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INHIBITION OF CREB, NF-κB, AND INTERLEUKIN-2: A MECHANISM OF IMMUNE SUPPRESSION BY CANNABINOL

By

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ABSTRACT

INHIBITION OF CREB, NF-κB, AND INTERLEUKIN-2: A MECHANISM OF IMMUNE SUPPRESSION BY CANNABINOL

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Immune suppression by cannabinoid compounds has been widely demonstrated in a variety of experimental models. The identification of CB1 and CB2 cannabinoid receptors on leukocytes has provided a putative mechanism of action for immune modulation by cannabinoids. CB1 and CB2 are G-protein coupled receptors, and ligand binding to either receptor negatively regulates adenylate cyclase. Cannabinol (CBN), a cannabinoid with minimal CNS activity, is one ligand that exhibits higher binding affinity for the CB2 receptor. In light of the CB2 selectivity of CBN, the objectives of this research project were (1) to examine the effects of CBN on immunocompetence, cAMP-mediated signal transduction, and IL-2 expression; (2) to identify specific CREB/ATF and NF-κB/c-Rel proteins modulated by CBN in activated thymocytes; (3) to characterize the inhibition of CREB and NF-κB transcription factor activation by CBN, and (4) to determine the role of cAMP and PKA in the CBN-mediated inhibition of IL-2, CREB, and NF-κB following T-cell activation.

Analysis of *in vitro* immune function endpoints demonstrated that CBN possessed immunomodulatory activity that was comparable to that of Δ^9-THC. An evaluation of the cAMP signaling cascade in the presence of CBN showed a concentration-dependent inhibition of adenylate cyclase and PKA activity. These alterations in upstream signaling correlated with the inhibition of transcription factor binding to CRE and κB motifs in splenocytes and thymocytes.

An inhibition of IL-2 expression at both the mRNA and protein level was demonstrated by CBN. The identification of the specific CREB/ATF and NF-κB/c-Rel dimers modulated by CBN following T-cell activation was accomplished using both supershift and EMSA-Western techniques. A CREB-1 homodimer was identified as the major CRE binding complex affected by CBN treatment whereas the inducible κB binding complex was shown to be a p65/c-Rel heterodimer. In addition, CBN inhibited the phosphorylation of CREB-1 and prevented the degradation of IκB-α suggesting that CBN inhibited CREB and NF-κB activation through an inhibition of phosphorylation.

The role of cAMP and PKA in the inhibition of IL-2, CREB, and NF-κB by CBN was addressed by using the membrane permeable cAMP analog dibutyryl cAMP or the specific PKA inhibitor H89. Co-stimulation of thymocytes with dibutyryl cAMP and PMA/Io could not reverse the inhibition of CREB phosphorylation, CRE binding, or κB binding produced by CBN. Furthermore, dibutyryl cAMP failed to reverse the inhibition of IL-2 protein secretion by CBN. Pretreatment of thymocytes with H89 resulted in minimal inhibition of CRE binding activity and CREB phosphorylation whereas κB binding and IκB-α degradation were unaffected by H89. In addition, H89 produced only a modest inhibition of thymocyte IL-2 protein. These results demonstrated a modest involvement of the cAMP/PKA pathway in the CBN-mediated inhibition of CREB, NF-κB, and IL-2 in activated thymocytes.

Together this series of studies established that CBN exhibits immunosuppressive activity thereby implicating the involvement of the CB2 receptor in immune modulation by cannabinoid compounds. This work is also the first to investigate the molecular mechanisms of cannabinoid-mediated immune suppression of T-cells. Furthermore, these findings suggest that signaling pathways other than the cAMP cascade significantly contribute to the modulation of CREB, NF-κB and IL-2 by cannabinol in mouse thymocytes.

I would like to dedicate this dissertation to my mom and dad. They have always encouraged me to follow my dreams and attain my goals. Earning my doctorate degree has been the biggest challenge of my life, and I couldn't have done it without the support of my parents. Their love and encouragement means more to me than they will ever know.

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ABBREVIATIONS

$[^3H]$	tritium
°C	degrees celcius
Δ	delta
Δ^9-THC	delta-9-tetrahydrocannabinol
AFC	antibody forming cell
AIDS	acquired immunodeficiency syndrome
AP-1	activator protein-1
ATF	activating transcription factor
ATP	adenosine triphosphate
βARK	beta-adrenergic receptor kinase
B-cell	B lymphocyte
BCS	bovine calf serum
BSA	bovine serum albumin
Ca^{+2}	calcium
CaM	calmodulin
CaMKIV	calcium/calmodulin-dependent protein kinase type four
cAMP	cyclic adenosine 3':5'-monophosphate
CAT	chloramphenicol aminotransferase
CB	cannabinoid receptor
CBD	cannabidiol
CBN	cannabinol
CBP	CREB binding protein
CD	cluster designation
CD28RE	CD28 response element

CHO	chinese hamster ovary
CNS	central nervous system
CO_2	carbon dioxide
Con A	concanavalin A
cpm	counts per minute
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
DAG	diacylglycerol
DBcAMP	dibutyryl cAMP
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBSS	Earle's balanced salt solution
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FBS	fetal bovine serum
FSK	forskolin
g	gravity
GDP	guanosine diphosphate
Gi	inhibitory G-protein
G protein	guanine-nucleotide-binding protein
Gs	stimulatory G-protein
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HPB-ALL	human peripheral blood-acute leukemia lymphocyte

hr	hour(s)
HSV	herpes simplex virus
ICER	inducible cAMP early repressor
IFN-γ	interferon-gamma
IgM	immunoglobulin M
IκB-α	inhibitor of nuclear factor-κB
IKK	I-kappa B kinase
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
iNOS	inducible nitric oxide synthase
Io	ionomycin
IP3	inositol 1,4,5-triphosphate
IS	internal standard
JAK	janus kinase
JNK	c-jun N-terminal kinase
κB	kappa B
kD	kilodalton
KID	kinase inducible domain
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
μg	microgram
MHC	major histocompatibility complex
min	minutes
μl	microliter
μM	micromolar
mM	millimolar

mRNA	messenger ribonucleic acid
N_2	nitrogen
NA	naive
NEMO	NF-kappa B essential modulator
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor for immunoglobulin κ chain in B cells
ng	nanogram
NIK	NF-kappa B inducible kinase
NK	natural killer
NLS	nuclear localization signal
nM	nanomolar
nm	nanometer
NO	nitric oxide
O_2	oxygen
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating-cell nuclear antigen
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PI 3-K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKI	protein kinase A inhibitor
PLA_2	phospholipase A_2
PLC	phospholipase C

PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonyl fluoride
RHD	Rel homology domain
RIA	radioimmunoassay
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
SAPK	stress-activated protein kinase
SAR	structure activity relationship
SDS	sodium dodecyl sulfate
SE	standard error
sec	second(s)
SPLC	splenocyte
sRBC	sheep red blood cells
STAT	signal transducers and activators of transcription
TBE	Tris-boric acid-EDTA
TBS	tris buffered saline
T_c	cytotoxic T lymphocyte
T-cell	T lymphocyte
TCR	T cell receptor
TFII	transcription factor type II
T_h	helper T lymphocyte
THC	tetrahydrocannabinol
THMC	thymocyte
TNFα	tumor necrosis factor alpha
TRE	TPA responsive element
VH	vehicle

x	times

INTRODUCTION

I. Cannabinoid Background

A. Structure and biological effects of cannabinoids

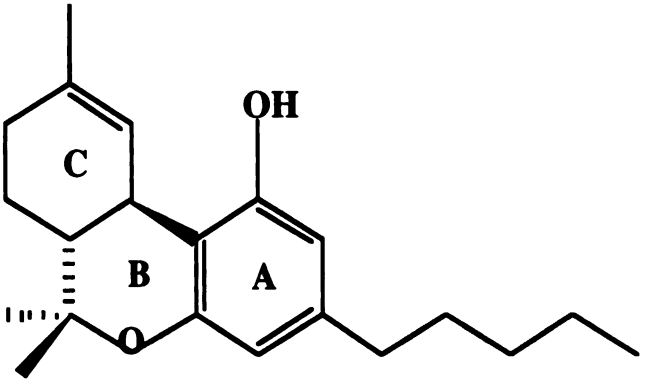
Cannabinoid compounds are derived from the *Cannabis sativa* plant which is more commonly known as marijuana. More than 60 different cannabinoids have been isolated from cannabis including Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), and cannabidiol (CBD). Of these constituents, Δ^9-THC is the primary psychoactive component of marijuana (Mechoulam, 1970) and is therefore the most extensively studied cannabinoid. By comparison, CBN exhibits only minimal psychotomimetic activity, and CBD is an inactive cannabinoid congener.

Δ^9-THC produces diverse biological effects in animals and humans. The predominant effect of cannabinoids is an alteration of central nervous system (CNS) function. Specifically, cannabinoid intoxication is marked by hypothermia, catalepsy, antinociception, and changes in spontaneous activity. Δ^9-THC also produces changes in mood, decreases psychomotor skills, and modifies cognition and memory (Dewey, 1986; Pertwee, 1988). In general, Δ^9-THC creates a state of CNS depression that is initially accompanied by a state of hyperexcitability. For example, mice exposed to low doses of Δ^9-THC become sedated and hypersensitive to external auditory or tactile stimuli (Dewey, 1986). Behavioral changes have also been linked to cannabinoid exposure. Acute administration of Δ^9-THC has demonstrated a decrease in stimulus-controlled behavior in several animal models (Black *et al.*, 1970; Carlini, 1968; Ferraro and Grilly, 1972). Similarly, an amotivational syndrome characterized by general apathy is often associated with chronic marijuana use. In some cases, high doses of Δ^9-THC have resulted in acute paranoia, panic reactions, and delusions in humans (Hollister, 1986).

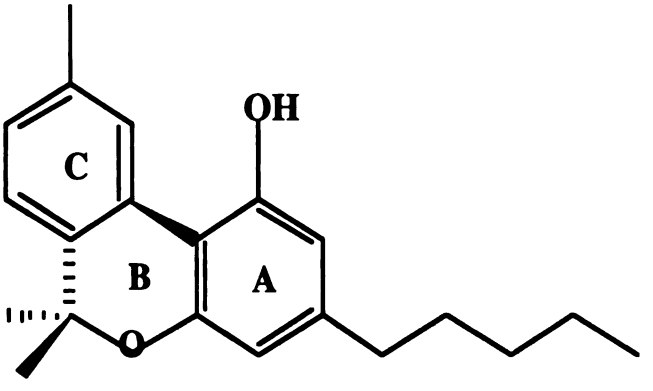
Cannabinoids also generate effects in a variety of peripheral organ systems. A number of endocrine changes have been described following Δ^9-THC exposure. These

include reduced secretion of prolactin, follicle-stimulating hormone and leutinizing hormone as well as the stimulation of ACTH release (Block *et al.*, 1991; Marks, 1973; Smith *et al.*, 1979). Furthermore, a single injection of Δ^9-THC can decrease serum levels of thyrotropin, triiodothyroxine, and thyroxine (Hillard *et al.*, 1984). Several studies have also reported alterations of the male reproductive tract by cannabinoids. For instance, Δ^9-THC causes a reduction in spermatogenesis, a regression of Leydig cells, and an alteration of sperm morphology (Dixit *et al.*, 1974; Husain and Lamb, 1984). Immunosuppression is another well-characterized effect of cannabinoid compounds, and a general profile of Δ^9-THC activity within the immune system will be provided in a subsequent section. In addition, cannabinoids have potential therapeutic applications such as bronchodilation, reducing intraocular pressure, stimulating appetite, and relieving the nausea associated with chemotherapy (Hollister, 1986). Cannabinoid compounds are unique from other drugs of abuse in that they produce no physical dependence, lack respiratory-depressant activity, and possess relatively low toxicity.

Extensive structure activity relationship (SAR) studies have determined at least four structural requirements for cannabimimetic activity (Razdan, 1986). A benzopyran ring provides the backbone for cannabinoid compounds (Figure 1). The benzopyran structure is necessary for activity as demonstrated by the inactive cannabidiol, an open-ring compound. However, the benzopyran is not solely responsible for activity. The presence of a phenolic hydroxyl group at the C-1 position is also a definite structural requirement. Elimination or substitution of this hydroxyl group results in a significant loss of potency (Johnson *et al.*, 1981; Loev *et al.*, 1973). In addition, the length of the aliphatic side chain determines the potency of cannabinoid compounds. A three carbon side chain appears to be the minimal requirement while nine carbons or more leads to a reduction in activity. Furthermore, branching of the aromatic side chain



Δ^9-THC



Cannabinol

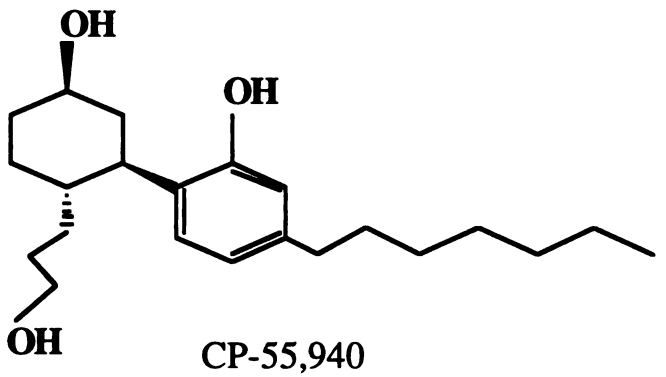
Figure 1. Structures of Δ^9-THC and Cannabinol. Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabinol (CBN) are natural cannabinoid compounds derived from *Cannabis sativa*. The structural backbone of classical cannabinoids is a benzopyran ring. Additional features necessary for activity include the phenolic hydroxyl group, the aliphatic side chain, and the presence of an alicyclic ring (C ring). Δ^9-THC and CBN are very similar in structure with the exception of the number of bonds in the C ring.

can enhance potency. Lastly, the attachment of an alicyclic ring (i. e., ring C) to the benzopyran backbone at the 3,4-position is critical for cannabimimetic activity.

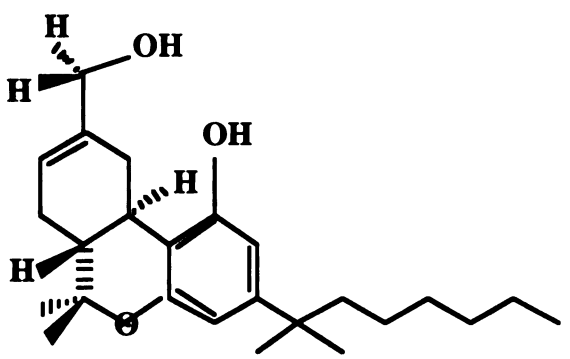
Several non-classical cannabinoid analogs have been synthesized which are more potent than Δ^9-THC and demonstrate high affinity binding to cannabinoid receptors (Figure 2). The CP-55,940 synthetic analogs produced by Pfizer are bicyclic and possess the essential structural requirements for activity. The dimethylheptyl HU-210/HU-211 analogs are structurally very similar to Δ^9-THC and exhibit a branched aliphatic side chain. Recently, Sterling Winthrop has developed aminoalkylindole compounds such as WIN-55,212. Although these analogs are structurally different from the classical cannabinoids, they exhibit pharmacological properties characteristic of the natural compounds (Compton *et al.*, 1992). Regardless of structural differences, the natural and synthetic cannabinoid compounds display enantiomer specificity with only the negative (-) isomer showing pharmacological activity (Little *et al.*, 1988). Interestingly, plant-derived Δ^9-THC and CBN differ only in the number of double bonds within the C ring (Figure 1). Although cannabinol is very similar in structure to Δ^9-THC, it lacks activity in the central nervous system. As a result, CBN has been historically considered a minimally active cannabinoid based on CNS endpoints.

