor antagonists (either alone or in combination) attenuated the CBD/PMA-induced enhancement of IL-2. This was an unexpected result because CBD-induced inhibition of PMA/Io-stimulated IL-2 was not prevented by the same concentration of antagonists in combination. Collectively, these results suggest the mechanism by which CBD inhibited PMA/Io-stimulated IL-2, and the mechanism by which CBD enhanced PMA-stimulated IL-2, are distinct. This result could also be explained by the theory mentioned above; that is, in response to PMA/Io activation, the expression of cannabinoid receptors was down-regulated (98). This explanation is only

plausible under the assumption that the cannabinoid effects are not solely mediated via the cannabinoid receptors. It is not known whether the PMA/CBD combination regulates cannabinoid receptor expression. Furthermore, it has not been definitively established whether the CBD-induced elevation in intracellular calcium was mediated via the cannabinoid receptors.

Conversely, perhaps cellular stimulation with PMA/Io up-regulates a receptor, distinct from CB1 or CB2, which mediates the cannabinoid-induced inhibition. One possibility is the vitamin D receptor, which, in response T cell activation, is induced within 24 h (334, 335). Interestingly, treatment of T cells with vitamin D inhibits proliferation in a serum concentration-dependent manner (336). Moreover, vitamin D inhibited PHA-induced IL-2 production (337). The mechanism by which vitamin D inhibited IL-2 production was determined to be mediated by a protein-protein interaction between NF-AT and the vitamin D receptor and in fact, the presence of vitamin D was not required (338). Thus, if cannabinoid treatment induced expression of the vitamin D receptor, this could contribute to the inhibition of IL-2 production. Similarly, induction of the glucocorticoid receptor by cannabinoids would also contribute to inhibition of IL-2. Part of the mechanism by which glucocorticoids inhibit IL-2 is sequestration of AP-1 proteins by the glucocorticoid receptor (339, 340). Interestingly, cannabinoids have been determined to bind to the hippocampal glucocorticoid receptor (341, 342). Collectively, these studies suggest that the mechanism of cannabinoid-induced immune modulation is both cannabinoid receptor-dependent and –independent.

VI. Summary

The studies performed in this dissertation addressed the following hypothesis: Immune modulation by cannabinoid compounds is mediated via cannabinoid receptors (CB1 and/or CB2), resulting in disruption of AP-1 transcription factor binding in the promoter region of immune system genes, such as IL-2. Cannabinoid compounds are immunosuppressive as demonstrated by the ability of cannabinoid compounds such as CBN, CBD and WIN-55212, to inhibit PMA/Io-stimulated IL-2 production. Interestingly, cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 production was not attenuated in the presence of the cannabinoid receptor antagonists, SR144528 and SR141716A. Furthermore, the inhibition of PMA/Io-stimulated IL-2 production was due, in part, to inhibition of transcription factor binding at the proximal AP-1 and distal NF-AT sites in the IL-2 promoter. Cannabinoid compounds inhibited nuclear protein expression of both c-fos and c-jun, which was neither due to inhibition of steady state mRNA levels nor to cytosolic retention of the proteins. This suggested that the inhibition of c-fos and c-jun protein expression was due to perturbation of post-translational modifications of the proteins, and in fact, cannabinoids inhibited the activation of ERK MAP kinases. With the demonstration that cannabinoid compounds inhibited PMA/Io-stimulated IL-2 in a cannabinoid receptor-independent manner, other potential targets in T cells were examined. Intracellular calcium modulation seemed a plausible target of inhibition in light of its critical role in T cell activation and NF-AT regulation. In fact, these studies show that cannabinoid compounds alter calcium regulation by elevating intracellular calcium levels. Furthermore, the cannabinoid-induced elevation in intracellular calcium was attenuated in the presence of the cannabinoid receptor

antagonists, either alone or in combination. Interestingly, several different pharmacological agents that increased intracellular calcium also inhibited IL-2 production in PMA/Io-stimulated SPLC. These findings suggest that the mechanism of cannabinoid-induced inhibition of PMA/Io-stimulated IL-2 likely involved several mechanisms including an elevation in intracellular calcium concentration, which was cannabinoid receptor-dependent.

The significance of these studies is that they support the notion that cannabinoids, traditionally thought to mediate their effects via cAMP, modulate several intracellular targets and possibly, extracellular targets as well. There is increasing evidence to support a minimal role for cAMP in cannabinoid-induced immunosuppression (343), including the fact that the cannabinoid receptors did not mediate all the effects of these compounds. Specifically, targets of cannabinoid compounds in PMA/Io-stimulated SPLC include inhibition of activation of ERK MAPK. This novel observation helps to explain the pleiotropic nature of these compounds. In addition, cannabinoids induced intracellular calcium in lymphocytes and this might contribute to the inhibition of IL-2 production in PMA/Io-stimulated SPLC. These studies suggest that the immune suppression induced by cannabinoid compounds is complex, multi-faceted, and most likely involves cannabinoid receptor-dependent and –independent mechanisms (Figure 55).