B. Medical use of marijuana

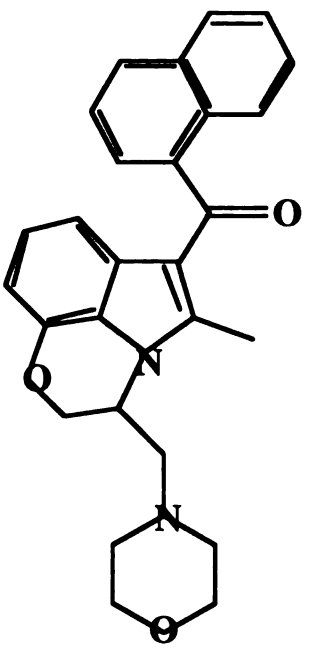
Marijuana has long been used as an herbal remedy for a variety of conditions such as convulsions, muscle tension, pain, and bronchoconstriction. Recently, California, Arizona, Alaska, Nevada, Oregon, and Washington have legalized the use of marijuana for medicinal purposes. The proposed medical uses include appetite stimulation, anti-emetic for chemotherapy, glaucoma, and multiple sclerosis. Dronabinol is an oral form of Δ^9-THC available by prescription for use as an appetite stimulant and an antinauseant.



CP-55,940



HU-210/HU-211



WIN-55,212

Figure 2. Structures of the synthetic cannabinoid analogs. CP-55,940 is a bicyclic cannabinoid analog with a 7 carbon aromatic side chain and an open benzopyran ring. These structural modifications increase the potency and affinity of CP-55,940 for cannabinoid receptors. HU-210/211 is structurally similar to Δ^9-THC and possesses a dimethyl-heptyl aromatic side chain which increases the potency of this cannabinoid analog. The aminoalkylindole, WIN-55,212, produces cannabinoid-like effects and binds to cannabinoid receptors.

The potential therapeutic use of cannabinoids has inspired interest in the development of new compounds with minimal side effects.

A comprehensive evaluation of the clinical research on medical uses of marijuana was recently written by Voth and Schwartz (1997). In this report, the authors assessed the available data concerning the use of crude marijuana and/or pure Δ^9-THC for multiple sclerosis, glaucoma, appetite stimulation, and cancer chemotherapy. Overall, the literature supported the therapeutic use of marijuana for stimulating appetite and for relieving the nausea of chemotherapy; however, insufficient data for multiple sclerosis and glaucoma were reported. Considerable clinical evidence exists for the use of marijuana as an antinauseant. In fact, Δ^9-THC was found to be most useful for patients that were refractory to other anti-emetic therapy. However, THC therapy has more significant side effects and toxicity than ondansetron and granisetron, the more common anti-nausea drugs. Appetite stimulation was also proven to be a useful medical application of marijuana. Δ^9-THC promotes the maintenance of weight and increases the appetite of AIDS patients. Low doses of Δ^9-THC can also stimulate the appetite of cancer patients.

Unfortunately, numerous side effects hinder the medical use of marijuana. The psychoactive effects of Δ^9-THC cause many patients to stop therapy. Predominant complaints include loss of concentration and memory, alterations in coordination, and distortion of reality. Several respiratory complications have also been associated with smoking marijuana as marijuana contains more tar and carcinogens than tobacco. Additionally, the long-term side effects of Δ^9-THC therapy remain relatively unknown. Therefore, the development of synthetic compounds which are therapeutic and lack undesirable side effects is of great interest.

C. General profile of immune modulation by Δ^9-THC

Immune suppression is a well characterized effect of cannabinoids as demonstrated by their ability to inhibit cell-mediated, humoral, and innate immune responses. As will be described below, these compounds have been shown to suppress B and T-cell function, macrophage and natural killer cell activity, as well as host resistance using various immunological models (Kaminski, 1994; Klein *et al.*, 1998; Munson and Fehr, 1983).

T-cells play a critical role in cell-mediated immunity and are responsible for antitumoricidal and antiviral activities. A number of studies have reported cannabinoid-mediated inhibition of T-cell function as assessed by a variety of functional endpoints. Clonal expansion of T-cells is central to cell-mediated immune responses, and *in vitro* administration of T-cell specific mitogens such as anti-CD3, PHA, and Con A can induce this proliferative response. Several investigators have demonstrated that Δ^9-THC can inhibit mitogen-induced T-cell proliferation of mouse spleen cells (Pross *et al.*, 1987; Pross *et al.*, 1990; Schatz *et al.*, 1993). A closer investigation of these findings determined that inhibition of proliferation by Δ^9-THC occurred only when the drug was present during clonal expansion (Specter *et al.*, 1990). Interestingly, a differential sensitivity of the T-cell subpopulations to Δ^9-THC has been observed *in vitro* with a reduction in $CD8^+$ but not $CD4^+$ cell numbers following mitogen stimulation (Pross *et al.*, 1990). A similar inhibition of clonal expansion by Δ^9-THC has been reported in PHA-stimulated human peripheral blood lymphocytes (Specter *et al.*, 1990). It has also been established that cannabinoids alter T-cell function at the level of cytokine secretion and responsiveness. Splenocytes stimulated with either PHA, LPS, or Con A produced significantly less interferon in the presence of Δ^9-THC than the untreated controls (Blanchard *et al.*, 1986). Additionally, Δ^9-THC has been shown to decrease the responsiveness of T-cells to IL-2 (Kawakami *et al.*, 1988). Decreases in NK cell cytolytic activity and the development of lymphokine-activated killer cells has also been

attributed to decreased IL-2 responsiveness (Kawakami *et al.*, 1988). Furthermore, we have recently reported that IL-2 transcription is altered by cannabinoid compounds as detected by significant decreases in IL-2 mRNA and protein (Condie *et al.*, 1996). Together these findings suggest that Δ^9-THC interferes with induction of IL-2 as well as promotion of several IL-2 dependent processes.

B-lymphocytes participate in acquired immune responses and are the primary effector cells of humoral immunity. Recognition and binding of specific antigen by surface immunoglobulin stimulates B-cells to proliferate and differentiate into antibody secreting plasma cells. Δ^9-THC and the synthetic cannabinoids CP-55,940 and WIN55,212 produce a modest stimulatory effect on B-cell proliferation at nanomolar concentrations (Derocq *et al.*, 1995). In contrast, Klein and coworkers have reported a dose-dependent inhibition of LPS-stimulated splenocyte proliferation by Δ^9-THC (Klein *et al.*, 1985). Both of these studies described THC-mediated alterations of lymphoproliferation; however, direct changes in B-cell effector function have not been definitively established following Δ^9-THC exposure. Rather, the effects of Δ^9-THC on humoral immune responses are characterized as indirect and result from alterations in accessory cell function (i.e., T helper cells). The IgM antibody forming cell (AFC) response is routinely employed to measure humoral immunity. Sheep red blood cells (sRBC) are commonly used as the T-dependent antigen, and the generation of this antibody reponse requires functional accessory T cells and antigen-presenting cells. Δ^9-THC has been shown to inhibit the AFC humoral response to T-cell dependent antigens both *in vivo* and *in vitro* (Schatz *et al.*, 1993; Smith *et al.*, 1978; Watson *et al.*, 1983). The IgM AFC response can also be initiated to the T-cell independent antigens DNP-Ficoll and LPS; however, Δ^9-THC does not inhibit these responses *in vivo* or *in vitro* (Schatz *et al.*, 1993). These findings suggest that the inhibition of humoral immune responses by Δ^9-THC occurs primarily through the inhibition of T-lymphocyte accessory cell function. Interestingly, the timing of antigen sensitization in relationship to

cannabinoid exposure appears to be critical for Δ^9-THC-induced inhibition of humoral responses. Studies investigating the relationship between antigen sensitization and Δ^9-THC exposure have determined that Δ^9-THC administration surrounding the time of sensitization with sRBC produces the greatest magnitude of inhibition (Schatz *et al.*, 1992; Schatz *et al.*, 1993). These findings were interpreted as suggesting that suppression of immune function by cannabinoid compounds is likely mediated through the alteration of an early T-cell activation event.

Macrophages are an integral part of the immune system and participate in both innate and acquired immune responses. These cells play an important role in innate immunity through phagocytosis of foreign antigen and production of tumor necrosis factor α. Additionally, macrophages possess intrinsic cytolytic activity which is partially mediated through the release of hydrolytic enzymes, reactive oxygen species, and nitric oxide (NO·). Macrophages also function as antigen presenting cells in acquired immunity by presenting antigen to T-lymphocytes. A variety of changes in macrophage function have also been attributed to Δ^9-THC. Analysis of functional endpoints indicative of macrophage activation demonstrated a dose-dependent inhibition of macrophage spreading and decreased phagocytosis by Δ^9-THC (Lopez-Cepero *et al.*, 1986). Chronic exposure to marijuana was shown to cause irregular cell surface morphology, increased vacuolization, and altered protein expression following activation of macrophages (Cabral *et al.*, 1991). Functional studies further demonstrated that Δ^9-THC inhibits the extrinsic activity of macrophages in a dose dependent manner whereas intrinsic activity (uptake of the herpes virus) was unaffected (Cabral and Vasquez, 1992). In addition, the production of TNFα from activated macrophages was repressed by Δ^9-THC treatment (Zheng *et al.*, 1992). Subsequent studies have reported that the Δ^9-THC-mediated alteration of TNFα occurs at the level of maturation and secretion from macrophages rather than through a decrease in TNFα mRNA expression (Fischer-Stenger *et al.*, 1993; Zheng and Specter, 1996b). It was evident that macrophage effector function

was compromised by Δ^9-THC; therefore, studies were performed to characterize the mechanism(s) underlying these functional changes. An inhibition of early signaling events by cannabinoids has been demonstrated in activated macrophages. Δ^9-THC markedly prevented the tyrosine phosphorylation of two specific proteins (p77 and p82) in peritoneal macrophages stimulated with lipopolysaccharide (Zheng and Specter, 1994); however, the identity and function of these proteins remains unclear. Similarly, the IFNγ-induced phosphorylation of the STAT1α transcription factor was inhibited by Δ^9-THC in murine macrophages (Zheng and Specter, 1996a). Recently, Δ^9-THC was found to produce a dose-dependent inhibition of nitric oxide (NO·) production in response to LPS plus IFN-γ treatment (Coffey *et al.*, 1996). Maximal inhibition of NO· was detected only when Δ^9-THC was present prior to macrophage stimulation suggesting that cannabinoids may interfere with activation signals. Jeon and coworkers also reported a Δ^9-THC-mediated reduction in NO· levels in the macrophage cell line, RAW 264.7 (Jeon *et al.*, 1996). Furthermore, Δ^9-THC was shown to inhibit adenylate cyclase activity and transcription of the inducible nitric oxide synthase (iNOS) gene (Jeon *et al.*, 1996). Interestingly, the inhibition of NO· by Δ^9-THC was reversed by membrane permeable cAMP analogs in both studies (Coffey *et al.*, 1996; Jeon *et al.*, 1996). Together these findings provide insight into the mechanism of inhibition by Δ^9-THC and indicate that cannabinoids can modulate the cytolytic function of activated macrophages at the molecular level.

Examination of Δ^9-THC effects on host resistance have determined that cannabinoid compounds can markedly suppress resistance to herpes simplex virus type 2 (HSV-2) and *Listeria monocytogenes* (Cabral *et al.*, 1986a; Morahan *et al.*, 1979) as measured by a decrease in the time to lethality. Additional studies with HSV-2 demonstrated an increase in frequency and severity of lesions in guinea pigs receiving Δ^9-THC (Cabral *et al.*, 1984). Subsequent work has revealed a decrease in proliferative responses and a reduction in IFN α and β release by Δ^9-THC during HSV-2 infection

(Cabral *et al.*, 1987; Cabral *et al.*, 1986b) thereby providing a possible mechanism for compromised host resistance following Δ^9-THC exposure. Additionally, increases in IL-6 mRNA were detected in Δ^9-THC treated mice infected with *Legionella pneumophila* (Smith *et al.*, 1997) suggesting that acute phase proteins may play a role in the decreased survival of infected animals.

In summary, considerable evidence exists for the immunosuppressive activity of cannabinoid compounds. Δ^9-THC alters numerous immunological responses; however, T-lymphocytes appear to be particularly sensitive to inhibition by cannabinoids. In addition, the precise molecular changes responsible for cannabinoid-mediated immune suppression have not been extensively characterized.

D. Cannabinoid Receptors

1. Receptor subtypes and signal transduction pathways

Historically, the mechanism of action for cannabinoids has been attributed to intercalation and disruption of the plasma membrane due to the lipophilic nature of these compounds. Over the last decade, however, a large body of evidence has accumulated supporting the involvement of receptors in mediating the physiological effects of cannabinoids. First and foremost, binding studies performed in neuronal tissue demonstrated specific and saturable binding by cannabinoid compounds (Harris *et al.*, 1978). Furthermore, stereoselective differences in biologic activity were observed among cannabinoid enantiomer pairs although the degree of lipophilicity was equivalent between (+) and (-) isomers (Thomas *et al.*, 1990). Accordingly, early studies performed by Howlett and co-workers demonstrated that cannabinoids negatively regulate adenylate cyclase activity in neuronal cells (Howlett, 1985; Howlett and Fleming, 1984). This was a significant finding considering the specific association of adenylate cyclase and G-protein receptors. The above findings were ultimately supported by the isolation and cloning of a novel G-protein coupled receptor (CB1) from a rat brain cDNA library

(Matsuda *et al.*, 1990). The CB1 receptor has also been cloned in mouse (Chakrabarti *et al.*, 1995) and human (Gerard *et al.*, 1990) and exhibits a highly conserved sequence identity at the amino acid level among these species. A splice variant of the CB1 receptor, termed CB1A, has also been described in human tissues and differs from CB1 at the amino terminal tail (Shire *et al.*, 1995). Recently, a second cannabinoid receptor (CB2) has been cloned from HL60 cells, a promyelocytic leukemia cell line (Munro *et al.*, 1993). Interestingly, CB1 and CB2 share only 44% identity which increases to a modest 68% when comparing the transmembrane domains which are thought to constitute the putative ligand binding portion of the receptor. Despite significant differences in the amino acid sequence between the two forms of cannabinoid receptors, most natural and synthetic receptor ligands exhibit similar binding affinities for both CB1 and CB2. Cannabinol, however, is one ligand capable of discriminating between the two receptors exhibiting significantly greater binding affinity for CB2 than for CB1 (Munro *et al.*, 1993; Schatz *et al.*, 1997).

Ligand binding to either CB1 or CB2 produces a marked inhibition of adenylate cyclase activity thereby lowering intracellular cAMP levels (Condie *et al.*, 1996; Howlett, 1985; Howlett and Fleming, 1984; Jeon *et al.*, 1996). The cannabinoid-mediated inhibition of adenylate cyclase is abrogated by pertussis toxin demonstrating that cannabinoid receptors couple to a G_i-like protein (Howlett *et al.*, 1986; Kaminski *et al.*, 1994). Initially, the inhibition of adenylate cylcase by cannabinoids was described in neuroblastoma cell lines (Howlett, 1985) and has since been extended to several cell types including rat Sertoli cells (Heindel and Keith, 1989), human leukemic cells (Rowley and Rowley, 1989), murine splenocytes (Kaminski *et al.*, 1992), RAW 264.7 cells (Jeon *et al.*, 1996), and CHO cells transfected with cannabinoid receptors (Felder *et al.*, 1993; Vogel *et al.*, 1993). Although modulation of cAMP production by cannabinoid receptors has been the most extensively studied, additional receptor-mediated signaling mechanisms have been characterized for the CB1 receptor. For example, cannabinoid

compounds can repress calcium influx through N-type calcium channels in neuroblastoma-glioma cells through a pertussis toxin sensitive mechanism (Caufield and Brown, 1992; Mackie and Hille, 1992). Similarly, transfection of CB1 into the At20 pituitary cell line established the inhibition of Q-type calcium channels as well as the stimulation of potassium influx by cannabinoids (Felder *et al.*, 1995). In contrast, transfection of the CB2 receptor into either At20 or CHO cells produced no effect on calcium or potassium channels (Felder *et al.*, 1995), thus it currently appears that modulation of ion channels by cannabinoid receptors is a unique characteristic of the CB1 subtype. Additionally, the coupling of CB1 and CB2 to the mitogen-activated protein (MAP) kinase pathway has been described in unstimulated CHO cells transfected with either receptor subtype (Bouaboula *et al.*, 1995; Bouaboula *et al.*, 1996).

2. Tissue distribution of CB1 and CB2

The tissue and cell-type distribution of CB1 and CB2 have not yet been comprehensively characterized; however, CB1 appears to be primarily expressed within the CNS whereas CB2 seems to be predominantly expressed within the immune system. CB1 was originally isolated from the rat cerebellum (Matsuda *et al.*, 1990) and has since been detected in human brain (Gerard *et al.*, 1990; Herkenham *et al.*, 1990) and testis (Gerard *et al.*, 1991). Herkenham and coworkers have reported dense expression of the CB1 receptor specifically in the hippocampus, cerebellum, and basal ganglia outflow nuclei regions of the brain (Herkenham *et al.*, 1990). Studies investigating the level of CB1 expression within the immune system have determined that this receptor subtype is only modestly expressed on immunocompetent cells (Bouaboula *et al.*, 1993; Kaminski *et al.*, 1992; Schatz *et al.*, 1997). More specifically, RT-PCR analysis has detected mRNA for CB1 in human T-cells, B-cells, and monocytes (Bouaboula *et al.*, 1993) as well as mouse spleen (Kaminski *et al.*, 1992), but not in mouse thymus (Schatz *et al.*, 1997). Alternatively, CB2 appears to be the predominant cannabinoid receptor associated with

the immune system. CB2 expression was first detected in HL60 cells and rat spleen (Munro *et al.*, 1993) and has recently been identified in primary cells of the mouse spleen and thymus (Schatz *et al.*, 1997). Moreover, CB2 has also been detected in several cell lines including the EL-4.IL-2, HPB-ALL, and Jurkat E6-1 T-cell lines (Condie *et al.*, 1996); the macrophage cell line, RAW264.7 (Jeon *et al.*, 1996); and the RBL-2H3 mast cell line (Facci *et al.*, 1995).

Cannabinoid receptor expression in the immune system was first identified in mouse spleen cells based on the following lines of evidence: (a) specific and saturable binding of [^{3}H]-CP-55,940, a high affinity cannabinoid receptor ligand; (b) significant inhibition of adenylate cyclase activity; (c) stereoselective inhibition of humoral immune responses; and (d) detection of CB1 mRNA trancripts by RT-PCR (Kaminski *et al.*, 1992; Schatz *et al.*, 1992). As previously mentioned, CB2 has been characterized and determined to be the prominent cannabinoid receptor subtype expressed on immunocompetent cells. Cannabinoid-mediated inhibition of adenylate cyclase activity has been shown in a variety of lymphoid models including mouse splenocytes, EL-4 and HPB-ALL T-cell lines, RAW264.7 cells, and purified T- and B-lymphocytes (Condie *et al.*, 1996; Jeon *et al.*, 1996; Schatz *et al.*, 1992; Schatz *et al.*, 1997) demonstrating the functional expression of cannabinoid receptors on leukocytes. Interestingly, similar studies performed in the human Jurkat E6-1 T-cell line failed to detect modulation of adenylate cyclase by cannabinoid compounds. Further investigation of this cell line revealed three aberrantly sized mRNA transcripts for CB2 suggesting that the CB2 receptors expressed in Jurkat E6-1 cells are not functional. These findings further demonstrate that the modulation of adenylate cyclase by cannabinoid compounds is a receptor-mediated event. Recently, a novel role for the CB2 receptor in the differentiation of B-lymphocytes was proposed. Analysis of protein expression demonstrated a significant upregulation of CB2 initially following CD40 activation of

both naive and memory B-cells, and a downregulation of CB2 receptor mRNA and protein during the process of differentiation (Carayon *et al.*, 1998).

II. Intracellular Signal Transduction

A. G-protein coupled receptor signals

As already discussed, cannabinoid receptors are members of the G-protein coupled receptor superfamily. This family of receptors transmits information from the cell surface to the nucleus through a G-protein trimeric complex composed of alpha (α), beta (β), and gamma (γ) subunits. Signaling from G-protein coupled receptors initially utilizes a common mechanism which can ultimately interact with numerous effector molecules. During the inactivated state, the α subunit is bound to GDP and remains associated with the $\beta\gamma$ dimer. Ligand binding to G-protein coupled receptors stimulates the activation of Gα by exchanging GDP for GTP which results in the dissociation of Gα from G$\beta\gamma$. In general, the activated Gα subunit directly interacts with an effector to either stimulate or inhibit its activity typically through $G\alpha_s$ or $G\alpha_i$, respectively. The Gα subunit has been shown to positively or negatively modulate a variety of effectors including adenylate cylcase, potassium and calcium ion channels, phospholipase A_2, and phospholipase C (Hepler and Gilman, 1992). The best characterized effector coupled to cannabinoid receptor G-proteins is adenylate cyclase, and engagement of the inhibitory G-protein (G_i) by cannabinoid ligands results in an inhibition of adenylate cyclase activity. The attenuation of Gα signaling is mediated through the activation of specific GTPases which convert the GTP bound to Gα into GDP thereby inactivating the α subunit.

It is now well established that $\beta\gamma$ dimers of the G-protein can initiate and transmit independent signals through an interaction with several of the known effectors of G-protein coupled receptors. For example, $\beta\gamma$ subunits were originally found to stimulate potassium channels of the heart by increasing the opening frequency of these channels

(Logothetis *et al.*, 1987). βγ subunits can also modulate the metabolism of phospholipids through effects on phospholipase A_2 (PLA_2) and phospholipase C (PLC) activity. Specifically, stimulation of PLA_2 by the βγ subunit of transducin has been observed in bovine retina (Jelsema and Axelrod, 1987). The beta component of transducin was identified as an isoform common to other G-protein complexes; therefore, it is likely that additional Gβγ units may be able to regulate PLA_2 activity. Similarly, βγ subunits purified from retina and brain can stimulate the activity of soluble PLC isolated from human HL60 cells (Camps, 1992). Transient transfection of PLC beta isoforms into COS-1 cells further revealed the activation of PLCβ1 and PLCβ2 by free βγ dimers with the β2 isozyme displaying greater sensitivity to Gβγ stimulation (Camps *et al.*, 1992). The modulation of specific PLC isoforms by βγ has also been reported with PLCβ1 and PLCβ3 from rat brain and PLCβ2 from HL60 cells (Smrcka and Sternweis, 1993). In addition to regulating enzyme activity, βγ subunits can inhibit calcium influx through N-type voltage-gated calcium channels. This alteration in calcium is mediated by different combinations of βγ subunits (i.e., β1γ3 or β3γ4) that are activated by somatostatin and M4 muscarinic receptors, respectively (Kleuss *et al.*, 1992; Kleuss *et al.*, 1993). Recently, the mechanism by which βγ dimers modulate calcium channel activity has been shown to occur through the direct interaction of Gβγ with the pore-forming α_1 subunit of the channel (DeWaard *et al.*, 1997). A majority of the aforementioned results are general and were obtained using brain βγ units to characterize Gβγ:effector interactions. However, specific effects have been attributed to the βγ subunit of the G_i protein once detached from $G\alpha_i$. For example, the βγ dimer of G_i can stimulate MAP kinase activity in both Rat-1 fibroblasts and COS-7 cells transfected with G_i-coupled receptors (Koch *et al.*, 1994). The effect on MAPK was dependent on the activation of Ras, and further examination described a role for the phosphoinositide 3-kinase in the activation of MAPK by $G_i\beta\gamma$ (Lopez-Ilasaca *et al.*, 1997). The activation of SAPK and p38 by these βγ subunits has also been demonstrated recently and appears to involve Rac1 and Cdc42,

members of the Rho protein family (Coso *et al.*, 1995; Minden *et al.*, 1995). Despite the extensive evidence for βγ signaling following G-protein coupled receptor activation, the effects of βγ subunits interacting with cannabinoid receptors have yet to be characterized.

Several variations of effector modulation by βγ subunits have been described including regulation solely by βγ, Gβγ effects independent of Gα, or simultaneous regulation of the effector by α and βγ subunits (Clapham and Neer, 1993; Smrcka and Sternweis, 1993). Two models have been proposed for the latter which describe modulation of the effector by the entire αβγ complex or through separate α and βγ interacting sites on the effector molecule (Clapham and Neer, 1993). As mentioned above, hydrolysis of α-GTP to α-GDP terminates signaling mediated by the α subunit. This regulatory mechanism also indirectly functions to attenuate βγ dimers because the activation state of Gα dictates the activity of Gβγ (Clapham and Neer, 1993). Alternatively, the presence of the βγ subunit significantly increases the affinity of Gα for GDP (Higashijima *et al.*, 1987) which facilitates inactivation of the G-protein. Thus, it appears that these mechanisms enable Gα and Gβγ to cross-regulate the activity of one another. In addition to modulating G-protein activity, βγ dimers are involved in the regulation of the receptors. Agonist-dependent phosphorylation of receptors is a well known desensitization mechanism, and a classic example is the phosphorylation of the β-adrenergic receptor by a β-adrenergic receptor kinase (βARK) (Stadel *et al.*, 1983). Interestingly, βγ subunits induce significant increases in the βARK-mediated phosphorylation of active muscarinic and β_2-adrenergic receptors (Haga and Haga, 1992; Inglese *et al.*, 1992). This resulted from a Gβγ directed localization of the kinase to the membrane as opposed to an increase in kinase activity. It has now become apparent that one signal may activate multiple pathways and that G-protein signal transduction is a complex process.

B. The cAMP signaling cascade

1. Protein family members

The cAMP signaling pathway is comprised of several components that serve to transmit signals from the cell surface to the nucleus. The transcription factors involved in cAMP signal transduction have been identified as the CREB/ATF proteins. The CREB/ATF family is composed of several members including CREB1, CREB2, ATF1, ATF2, and CREM (Papavassiliou, 1994). These proteins are constitutively expressed in the nucleus and can form homo- or heterodimers with each other. The CREB/ATF transcription factors have been classified as members of the BZip superfamily of proteins, as they possess the characteristic BZip structure consisting of a basic amino acid region and a leucine zipper motif at their C-terminus. These regions are highly conserved and enable DNA binding and dimerization, respectively (Lalli and Sassone-Corsi, 1994). In addition to the BZip region, CREB/ATF proteins contain a transcriptional activation sequence consisting of a kinase-inducible domain (KID) and two flanking glutamine-rich regions (Gonzalez *et al.*, 1991; Habener, 1990). The KID possesses phosphorylation sites for several kinases; however, the specific phosphorylation of Ser-133 within this domain is critical for the transcriptional activity of CREB. CREB-1, ATF-1, and CREMτ have been characterized as transcriptional activators (Papavassiliou, 1994) whereas CREB-2, ICER (inducible cAMP early repressor), and the CREMα, -β, -γ isoforms have been shown to repress gene expression (Karpinski *et al.*, 1992; Laoide *et al.*, 1993; Molina *et al.*, 1993). The mode of inhibition by CRE repressor proteins has been proposed to occur either by direct binding to CRE motifs or through interactions with activators to quench their activity (Lalli and Sassone-Corsi, 1994). ICER is unique among the repressors as its expression is induced by cAMP (Molina *et al.*, 1993).

2. Activation and regulation of the cAMP cascade

G-protein stimulation of adenylate cyclase stimulates the conversion of ATP into cAMP. cAMP-dependent protein kinase, also known as protein kinase A (PKA), is the fundamental kinase component of the cAMP pathway. In the absence of cAMP, PKA exists as a tetramer complex composed of two regulatory subunits and two catalytic subunits (R_2C_2). Two isoforms of the regulatory subunit, termed RI and RII, have been characterized and exhibit different subcellular localization. RI has been found to reside in the cytosol whereas RII is often associated with the plasma membrane (Scott and McCartney, 1994). The primary function of cAMP is to bind cooperatively to A and B sites of the regulatory subunit which are located near the C-terminus of the protein. The binding of cAMP to these sites alters the affinity of the regulatory dimer for the catalytic units resulting in the dissociation of active catalytic units (Taylor *et al.*, 1990). The soluble catalytic subunits phosphorylate serine or threonine residues of several target proteins located within the PKA recognition sequence identified as X-Arg-Arg-X-Ser/Thr-X. The catalytic subunit of PKA can translocate to the nucleus where phosphorylation of additional regulatory proteins occurs (Meinkoth *et al.*, 1993). The CREB/ATF family of transcription factors is a critical nuclear target of PKA-mediated phosphorylation which enables their binding to palindromic cAMP response elements (CRE: 5'-TGACGTCA-3') located in the promoter region of cAMP responsive genes (Figure 3). CREB-1, the best characterized within this family, is phosphorylated on Ser-133 which induces a conformational change facilitating DNA binding and subsequent induction of transcription (Gonzalez and Montminy, 1989; Yamamoto *et al.*, 1989). PKA phosphorylation sites have also been identified in the ATF-1 and CREM proteins (de Groot *et al.*, 1993; Hai *et al.*, 1989).