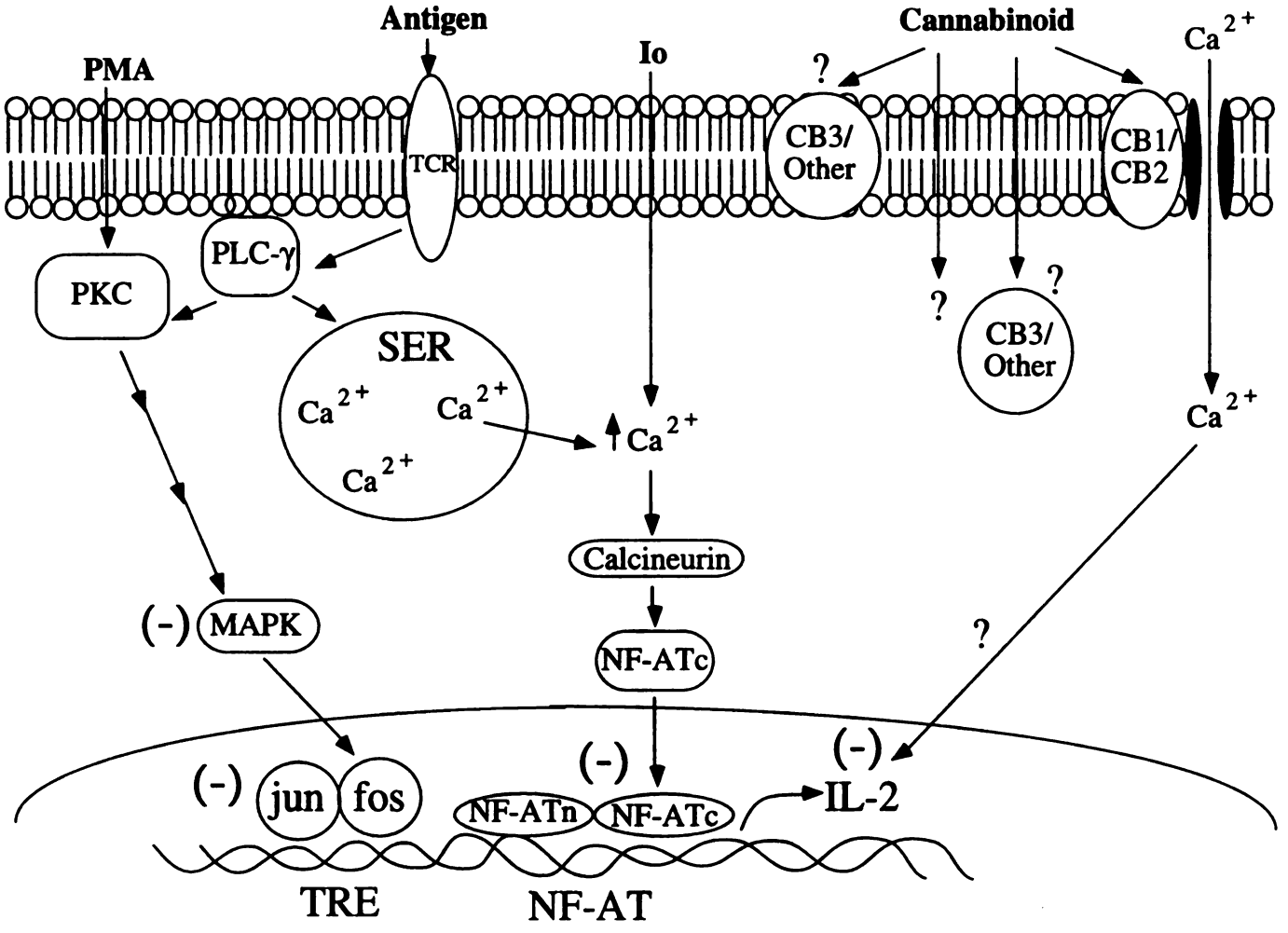


Figure 55. Schematic representation of cannabinoid effects in T lymphocytes. (-) represents those cellular targets in PMA/Io-stimulated SPLC that are inhibited by cannabinoid compounds. The mechanism of immune modulation by cannabinoids likely involves both cannabinoid receptor-dependent and –independent mechanisms.

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