Early studies examining the attenuation of CREB activity focused on the levels of CREB protein. Analysis of CREB following extended forskolin stimulation (12 hr) showed that protein levels remained unchanged (Gonzalez and Montminy, 1989);

Figure 3. Schematic representation of the cAMP signaling cascade. Signals are transmitted from membrane receptors to the nucleus through an intial receptor:G-protein interaction. Ligand binding to the receptor releases the Gα subunit which stimulates adenylate cyclase to convert ATP into cAMP. Once produced, cAMP binds to the regulatory subunits (R) of PKA thereby releasing two active catalytic subunits (C) of the enzyme to phosphorylate target proteins. The catalytic subunits of PKA translocate into the nucleus and phosphorylate the CREB/ATF family of transcription factors which form homo- or heterodimers and bind to CRE sequences in the promoter region of cAMP-responsive genes.

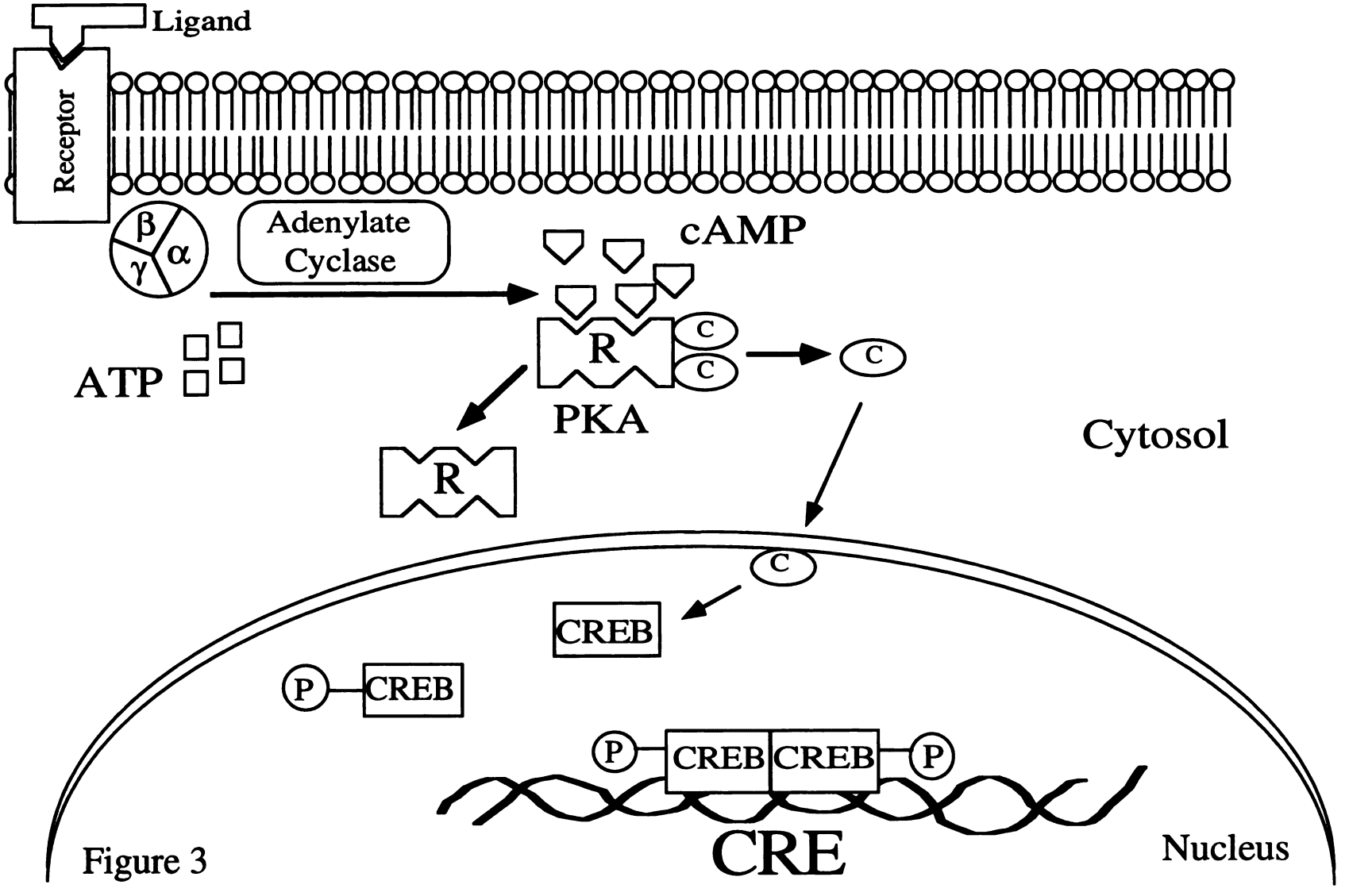

Ligand
Receptor
β
γ
α
Adenylate Cyclase
cAMP
ATP
R
C
C
C
PKA
R
Cytosol
C
CREB
P
CREB
P
CREB
CREB
P
CRE
Nucleus

Figure 3

therefore, degradation of CREB did not appear to be a regulatory mechanism. Consistent with the post-translational modification of CREB, activation has been reported to peak at 30-60 min and declines rapidly thereafter (Seasholtz *et al.*, 1995) implicating dephosphorylation as a potential negative regulatory mechanism. Okadaic acid is a common inhibitor of protein phosphatase activity, and the inactivation of CREB can be prevented in the presence of okadaic acid. The characterization of the specific protein phosphatase inhibited in this study identified protein phosphatase 1 (PP-1) as the enzyme responsible for dephosphorylating CREB (Hagiwara *et al.*, 1992). Similarly, inhibition of liver cell protein phosphatase 2A (PP-2A) with okadaic acid enhanced CREB phosphorylation and CRE-mediated gene expression suggesting that nuclear PP-2A may also be involved in the regulation of CREB activity (Wadzinski *et al.*, 1993). Thus, dephosphorylation was considered to be the primary mechanism of terminating cAMP-induced activation of the CREB/ATF family of proteins. However, the existence of repressors and their role in regulating CRE activity can not be disregarded. As previously mentioned, ICER protein expression is induced by cAMP and functions to down-regulate cAMP responsive genes establishing a negative feedback loop for the regulation of cAMP signal transduction at the nuclear level.

In addition to the phosphorylation state of CREB, regulation of the upstream components of the cAMP pathway has been described. Several mechanisms, both direct and indirect, are known to modulate the activity of PKA. PKI, the endogenous inhibitor of PKA, is ubiquitously expressed and primarily serves to sequester the active PKA catalytic units. PKI also transports the nuclear catalytic subunits into the cytosol thereby promoting reassociation with the regulatory dimer (Wen *et al.*, 1995). Furthermore, the overexpression of R subunits regulates PKA activity by facilitating formation of the tetrameric holoenzyme once basal levels of cAMP are attained (Amieux *et al.*, 1997). Termination of the cAMP signaling cascade is also mediated by the enzymes regulating cAMP synthesis. The desensitization of adenylate cyclase occurs following an initial

stimulus rendering it insensitive to subsequent signals (Mons *et al.*, 1995), and phosphodiesterases are stimulated to degrade and inactivate cAMP molecules (Smith *et al.*, 1996). It is likely that a combination of these regulatory mechanisms ensures that cAMP/PKA signal transduction is tightly controlled.

3. Kinetics of cAMP signal transduction

The kinetics of the cAMP pathway have been termed 'burst-attenuation' kinetics and can be divided into three specific phases which possess distinct regulatory mechanisms. The first phase in cAMP signal transduction is traditionally characterized by a rapid and transient burst of cAMP production. The nuclear translocation of PKA catalytic units and the Ser-133 phosphorylation of the CREB protein is also included in this initial phase (Hagiwara *et al.*, 1993). Peak activation of the cAMP cascade occurs between 30-60 min post-stimulation as demonstrated by maximum induction of CRE DNA binding complexes.

The second stage of cAMP signaling kinetics is known as the attenuation phase and is identified by the termination of cAMP-mediated signals. The principal event of this phase is the dephosphorylation of CREB at Ser-133 by PP-1 (Hagiwara *et al.*, 1992) which facilitates the steady decline of the signal to prestimulation status by 4-6 hr. Okadaic acid, a broad spectrum phosphatase inhibitor, prevents the attenuation and prolongs both the phosphorylation state of CREB and the expression of cAMP regulated genes (Hagiwara *et al.*, 1992).

The final phase of cAMP kinetics is the refractory period during which cells are unresponsive to additional cAMP stimulation. The refractory phase was originally identified using the thyroid follicular cell line, FRTL-5. Treatment of these cells with thyroid stimulating hormone increased cAMP levels initially; however, subsequent forskolin stimulation failed to induce CREB phosphorylation or CRE reporter activity (Armstrong *et al.*, 1995). This phase develops approximately 6-8 hrs following

stimulation and may last up to 3-5 days. A characterization of the underlying mechanism of cAMP unresponsiveness revealed a significant decrease in the translation of PKA catalytic subunits rather than a stimulation of phosphatase activity or an increase in the regulatory subunits of PKA (Armstrong *et al.*, 1995).

4. CBP, a transcriptional coactivator

The final step in cAMP responsive gene expression occurs at the level of the transcriptional coactivator identified as CREB binding protein (CBP). CBP is a large 265 kD nuclear protein that binds to CREB along with components of the basal transcriptional machinery including TFIIB, TFIID, and the RNA polymerase complex (Bisotto *et al.*, 1996; Kee *et al.*, 1996; Kwok *et al.*, 1994). Initial studies established a specific interaction between CBP and the Ser-133 phosphorylated form of CREB (Chrivia *et al.*, 1993). This was further demonstrated by the inability of CBP expression alone or in combination with a CREB Ser-133 mutant to induce transcription (Kwok *et al.*, 1994). The CREB binding domain of CBP interacts with the KID region of CREB and is composed of 200 amino acids located near the N-terminus of the protein (Goldman *et al.*, 1997). Thus, CBP is only recruited by activated CREB and provides a functional link between CRE DNA binding proteins and the transcriptional enzymes essential for gene expression. However, it is notable that interactions between CREB and CBP are insufficient for transcriptional induction indicating the involvement of additional regulatory mechanisms (Sun and Maurer, 1995). Chawla and coworkers recently identified a transcriptional activator domain within CBP that was regulated by nuclear calcium and CaM kinase IV (Chawla *et al.*, 1998). This region was also sensitive to increases in cAMP suggesting that CBP-mediated transcription is stimulated by both calcium and cAMP signals. Currently, increases in intracellular cAMP result in the following sequence of events: (1) phosphorylation of CREB and binding to the CRE motif; (2) recruitment and activation of CBP; and (3) induction of target gene expression.

Interestingly, an association between CBP and other transcription factors has also been demonstrated. CBP can interact with p65, c-jun, c-fos, Elk-1, c-myb, and members of the steroid hormone receptor family (Goldman *et al.*, 1997; Zhong *et al.*, 1998). Therefore, CBP may integrate signals from multiple pathways as a universal mechanism of conjoining activated transcription factors with the transcriptional machinery.

5. Cross-talk between the PKA and PKC pathways

Several lines of evidence have demonstrated cross-talk between the PKA and PKC pathways at multiple levels within the signaling cascades. Early studies have demonstrated the phosphorylation of the catalytic subunit of adenylate cyclase following phorbol ester treatment of frog erythrocytes (Yoshimasa *et al.*, 1987). PMA can also stimulate specific types of adenylate cyclase in 293 cells transiently expressing enzyme types 1, 2, or 3 (Jacobowitz *et al.*, 1993). The activation of type 2 adenylate cyclase by PMA was thought to be mediated by phosphorylation of adenylate cyclase by PKC because staurosporine markedly blocked the activation of adenylate cyclase by phorbol esters (Yoshimura and Cooper, 1993). Similarly, a rapid and transient increase in intracellular cAMP levels following PMA/Io stimulation of mouse splenocytes has been described (Kaminski *et al.*, 1994) demonstrating that PKA/PKC cross-talk occurs in lymphoid cells. Increases in cAMP following PMA stimulation have also been reported in vascular smooth muscle cells further suggesting that activation of PKC may potentiate the cAMP pathway (Ren *et al.*, 1996). The MAPK pathway is one downstream target of PKC, and the activation of MAPK (specifically ERK1) has been shown following increases in intracellular cAMP in PC12 cells (Frodin *et al.*, 1994). This cAMP effect was also synergistic with phorbol ester stimulation of ERK1 activity. Furthermore, cAMP elevating agents such as forskolin, dibutyryl cAMP, or isoproterenol activated MAPK as early as 8 min post-stimulation in rat cardiomyocytes (Yamazaki, 1997).

Interestingly, stimulation of MAPK by cAMP was dependent on calcium in these cells and could not be detected under calcium-free conditions.

Cross-talk between these two signaling pathways also occurs at the transcription factor level. Along with the CREB/ATF proteins, c-fos and c-jun are members of the leucine zipper superfamily of transcription factors. Fos and Jun dimerize to form AP-1 which binds to DNA sequences known as TRE sites (5'-TGACTCA-3') in the promoter region of various genes. AP-1 is one of the DNA binding proteins situated downstream of PKC and can bind to TRE sites following phorbol ester stimulation. As members of the leucine zipper family, c-fos and c-jun could potentially form 'cross-family' dimers with other leucine zipper proteins, namely the CREB/ATF transcription factors. It should also be noted that the TRE motif differs from the CRE by only one base pair. Indeed, several studies have established that CREB is capable of forming heterodimers with Jun which then bind to AP-1 sites on DNA (Chatton *et al.*, 1994; Hai and Curran, 1991; Ivashkiv *et al.*, 1990). Furthermore, CREB, Fos and Jun were all detected in the protein complex bound to the AP-1 proximal site of the IL-2 promoter in both immature and mature T-lymphocytes (Chen and Rothenberg, 1993). Cross-family dimerization is not limited exclusively to TRE sites as a similar interaction has been observed with a CREB/cJun heterodimer binding to a CRE motif (Benbrook and Jones, 1990). Together these observations imply that activation of CREB/ATF and AP-1 proteins can regulate gene expression through both CRE and TRE motifs. Additionally, a CRE site exists within the c-*fos* promoter (Sheng *et al.*, 1990) suggesting that CREB/ATF proteins are involved in regulating c-fos expression thereby providing another level of interaction between the PKA and PKC pathways.

C. The NF-κB signaling pathway

1. Family members

The NF-κB/c-Rel proteins are members of the Rel family of transcription factors. These proteins contain a Rel homology domain (RHD) consisting of approximately 300 amino acids which contains the necessary sequences for DNA binding and dimerization (Verma *et al.*, 1995). A nuclear localization signal (NLS) is also present in the N-terminal portion of the protein to direct NF-κB into the nucleus upon cellular activation. The current members of the NF-κB/c-Rel family are p50/p105, p65, c-Rel, RelB, and p52/p100 (Ghosh *et al.*, 1998). These proteins can form homodimers or heterodimers with one another and bind to κB sequences (GGGACTTTCC) in the promoter regions of responsive target genes (Ghosh and Baltimore, 1990). Extensive characterization has been done to determine the transcriptional effects of various dimer combinations. In fact, most NF-κB dimers have been classified as transcriptional activators including the heterodimers of p50/p65, p50/c-rel, p65/c-rel and the p65/p65 homodimer. However, the p50/p50 and p52/p52 homodimers have been described as repressors of gene transcription (Brown *et al.*, 1994; Hansen *et al.*, 1994; Plaskin *et al.*, 1993).

2. The NF-κB inhibitor, IκB

IκB functions as an inhibitor of NF-κB activity by sequestering this transcription factor in the cytosol of quiescent cells. Several IκB molecules have been indentified to date including IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105, p100, and IκBR (May and Ghosh, 1998; Miyamoto and Verma, 1995). The structure of the IκB proteins contains several ankryin repeat motifs which are necessary for noncovalent protein-protein interactions with NF-κB dimers (Inoue *et al.*, 1992). The interaction between IκB and NF-κB functions to maintain NF-κB dimers in an inactive form by masking their nuclear localization signal and preventing nuclear translocation. Of the IκB family members, IκB-α was the first to be identified and is therefore the most extensively characterized

inhibitor protein. IκB-α is a 37 kD protein and binds to dimers containing p65 and/or c-Rel proteins (Verma *et al.*, 1995). Several recent studies have also begun to investigate the function of IκB-β during NF-κB activation. IκB-β is a 45 kD protein and can regulate p50/p65 and p50/c-Rel complexes also (Ghosh *et al.*, 1998); however, distinct differences in signal response kinetics exist between IκB-α and IκB-β. Specifically, IκB-α is rapidly degraded in response to activating stimuli and reappears to terminate gene expression (Arenzana-Seisdedos *et al.*, 1995). In contrast, the regeneration of IκB-β occurs only upon removal of the activation stimulus, therefore the duration of responses regulated by IκB-β is prolonged (Thompson *et al.*, 1995). An additional mechanism also contributes to the persistent activation by IκB-β. Unphosphorylated IκB-β reportedly binds to cytosolic NF-κB to protect it from IκB-α (Suyang *et al.*, 1996). This NF-κB:IκB-β complex translocates to the nucleus where NF-κB activates gene expression. The degradation of IκB-ε also follows slower kinetics. An initial characterization of IκB-ε has suggests the selective modulation of dimers containing only p65 or c-Rel (Whiteside *et al.*, 1997). It is thought that IκB-ε regulates a specific subset of dimers and may serve a specialized function. Bcl-3, another IκB family member, resides in the nucleus rather than the cytosol to interact with homodimers of p50/p50 or p52/p52 (Nolan *et al.*, 1993). Another unique feature of Bcl-3 is its ability to bind to κB sites on DNA as a Bcl-3:p50/p50 complex which results in gene activation (Bours *et al.*, 1993). Additional insight into the mechanism of NF-κB regulation by Bcl-3 or other members of the IκB family is presently unknown.

3. Induction and regulation of NF-κB

A diverse group of stimuli are known to induce NF-κB DNA binding activity in a variety of cell types. Such stimuli include pro-inflammatory cytokines, lipopolysaccharide, PMA, UV light, reactive oxygen species, antigen, and viral proteins (Barnes and Karin, 1997). As mentioned above, NF-κB exists in the cytosol of

unstimulated cells bound to the IκB proteins. Originally, the mechanism by which NF-κB dissociated from IκB was unclear. Early studies demonstrated that PMA was a potent inducer of NF-κB, thus phosphorylation by PKC was considered important for activation (Baeuerle *et al.*, 1988). PKA was also shown to activate NF-κB through a phosphorylation event (Muroi and Suzuk, 1993; Shirakawa *et al.*, 1989; Shirakawa and Mizel, 1989). Evidence supporting PKA-mediated phosphorylation of IκB includes the induction of NF-κB DNA binding by forskolin or dibutyryl cAMP and inhibition of NF-κB binding activity in the presence of the PKA inhibitor H89 (Muroi and Suzuk, 1993). Further characterization of the stimuli-induced phosphorylation mechanism uncovered the specific phosphorylation of IκB-α on two serine residues (Ser 32 and Ser 36) (Brockman *et al.*, 1995; Brown *et al.*, 1995). Conversely, peptide aldehyde inhibitors of the 26S proteosome (i.e. calpain I or MG-132) prevent the degradation of IκB-α (Brown *et al.*, 1995; Lin *et al.*, 1995). These findings suggested that additional regulatory mechanisms are involved in the release of NF-κB from IκB. Chen and co-workers discovered that IκB-α was ubiquitinated in HeLa cell extracts following phosphorylation, and the ubiquitinated IκB-α remained associated with the NF-κB dimer (Chen *et al.*, 1995). These studies also described the degradation of the ubiquitinated form of IκB-α by the 26S proteosome. It is notable that the initial phosphorylation of IκB-α is essential for subsequent ubiquitination and degradation. In summary, a specific series of events is required for NF-κB activation (Figure 4): (1) phosphorylation of IκB-α on Ser 32 and Ser 36; (2) ubiquitination of neighbor lysine residues; (3) degradation of IκB-α by the 26S proteosome; and (4) nuclear translocation of NF-κB to bind κB sequences in DNA.

Recently, a large cytosolic IκB kinase complex has been identified (Mercurio *et al.*, 1997; Regnier *et al.*, 1997; Zandi *et al.*, 1997). Two IκB kinases, designated IKKα and IKKβ, have been characterized as subunits of the larger IκB regulatory complex (DiDonato *et al.*, 1997). IKKα and IKKβ can form heterodimers and function to

Figure 4. Schematic representation of the NF-κB signaling pathway. In quiescent cells, NF-κB is retained in the cytosol bound to the inhibitor, IκB-α. Following an activation stimulus, IκB-α is phosphorylated by IκB kinases within the IκB kinase complex. The phosphorylation triggers the ubiquitination (Ub) and subsequent degradation of IκB-α by the 26S proteosome. IκB-α degradation enables the nuclear translocation and DNA binding of NF-κB to κB sequences in the promoter region of target genes.

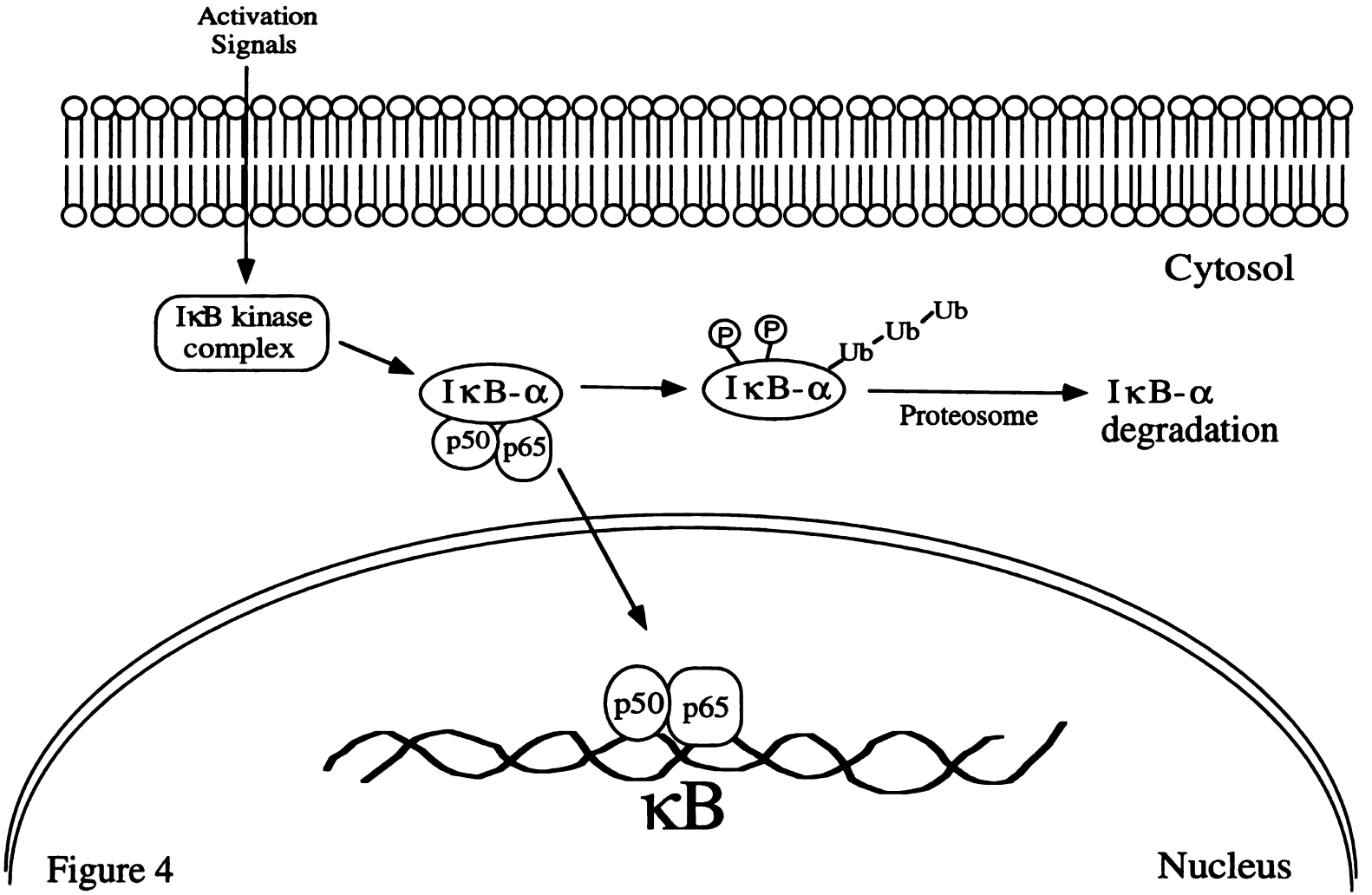

Activation
Signals
Cytosol
IκB kinase
complex
IκB-α
p50
p65
P
P
Ub
Ub
Ub
IκB-α
Proteosome
IκB-α
degradation
p50
p65
κB
Nucleus

Figure 4

phosphorylate IκB proteins in response to NF-κB inducing stimuli (DiDonato *et al.*, 1997; Woronicz *et al.*, 1997). In addition to the IKK molecules, a protein named NF-κB Essential Modulator (NEMO) has been described as a critical component of the IκB kinase complex (Yamaoka *et al.*, 1998). NEMO appears to be essential for kinase complex formation and necessary for NF-κB activation by PMA, LPS, and IL-1. Current research efforts have focused on the elucidation of the mechanisms involved in the regulation of the IκB kinase complex. Initially, Malinin and coworkers reported the involvement of a MEKK-1 related kinase termed NF-κB-inducing kinase (NIK) in the activation of NF-κB following TNFα and IL-1 stimulation (Malinin *et al.*, 1997). Recently, NIK has been shown to preferentially phosphorylate IKKα following cytokine stimulation which results in the activation of IKKα kinase activity (Ling *et al.*, 1998). A specific activation of IKKβ by MEKK1 has also been described (Nakano *et al.*, 1998) further demonstrating the differential activation of IKKα and IKKβ by upstream kinases. These studies suggest that MEKK1 and NIK can activate the IκB kinase complex independent of each other. Although the complete regulation of the IκB kinase complex is presently unclear, it has been speculated that the multi-subunit complex may function to integrate signals from a variety of NF-κB activation pathways (May and Ghosh, 1998).

Additional regulation of the NF-κB dimer has been described at the level of the cytosolic NF-κB:IκB complex. The catalytic subunit of PKA (PKAc) is associated with the complex and remains inactive when bound with the NF-κB:IκB proteins (Zhong *et al.*, 1997; Zhong *et al.*, 1998). Upon degradation of IκBα, PKAc becomes activated and phosphorylates the p65 component of the NF-κB dimer. This function of PKA in the regulation of NF-κB has therefore been characterized as a cAMP-independent mechanism. The post-translational modification of p65 by PKAc can significantly increase p65 DNA binding and to potently augment the transactivating activity of p65 (Zhong *et al.*, 1997; Zhong *et al.*, 1998).

Attenuation of NF-κB gene expression occurs through an autoregulatory feedback loop. This is mediated through the binding of NF-κB to κB sequences in the promoter region of IκB-α resulting in an increased expression of the IκB-α gene. Following its degradation, IκB-α rapidly reappears and has been detected as early as 90 min post-stimulation (Henkel *et al.*, 1993). The newly synthesized IκB-α reforms a complex with cytosolic NF-κB dimers rendering them inactive. IκB-α can also extinguish the activation signal by entering the nucleus and removing the NF-κB bound to DNA (Arenzana-Seisdedos *et al.*, 1995).

4. Immune response genes regulated by NF-κB

Several pathological conditions have been associated with chronic or abberant NF-κB activation including cancer, atherosclerosis, septic shock, and inflammatory conditions. With respect to the immune system, NF-κB regulates many of the genes involved in inflammation and leukocyte activation. Examples of genes up-regulated by NF-κB include acute phase response proteins, cytokines and chemokines, adhesion molecules, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) (Barnes and Karin, 1997). The prominent involvement of NF-κB in regulating inflammatory genes has generated substantial interest in NF-κB regulation as a potential therapeutic strategy. The current therapy for inflammatory disease conditions utilizes glucocorticoids as immunosuppressive agents. Interestingly, glucocorticoids modulate the transcription of inflammatory response genes, in part, by altering the activation of NF-κB. This occurs at the molecular level through several independent mechanisms. A direct interaction between the glucocorticoid receptor and NF-κB has been demonstrated in the nucleus which causes sequestration of NF-κB thereby preventing its binding to κB sites in DNA (Ray and Prefontaine, 1994; Scheinman *et al.*, 1995b). The glucocorticoid receptor also utilizes the negative feedback loop involved in NF-κB regulation by binding to the κB sites in the promoter of IκB-α and initiating the synthesis of new IκB-α

(Scheinman *et al.*, 1995a). As previously stated, IκB-α sequesters NF-κB into the inactive form located in the cytosol. However, the abundance of negative side-effects associated with chronic glucocorticoid therapy has heightened interest into the development of molecularly specific inhibitors of NF-κB.

III. T-lymphocyte Background

A. T-cells and the immune system

T lymphocytes perform a multitude of biological functions and are important effector cells of the acquired immune response. Two populations of T-cells exist and are distinguished by the differential expression of CD4 or CD8 coreceptors on their surface. These T-cells can also be identified by their immunologic activities with $CD4^+$ lymphocytes usually referred to as T helper (T_h) cells and $CD8^+$ lymphocytes typically known as T cytotoxic (T_c) cells. More specifically, T_h cells produce and secrete cytokines in response to stimulation while T_c cells eliminate virus-infected and tumor cells. Two distinct subpopulations of T helper cells have been characterized (i.e., T_h1 and T_h2), and each class displays a specific cytokine profile. T_h1 lymphocytes synthesize IL-2 and IFN-γ and are involved in cell-mediated immunity whereas T_h2 cells produce IL-4, IL-5, and IL-10 to facilitate humoral immune responses (Mosmann *et al.*, 1986). Additionally, T_h1 and T_h2 subsets can cross-regulate each other with IFN-γ down-regulating T_h2 cells and IL-10 inhibiting T_h1 activity.

The stimulation of T-cell effector function depends upon antigen activation of the T-cell receptor expressed on the surface of mature T-cells. The T-cell receptor (TCR) is a heterodimer of α and β proteins and associates with a multi-unit CD3 complex consisting of delta, epsilon, gamma, and zeta polypeptide chains. The CD3 chains are organized as specific dimers including a gamma/epsilon heterodimer, a delta/epsilon heterodimer, and a zeta homodimer. The principal role of the CD3 complex is the transmission and amplification of activation signals from the TCR to the nucleus.

B. T-cell development

T-cell development is a complex, multi-step process that originates in the bone marrow and continues in the thymus. Hematopoietic stem cells are the lymphoid precursors within the bone marrow which give rise to several different lymphoid lineages. The earliest step in the developmental process is the migration of T-cell progenitors from the bone marrow to the thymus by entering the bloodstream. Once in the thymus, pro-thymocytes express the Thy-1 surface marker indicating these progenitor cells are committed to the T-cell lineage (Rodewald *et al.*, 1994). The expression of the receptor tyrosine kinase c-kit is also detected on thymus progenitors (Hattori *et al.*, 1996); therefore, the earliest thymocytes are denoted as Thy-1^+c-kit^+ cells. The progression of T-cell development is generally monitored by changes in surface molecule expression, and subpopulations of thymocytes are often identified by such nomenclature (Figure 5).

1. Double negative thymocytes

During the first stage of development in the thymus, immature T-cells are considered double negative thymocytes as they lack expression of CD4 and CD8 co-receptors (i.e., $CD4^-CD8^-$). These double negative thymocytes are located in the subcapsular region of the thymus. The $CD4^-CD8^-$ cells also lack any form of a T-cell receptor initially; however, they do express multiple surface markers including CD44, CD25, and c-kit (Ceredig *et al.*, 1985; Godfrey *et al.*, 1992; Raulet, 1985; Trowbridge *et al.*, 1985). CD25 is the alpha subunit of the interleukin-2 receptor and CD44 is an adhesion molecule. In fact, four subsets of double negative thymocytes have been identified through the differential expression of c-kit, CD25, and CD44 (Godfrey *et al.*, 1993; Pearse *et al.*, 1989). The first two subsets differ only in the level of CD25 expression with the earliest thymocytes being c-$kit^+CD44^+CD25^-$ and the second population being c-$kit^+CD44^+CD25^+$. With the loss of c-kit and a decrease in CD44, the third subset of double negative thymocytes ($CD25^+CD44^{-/low}$) acquires a pre-TCR

Figure 5. The ordered progression of T-lymphocyte development. Immature T-cells progress through three major stages of development within the thymus. Each phase is defined by the surface expression of the CD4 and CD8 coreceptors. The expression of other specific molecules unique to each phase has also been described as depicted in the figure. Thymocytes in the first stage of development are denoted as double negative due to the lack of CD4 and CD8 expression (CD4-CD8-). Double negative thymocytes possess an early form of the TCR known as the preTCR. Acquisition of CD4 and CD8 molecules occurs during the second developmental stage to produce double positive thymocytes (CD4+CD8+). The α and β chains of the TCR can be detected at the double positive stage. Positive and negative selection of double positive cells generates single positive thymocytes expressing either the CD4 or CD8 coreceptor (CD4+CD8-; CD4-CD8+). Single positive thymocytes exit the thymus and become the mature T-cell population.

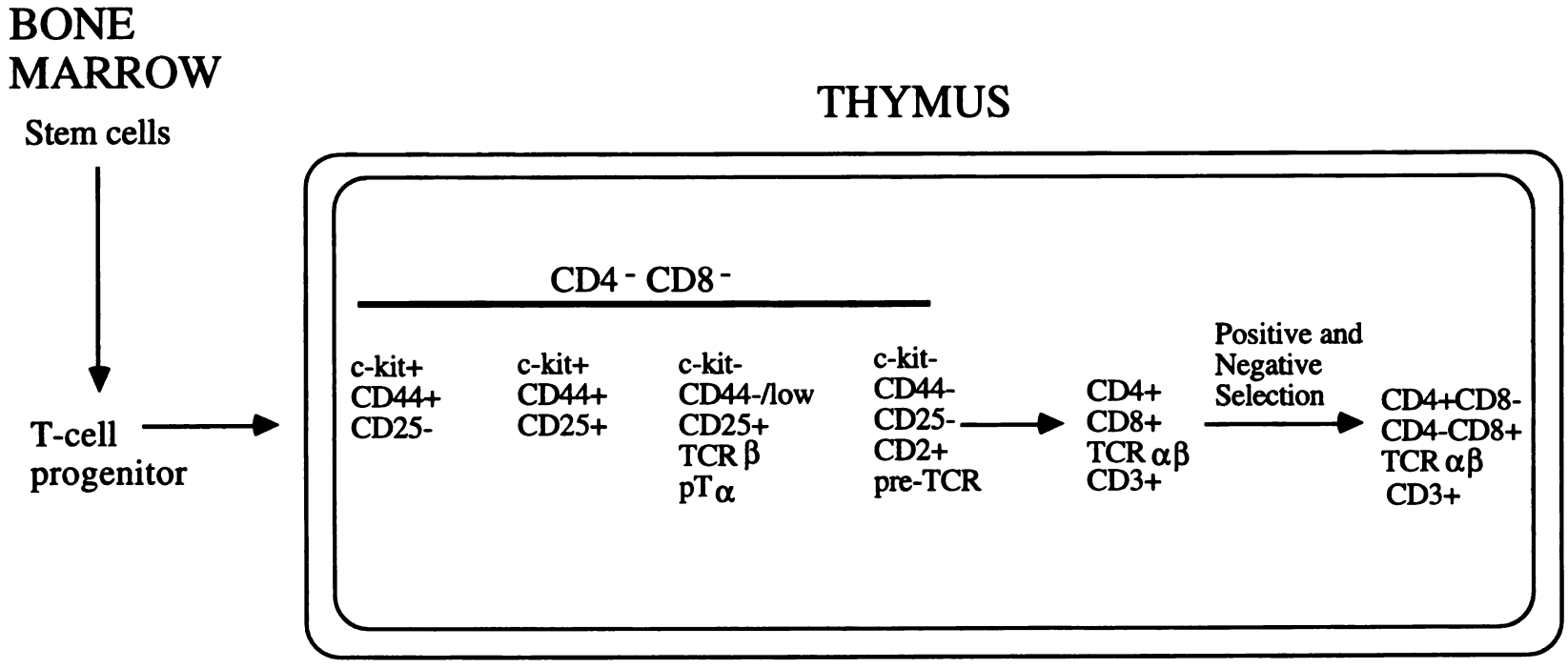

BONE
MARROW
Stem cells
T-cell
progenitor
THYMUS
CD4 - CD8 -
c-kit+
CD44+
CD25-
c-kit+
CD44+
CD25+
c-kit-
CD44-/low
CD25+
TCR β
pTα
c-kit-
CD44-
CD25-
CD2+
pre-TCR
CD4+
CD8+
TCR αβ
CD3+
Positive and
Negative
Selection
CD4+CD8-
CD4-CD8+
TCR αβ
CD3+

Figure 5

through the rearrangement of the TCR β subunit gene (Dudley *et al.*, 1994; Mallick *et al.*, 1993). Further analysis of TCRβ in $CD4^-CD8^-$ murine thymocytes determined the association of a 33 kD peptide that functions as a partner for TCRβ in the absence of TCRα (Groettrup *et al.*, 1993; Groettrup and von Boehmer, 1993). This additional polypeptide was appropriately identified as pTα (Saint-Ruf *et al.*, 1994). Thus, the pre-TCR on developing thymocytes is a heterodimer composed of TCRβ and pTα peptides. The significance of pTα expression was demonstrated by Fehling and coworkers using $pT\alpha^{-/-}$ mice. These animals exhibited a 10-fold decrease in thymocyte number along with compromised development of T-cells expressing the formal αβTCR (Fehling *et al.*, 1995).

Consistent with a fundamental role for the pre-TCR during development, several reports have demonstrated the signaling potential of the thymocyte TCR. The pre-TCR associates with a CD3 complex that differs from the mature TCR:CD3. Select protions of the CD3 complex have been shown to weakly interact with the preTCR, namely the epsilon, gamma, and zeta chains of CD3 (Borst *et al.*, 1996; Groettrup *et al.*, 1992; Punt *et al.*, 1991). Biochemical analysis of the CD3 expressed on immature thymocytes has revealed an impairment in the production of double positive cells in the absence of CD3ζ (Love *et al.*, 1993; Malissen *et al.*, 1993; Ohno *et al.*, 1993). Furthermore, cross-linking of CD3ε or CD3ζ initiated signals important for the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocytes (Shinkai *et al.*, 1995). Although the specific details of pre-TCR signaling have yet to be elucidated, an involvement of the tyrosine kinase $p56^{lck}$ has been suggested. This is supported by the disruption of αβ thymocyte development in mice with targeted mutations in the lck gene (Molina *et al.*, 1992). Similar effects were also detected in a dominant-negative $p56^{lck}$ transgenic model (Levin *et al.*, 1993). Moreover, normal maturation of $pT\alpha^{-/-}$ thymocytes occurs when an active form of lck is expressed (Fehling *et al.*, 1997) implicating $p56^{lck}$ in the transmission of pre-TCR signals. The participation of additional downstream signaling components, including

kinases and specific nuclear targets, is relatively uncharacterized at the present time (Rodewald and Fehling, 1998).

The final subset in the double negative sequence is the c-kit$^-$CD25$^-$CD44$^{-/low}$ thymocytes which possess a productive pre-TCR and demonstrate an increase in the CD2 surface molecule. It is now recognized that proper β gene rearrangement and synthesis of a functional pre-TCR is essential for triggering the proliferation and progression of late CD4$^-$CD8$^-$ thymocytes to the double positive stage.

2. Double positive thymocytes

The differentiation into double positive thymocytes is marked by the acquisition of CD4 and CD8 coreceptor molecules (i.e., CD4$^+$CD8$^+$). The pTα chain is also replaced by the rearranged α gene of the TCR during this stage of thymopoiesis (Petrie *et al.*, 1995; Wilson *et al.*, 1996) resulting in the formation of the αβTCR. Thymocytes unable to generate productive TCRαβ dimers fail to mature and subsequently undergo apoptotic cell death. CD4$^+$CD8$^+$ thymocytes also experience positive and negative selection which establishes the MHC restricted, self-tolerant TCR repertoire of mature T-cells. Positive selection examines the ability of newly synthesized αβTCR to recognize self-MHC I and II molecules on the cortical epithelial cells of the thymus (von Boehmer, 1994). Specific recognition of self-MHC by the TCR maintains thymocytes for further maturation. Studies utilizing a thymic organ culture system have demonstrated the importance of the MHC interaction in positive selection. For example, mice deficient in key components of either the MHC I or MHC II complex had diminished CD8$^+$ and CD4$^+$ positive selection, respectively (Tourne *et al.*, 1995; Zijlstra *et al.*, 1990). By comparison, negative selection is mediated by the macrophages and dendritic cells of the bone marrow. Specifically, these APCs present antigenic peptides to the CD4$^+$CD8$^+$ cells to determine the degree of antigen:TCR interactions. Thymocytes displaying either high affinity for the peptide/MHC complex or recognition of 'self' antigen are eliminated

by apoptosis (Nossal, 1994). In addition to the affinity of the TCR, negative selection also appears to be dependent on the concentration of the antigen. Several studies have shown that low peptide concentrations promote positive thymocyte selection while high concentrations result in negative selection (Alam *et al.*, 1996; Ashton-Rickardt *et al.*, 1994; Sebzda *et al.*, 1994). Thus, low affinity TCRs and low concentrations of antigen can rescue developing thymocytes from death by providing survival signals .

Despite recent advances in T-cell development, the molecular aspects of thymic selection are relatively unknown. A direct relationship between the quantity of TCR signals and selection has been described in CD3ζ knock-out mice expressing low levels of an HY-specific TCR. HY is a male-specific antigen that normally induces the negative selection of T-cells in males that recognize HY; however, positive selection of HY-specific T-cells occurred in the male mice of the $CD3\zeta^{-/-}$ model (Yamazaki *et al.*, 1997). The authors suggest these findings are due to decreased HY-TCR levels rather than the stimulation of a unique positive selection signal. Alternatively, the nature of the antigenic peptide has been proposed to modulate TCR-mediated signaling events. Two kinetic models, the kinetic proofreading model and the kinetic discrimination model, attribute variations in TCR signaling during selection to differences in peptide affinity (McKeithan, 1995; Rabinowitz *et al.*, 1996). For example, low affinity peptides may lead to incomplete formation of the TCR signaling complex whereas high affinity peptides are more likely to generate complete activation complexes. Therefore, alterations in signaling induced by differential ligand affinity may be involved in the overall selection of $CD4^+CD8^+$ thymocytes.

It is also unclear whether distinct signals exist for positive and negative selection processes. Recently, several transgenic studies have provided insight into the specific involvement of various signaling pathways. The expression of an inactive form of the $p56^{lck}$ tyrosine kinase demonstrated its participation in positive and negative selection (Hashimoto *et al.*, 1996). A similar role for the Zap-70 kinase in both selection processes

has also been shown in Zap-70 deficient mice (Negishi *et al.*, 1995). In contrast, calcium signals and the MAP kinase pathway appear to be involved only in positive selection. Overexpression of dominant negative forms of several components of the MAPK pathway, including Ras, Raf-1, and Mek-1, interferred with positive selection (Alberola-Ila *et al.*, 1995; O'Shea *et al.*, 1996; Swan *et al.*, 1995). Similarly, cyclosporin A inhibition of the calcium/calmodulin dependent phosphatase, calcineurin, significantly reduced positive selection (Wang *et al.*, 1995). Based on this initial evidence, several elements of the mature αβTCR signaling network seem to play selective roles in $CD4^+CD8^+$ thymocyte selection.

The final stage in T-cell development is the lineage commitment of selected thymocytes. At this point, double positive cells differentiate into single positive thymocytes that express only one of the surface coreceptors (i.e., $CD4^+CD8^-$ or $CD4^-CD8^+$). Consequently, the two major populations of mature T-cells are established and their effector function is determined. The $CD4^+CD8^-$ subset represents the T helper lymphocytes and the $CD4^-CD8^+$ subset serves as cytotoxic T-cells.

C. T-lymphocyte activation and associated signaling pathways

The activation of mature T-cells is initiated by T-cell receptor recognition of antigen presented by the major histocompatability complex (MHC) I or II expressed on the surface of antigen presenting cells (APC). The associated CD4 and CD8 coreceptors interact with antigen in the context of MHC II or MHC I, respectively, and activate the $p56^{lck}$ and $p59^{fyn}$ *src*-family tyrosine kinases. Upon activation, lck and fyn phosphorylate the immunoreceptor tyrosine-based activation motifs within the cytoplasmic regions of the TCR/CD3 complex which serve to recruit additional early signaling components, namely the Zap-70 and phosphoinositide (PI)-3 kinases (Carpenter and Cantley, 1996; Chan *et al.*, 1992). The tyrosine kinases also couple the TCR to multiple signaling pathways through the activation of phospholipase Cγ (PLC) and $p21^{ras}$

thereby initiating a complex cascade of biochemical events. PLC transmits a fundamental signal through the production of two second messengers which trigger pathways essential for T-cell activation. PLCγ hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which initiates calcium responsive signals and activates PKC.

The regulation of calcium by IP_3 occurs through an IP_3 receptor present on the membrane of the endoplasmic reticulum. Binding of IP_3 to the IP_3 receptor mobilizes calcium stores resulting in an increase in intracellular calcium levels. Following the initial burst of calcium, a depletion of the endoplasmic reticulum stores is sensed and extracellular Ca^{2+} enters the T-cell through an unidentified channel in the plasma membrane. The regulation of calcium levels in lymphocytes is mediated by calcium-release-activated calcium (CRAC) channels that differ from the traditional voltage-gated calcium channels (Hoth and Penner, 1992). Therefore, the process of modulating and maintaining Ca^{2+} levels in immune cells has been termed capacitative calcium entry (Berridge, 1995). The primary action of calcium is to activate Ca^{2+}/calmodulin dependent enzymes involved in transcription factor regulation including the serine phosphatase calcineurin and Ca^{2+}/CaM kinase IV. Calcineurin is the best characterized effector of the calcium signal and functions to regulate the activity of the NF-AT transcription factor family. Specifically, activated calcineurin dephosphorylates the cytosolic NF-AT protein allowing its nuclear translocation and induction of cytokine gene expression. Recently, several studies have demonstrated the importance of the Ca^{2+}/CaM kinase type IV (CaMKIV) during T-cell activation events. The involvement of CaMKIV was initially supported by its selective expression in the nucleus of T-lymphocytes along with an increase in its kinase activity following activation of the T-cell (Gringhius *et al.*, 1998; Means *et al.*, 1997). Furthermore, the expression of a kinase inactive form of CaMKIV in thymocytes resulted in a significant reduction of PMA/Io-induced IL-2 (Anderson *et al.*, 1997) suggesting a critical role for CaMKIV in IL-2

regulation. CaMKIV has also been shown to regulate transcription factors as evidenced by an increase in CREB phosphorylation and AP-1 activity in T-cells (Anderson *et al.*, 1997; Gringhuis *et al.*, 1997).

Once produced, diacylglycerol activates PKC; however, the specific contribution of PKC to T-cell activation is not well defined. An involvement of PKC is supported by considerable evidence using phorbol esters to activate PKC which results in transcription factor phosphorylation and gene expression. In fact, phorbol ester plus calcium ionophore (PMA/Ionomycin) is a stimulus that closely mimics activation of the TCR and is often used experimentally to stimulate T-cells *in vitro*. As depicted in figure 6, PMA activates PKC and ionomycin increases intracellular Ca^{2+} levels which together induce the signaling cascades necessary for T-cell activation. Multiple isozymes of PKC have been identified in several cell types, but the precise function of particular isoforms in T-cells is the focus of current investigation. Studies using activated isoforms have demonstrated that PKCε can stimulate NF-AT, AP-1, and NF-κB in the Jurkat cell line whereas PKCθ activates AP-1 in EL-4 cells (Baier-Bitterlich *et al.*, 1996; Genot *et al.*, 1995). In addition, PKCα has been shown to induce NF-AT activity (Berridge, 1997). Thus, it appears that several PKC isoforms may modulate the activity of DNA binding proteins and participate in T-cell activation. The involvement of PKC in the regulation of $p21^{ras}$ is controversial in T-cells as PKC-dependent and PKC-independent activation of Ras has been described (Izquierdo *et al.*, 1994; Ohtsuka *et al.*, 1996). The potential stimulation of Ras by PKC would suggest an upstream regulatory role for PKC in T-cell activation.

Activation of $p21^{ras}$ transmits signals to the nucleus through a kinase phosphorylation cascade involving Raf-1, MEK, and MAP kinase. Initiation of the Ras pathway occurs through the tyrosine phosphorylation and activation of the Raf-1 kinase by Ras-GTP. Once activated, Raf-1 phosphorylates serine residues on MEK-1 and MEK-2 which subsequently phosphorylate the MAPK kinases ERK-1 and ERK-2 on tyrosine

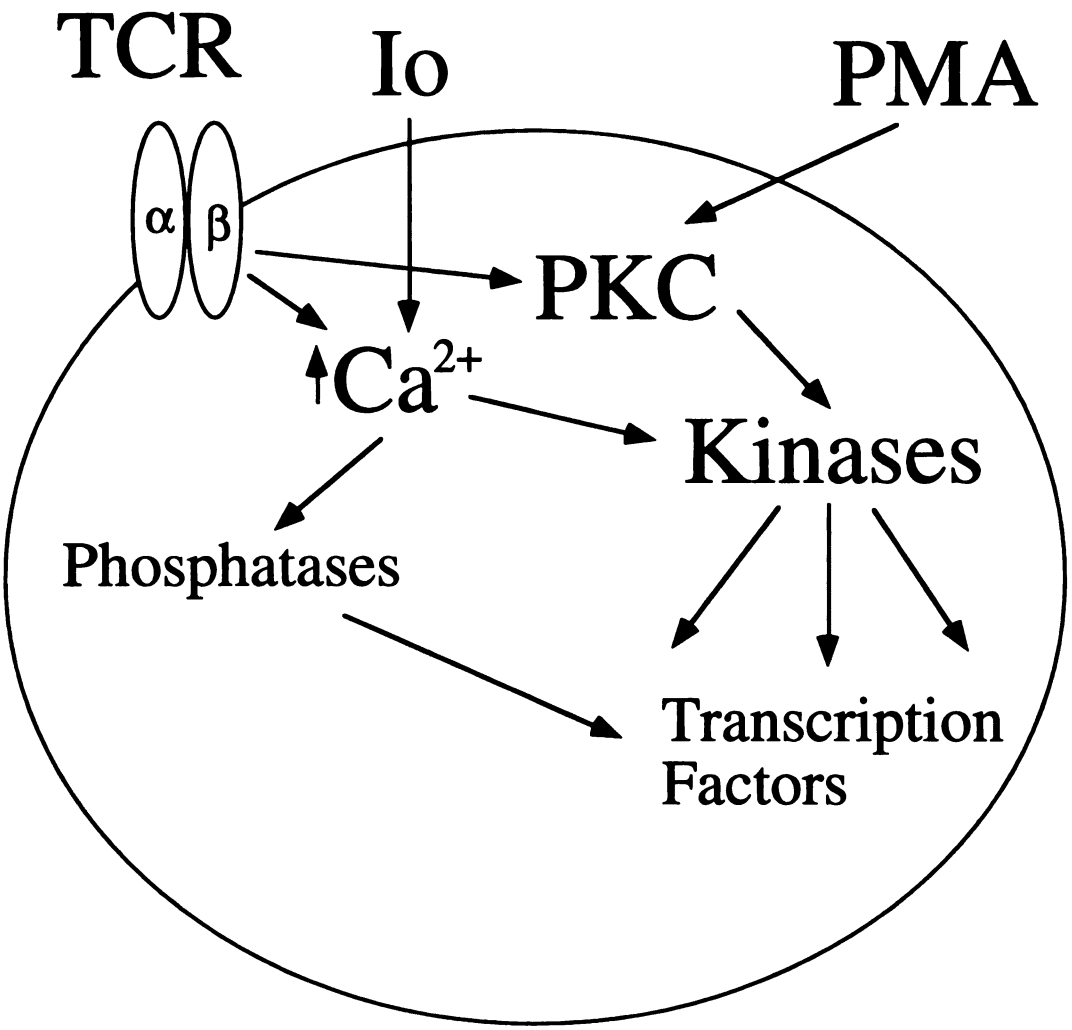


Figure 6. Activation of a T-cell by PMA plus Ionomycin. The phorbol ester and calcium ionophore combination of PMA/Ionomycin mimics signaling through the T-cell receptor and is used *in vitro* to activate T-cells. PMA activates PKC and ionomycin increases intracellular calcium levels. Together these signals activate kinase cascades and transcription factors essential for T-cell activation.

and threonine residues. The terminal event in the Ras cascade is the nuclear translocation of activated ERK-1 and ERK-2 and the phosphorylation of AP-1 and Elk-1 proteins (Graves *et al.*, 1995; Karin, 1995). The Ras pathway has also been described as an important signaling component of the TCR complex. For example, activation of T-lymphocytes with anti-CD3 antibody or the phytohemagglutinin mitogen was shown to induce Ras activity (Downward, 1990). Transfection of activated $p21^{ras}$ in the presence of increased intracellular Ca^{2+} stimulated the IL-2 promoter whereas expression of an inhibitory Ras mutant blocked the induction of IL-2 following TCR activation (Rayter *et al.*, 1992). Similarly, expression of a dominant-negative Ras in thymocytes abrogated TCR-mediated signals for proliferation and maturation (Sawn *et al.*, 1995). AP-1, the predominant downstream effector of the Ras pathway, is essential for the regulation of IL-2 expression. In addition to AP-1, Ras can synergize with calcium signals to activate NF-AT which results in IL-2 transcription (Woodrow *et al.*, 1993). Taken together, these studies have established a role for $p21^{ras}$ in T-cell activation and IL-2 production. Although the Ras signaling cascade is stimulated by the TCR, the regulation of $p21^{ras}$ in T-lymphocytes is poorly understood. As mentioned above, the activation of Ras by PKC is controversial due to conflicting results from independent studies. Recently, the modulation of MAPK activity by PI 3-kinase has been described in primary T-cells of the lymph node. Eder and coworkers demonstrated that activated PI 3-kinase was necessary for MEK-1 and ERK-2 activation (Eder *et al.*, 1998). Expression of a dominant-negative mutant of PI 3-kinase also inhibited the activation of AP-1 and NF-AT transcription factors and IL-2 expression in activated T-cells. These findings suggest that PI 3-kinase may regulate the Ras pathway through a PKC-independent mechanism in primary T-cells.

Complete activation of T-lymphocytes requires a second costimulatory signal delivered by CD28. CD28 is constitutively expressed as a homodimer on the surface of T-cells and interacts with B7 molecules on antigen presenting cells. Activation of CD28

delivers distinct signals to compliment those emanating from the TCR and is necessary to prevent a state of unresponsiveness known as anergy or tolerance (Harding *et al.*, 1992). Although the cytoplasmic domain of the CD28 costimulatory molecule lacks intrinsic enzyme activity, it does contain known motifs for protein-protein interactions (June *et al.*, 1994). The exact signaling events coupled to CD28 have not been completely resolved; however, evidence does exist for several potential mechanisms. CD28 costimulation induces the tyrosine phosphorylation of several cellular proteins including the cytoplamic tail of CD28 (Lu *et al.*, 1992; Pages *et al.*, 1994; Vandenberghe *et al.*, 1992). An association between the tyrosine kinases p56^{lck} and p59^{fyn} and CD28 has been shown in the Jurkat human T-cell line suggesting their involvement in CD28-mediated signaling (Hutchcroft and Bierer, 1994). Similarly, the activation of p72$^{ITK/EMT}$, a Tec family tyrosine kinase, occurs following ligation of the CD28 molecule (August *et al.*, 1994). Therefore, it is likely that multiple tyrosine kinases can mediate the early signals from CD28. The recruitment of PI 3-kinase to the cytoplasmic tail of CD28 has implicated its involvement in the CD28 signaling cascade. A role for PI 3-kinase is also supported by kinase inhibition studies which reported a decrease in IL-2 upon stimulation of resting T-cells in the presence of wortmannin, a PI 3-kinase inhibitor (Ward, 1995). Furthermore, mutation of essential tyrosine residues within the CD28 tail markedly diminished PI 3-kinase activation and IL-2 production induced by CD28 activation (Pages *et al.*, 1994). Anti-CD28 antibodies have been shown to activate the p21^{ras} pathway resulting in increased Raf-1 and MAPK activity suggesting the participation of these kinases in CD28-mediated signaling (Nunes *et al.*, 1994). The coupling of CD28 to the Ras signaling cascade may occur through the tyrosine phosphorylation of select adaptor molecules. For example, phosphorylation of the p62 protein is specific to CD28 activation, and p62 can associate with p21^{ras} (Nunes *et al.*, 1996) demonstrating a potential link between CD28 and MAPK pathway. Although the biochemical signals mediated by CD28 are still being dissected, the end result is the activation of transcription

factors. Evidence exists for the activation of both NF-κB and AP-1 following CD28 ligation suggesting that signaling through the TCR and CD28 can modulate some of the same DNA binding proteins (Emead *et al.*, 1996). In fact, it has been postulated that CD28-mediated signals may serve to increase the extent and/or the duration of TCR-mediated signals to achieve full activation of the T-cell. Ultimately, these signaling pathways culminate in the production and secretion of IL-2 as well as the induction of IL-2 receptor expression on the T-cell surface.

D. Interleukin-2 and the regulation of its expression

The hallmark of T-lymphocyte activation is the production and secretion of IL-2. IL-2 is a 15 kD glycoprotein produced only by activated T helper cells and is central to the generation of an immune response. IL-2 possesses pleiotropic activity and functions as an autocrine and paracrine factor by stimulating the proliferation and differentiation of several cell types within the immune system. IL-2 was initially characterized as a T-cell specific growth factor because it was essential for the clonal expansion and cell cycle progression of T-cells. Specifically, IL-2 stimulates advancement through G_1 into the S phase of the cell cycle following an antigen-induced transition from G_0 to G_1 (Stern and Smith, 1986). This cytokine also plays an important role in the differentiation of $CD4^+$ and $CD8^+$ T-cells into functional effector cells, an example being the stimulation of CTL cytolytic activity (Wagner and Rollinghoff, 1978). In addition to T-cell regulation, IL-2 plays a fundamental role in the proliferation and differentiation of B lymphocytes (Forman and Pure, 1991; Zubler *et al.*, 1984). For instance, examination of *in vitro* humoral immune responses detected an augmentation of antibody production in the presence of IL-2 (Watson *et al.*, 1979). Subsequent studies in murine B-cells determined that IL-2 could stimulate mRNA expression of the J chain and the secretory μ chain, both of which are necessary for the assembly of the IgM pentamer molecule (Blackman *et al.*, 1986; Nakanishi *et al.*, 1984). The positive effects of IL-2 on T- and B-cells demonstrate

its involvement in acquired immune responses; however, IL-2 can also modulate the cellular constituents of innate immunity. IL-2 functions to stimulate natural killer (NK) cell activity and promotes the derivation of lymphokine-activated killer cells from the NK cells. Additionally, an enhancement of the phagocytotic activity of macrophages has been described with IL-2 (Gomez *et al.*, 1998).

In order to elicit its biological effects, IL-2 must bind to IL-2 receptors (IL-2R) expressed on target cells. In fact, IL-2 receptor expression has been identified on Th_1 cells, $CD8^+$ cytotoxic T cells, NK cells, B cells, and macrophages (Gomez *et al.*, 1998). The complete IL-2R is composed of three separate polypeptides of varying sizes that have been established as the α, β, and γ chains . The trimeric complex of $\alpha\beta\gamma$ is considered to be the high affinity form of the IL-2 receptor. The α chain is a 55 kD protein, and its expression is rapidly induced by antigen or mitogen stimulation (Ascherman *et al.*, 1997). However, the presence of the α chain alone represents only a low affinity binding site for IL-2 (Wang and Smith, 1987). By comparison, a heterodimer of the β and γ chains produces an IL-2 binding site of intermediate affinity (Wang and Smith, 1987) whereas either chain alone has very low affinity for IL-2. The β chain of the receptor is 75 kD and possesses an extensive cytoplasmic region that is essential for IL-2-mediated signal transduction (Merida *et al.*, 1993). Similarly, the 64 kD γ chain is also involved in transmitting signals from the receptor, and several studies have shown that heterodimerization of IL-2Rβ and IL-2Rγ is required for IL-2R signaling (Nakamura *et al.*, 1994; Nelson *et al.*, 1994). The binding of IL-2 to its receptor activates the proximal signaling mediators recognized as the JAK tyrosine kinases. Specifically, the β and γ chains of the IL-2R interact with the JAK1 and JAK3 kinases which results in the tyrosine phosphorylation of target proteins (Miyazaki *et al.*, 1994; Nelson *et al.*, 1996). One such target of the JAK kinases is the STAT family of transcription factors that transmit cytokine-induced signals to the nucleus. The recruitment and activation of both Stat 3 and Stat 5 proteins has been demonstrated following engagement of the IL-2R

(Lin *et al.*, 1996; Stahl *et al.*, 1995). The down-regulation of high affinity receptors has been reported as a consequence of the IL-2:IL-2R interaction (Smith and Cantrell, 1985). This change is accompanied by a marked increase in the number of low affinity binding sites apparently as a mechanism for regulating cellular responses to IL-2.

Expression of the IL-2 gene is tightly regulated at the level of transcription; therefore, activation of the TCR is a prerequisite for IL-2 production. The 300 base pairs located directly upstream of the transcriptional start site are designated the minimal essential promoter region of the IL-2 gene (Serfling *et al.*, 1989). This portion of DNA is the least amount required for IL-2 mRNA synthesis and contains binding sites for a variety of transcription factors. As depicted in figure 7, the essential IL-2 promoter consists of two AP-1 sites, a pair of NF-AT sequences, two Octamer sites, an NF-κB sequence, and a CD28 responsive element (CD28RE) (Fraser *et al.*, 1991; Novak *et al.*, 1990). All of the transcription factors that bind to these sites are induced by T-cell activation with the exception of the octamer proteins. Moreover, the diversity of these DNA binding proteins illustrates the integration and cooperation of multiple signaling pathways for IL-2 expression.

The prototypic AP-1 complex is a heterodimer of Fos and Jun nuclear proteins and binds to TPA-responsive elements (TRE) following cellular activation. Two TRE motifs known as the proximal and distal AP-1 sites within the IL-2 promoter are located from -145 to -151 and -179 to -185 bp, respectively. DNA footprint protection of the AP-1p site (Jain *et al.*, 1992b) coupled with the detection of DNA bound Fos and Jun in activated T-cells (Vacca *et al.*, 1992) demonstrated that the proximal sequence was critical for induction of the IL-2 gene. Furthermore, mutation of the AP-1p sequence resulted in a substantial reduction in IL-2 induced by phorbol ester plus calcium ionophore (Jain *et al.*, 1992b). Similar analysis of the AP-1d site in these studies failed to detect significant changes in IL-2 expression. In addition to the proximal and distal TRE

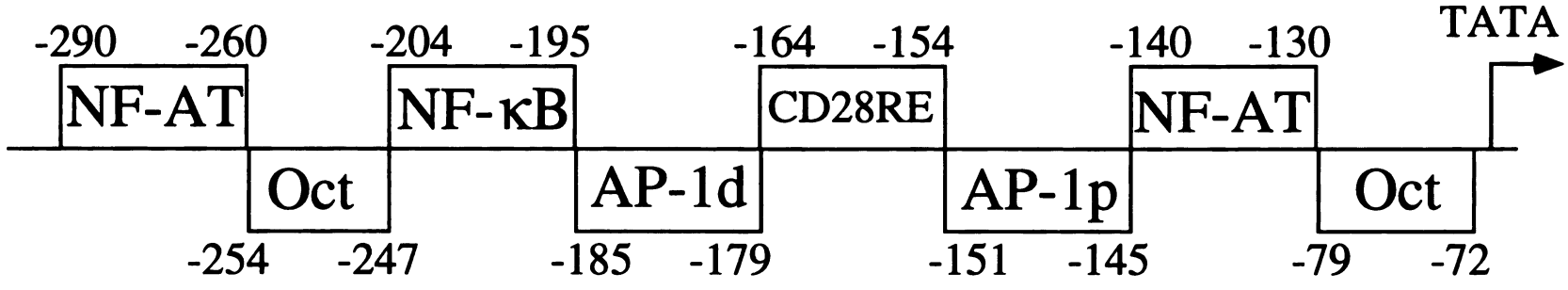


Figure 7. Minimal essential region of the IL-2 promoter. The region of the IL-2 promoter required for gene expression is approximately 300 bp upstream from the transcriptional start site. DNA binding sites for a variety of transcription factors including NF-κB, NF-AT, AP-1, and Octamer proteins are located within this region of the IL-2 promoter.

sequences, AP-1 participates in the regulation of IL-2 at both NF-AT and Octamer motifs as will be described below.

Two binding sites for NF-AT (nuclear factor of activated T-cells) are positioned at -130 to -140 bp and -260 to -290 bp upstream of the IL-2 transcriptional start site. Mutational studies have determined that the presence of both sequences is vital for IL-2 promoter activity (Boise *et al.*, 1993). T-cell activation induces a protein/DNA binding complex composed of NF-ATc and NF-ATn which display different subcellular localization. NF-ATc is dephosphorylated by calcineurin enabling its nuclear entry and DNA binding to NF-AT motifs (Flanagan *et al.*, 1991). The nuclear component (NF-ATn) of the protein complex is a Fos/Jun heterodimer (Jain *et al.*, 1992a) which functions to stabilize the binding of NF-ATc to the distal DNA site (Jain *et al.*, 1993). Interestingly, the NF-AT protein exhibits sequence homology to the NF-κB family of proteins (Nolan, 1994).

NF-κB/c-Rel transcription factors also play an important role in regulating the expression of IL-2 in activated T-cells. The recognized κB binding sequence is located between the AP-1d and one of the octamer motifs at base pairs -195 to -204 relative to the transcriptional start site. This κB site has been shown to be a significant regulatory element of the IL-2 promoter in primary T-cells (Hughes and Pober, 1996). Traditionally, the p65/p50 heterodimer binds the κB motif in activated T-cells to increase the expression of target genes. Several recent reports have also established that select members of the NF-κB family can bind to the CD28RE (-154 to -164) within the IL-2 promoter demonstrating further regulation of IL-2 by the NF-κB/c-Rel proteins (Butscher *et al.*, 1998; Ghosh *et al.*, 1993; Lai *et al.*, 1995; McGuire and Iacobelli, 1997). p50, p65, and c-Rel have all been identified in the CD28RE binding complex and the specific p65/c-Rel heterodimer was shown to be a potent transcriptional activator of the CD28RE (Ghosh *et al.*, 1993; Lai *et al.*, 1995).

The remaining DNA sequences of the IL-2 promoter are the binding sites for the octamer transcription factors (-72 to -79 bp and -247 to -254 bp). The Oct-1 protein is ubiquitously expressed in most cell types whereas Oct-2 is a lymphocyte specific protein. Interestingly, Oct-1 and Oct-2 bind to the octamer sequences with equal binding affinity and occupy adjacent DNA regions in resting cells. Examination of the DNA binding activity of Oct-1 in stimulated cells detected a necessary interaction with an octamer-associated protein (OAP^{40}) for IL-2 production (Ullman *et al.*, 1991). OAP^{40} was later described as the AP-1 heterodimer of Fos and Jun (deGrazia *et al.*, 1994; Ullman *et al.*, 1993) and was also shown to interact with the Oct-2 protein (deGrazia *et al.*, 1994).

E. The role of the cAMP pathway in T-cells

1. Inhibition by cAMP

The role of cAMP in immune function has conventionally been thought of as a negative regulatory signal based on studies utilizing high concentrations of membrane-permeable cAMP analogs or agents that dramatically elevate intracellular cAMP levels. This immunoinhibitory effect of cAMP has often been associated with a reduction in mitogen-stimulated lymphoproliferation and cell cycle arrest at the transition between G1 and the S phase (Goodwin *et al.*, 1977; Johnson *et al.*, 1988; Maraguchi *et al.*, 1984). The activation of PKA type I in T-cells has been shown to inhibit TCR-triggered proliferation (Skalhegg, 1992) providing a potential mechanism for dysregulation of T-cell replication by cAMP. An increase in cAMP also alters T-cell cytokine production by decreasing T_h1 cytokine levels and enhancing the expression of T_h2 cytokines. Several reports have described an inhibition of IL-2 and IFN-γ synthesis in activated mouse and human T-cells by forskolin, cAMP analogs, and PGE_2 (Betz and Fox, 1991; Didier *et al.*, 1987; Snijdewint *et al.*, 1993). By comparison, IL-4 was resistant to inhibition by cAMP and IL-5 was up-regulated by cAMP elevating agents in these T-cells. In addition, the presence of PGE_2 during the priming of naive $CD4^+$ T-cells inhibits the mRNA

expression of IL-2 and IFN-γ in a dose-dependent manner whereas IL-4 and IL-5 synthesis was not affected (Katamura *et al.*, 1995). Similar results were obtained with forksolin and dibutyryl cAMP in this study suggesting that cAMP may differentially regulate the development of T_h1 and T_h2 cytokine production.

Analysis of the molecular mechanisms of cAMP-mediated inhibition of T-cells has detected changes in the signaling cascades essential to T-cell activation. Several studies have reported that increased intracellular cAMP suppresses PLCγ activity and phosphoinositide turnover (Alava *et al.*, 1992; Bismuth *et al.*, 1988; Lerner *et al.*, 1988) indicating that cAMP can inhibit the initiation of upstream biochemical signals. An association of PKA I with the TCR/CD3 complex also occurs following T-cell activation (Skalhegg *et al.*, 1994). These authors speculated that the inhibitory effect of cAMP may be mediated by PKA phosphorylation interfering with TCR signaling pathways. Pretreatment of Jurkat cells with cholera toxin, which increases cAMP by direct activation of $G\alpha_S$, inhibits TCR-mediated activation of Ras resulting in decreased IL-2 promoter activity (Ohtsuka *et al.*, 1996). A similar inhibition of IL-2 has been demonstrated in EL-4 cells and occurs, in part, through the repression of NF-AT by cAMP (Tsuruta *et al.*, 1995). In addition to interfering with initial activation signals, cAMP also modulates the activity of downstream kinases. Recently, Hsueh and Lai observed that c-Jun N-terminal kinase (JNK) and MAP kinase display a diffferential sensitivity to cAMP-mediated inhibition in both EL-4 cells and splenic T-lymphocytes. The activity of JNK was selectively inhibited by cAMP and correlated with a repression of IL-2, but ERK activation remained unaffected by cAMP treatment (Hsueh and Lai, 1995). Furthermore, the inhibition of JNK was a delayed effect of cAMP and required protein synthesis for effective antagonism of kinase activity. In contrast, forskolin and dibutyryl cAMP were found to suppress the activation of ERK in phorbol ester or CD3 stimulated Jurkat and EL-4 T cell lines (Tamir *et al.*, 1996).

2. Stimulation by cAMP

Interestingly, those studies reporting an inhibition of immune function by cAMP analogs at high concentrations also found these analogs to be immunostimulatory at lower, physiological concentrations. A positive role of cAMP in lymphoid systems is further supported by the rapid and transient increase in intracellular cAMP levels following lymphocyte activation through the TCR or with mitogen (Hadden *et al.*, 1972; Kaminski *et al.*, 1994; Kammer, 1988; Kammer *et al.*, 1988; Russell, 1978). In contrast to inhibition by PKA, considerable evidence exists for a stimulatory role of PKA in T-cell activation and proliferation. Stimulation of human T lymphocytes with either anti-CD3 or phorbol ester/calcium ionophore resulted in an increase of type I PKA activity and phosphorylation of proteins associated with the T-cell plasma membrane (Laxminarayana *et al.*, 1993). Additionally, expression of antisense oligomers to the PKA regulatory or catalytic subunits produced an inhibition of basal PKA activity and IL-2 synthesis in activated mouse hybridoma cells (Sugiyama *et al.*, 1997). These findings implicate PKA as an antigen-responsive kinase involved in the propagation of TCR signals essential for T-cell lymphokine expression. A critical involvement of PKA in cell cycle progression has also been demonstrated through the induction of cell cycle arrest by inhibition of PKA and reversal of this arrest by low concentration of 8-bromo-cAMP (Grieco *et al.*, 1996). Moreover, direct addition of cAMP analogs (10-100 μM) to mouse spleen cultures was shown to increase the humoral immune response to sheep red blood cells as well as to enhance PMA/Io stimulated lymphoproliferation (Koh *et al.*, 1995). Furthermore, activation of human peripheral T-lymphocytes with mitogen induces CRE binding complexes (Wollberg *et al.*, 1994). Together this evidence implicates cAMP as a positive second messenger in lymphocyte activation and function at physiological concentrations.

Despite the lack of a consensus CRE sequence in the IL-2 promoter, several recent reports have described a fundamental role for the CREB/ATF proteins in IL-2

regulation (Barton *et al.*, 1996; Butscher *et al.*, 1998; Hsueh *et al.*, 1997). This was initially shown by a novel study in transgenic mice expressing a dominant-negative form of CREB. Expression of this abberant form of CREB produced a significant decrease in mitogen-stimulated proliferation and IL-2 production in thymocytes and induced cell cycle arrest in these T-cells (Barton *et al.*, 1996). Supershift studies have also identified CREB as part of the protein complex binding to the AP-1 proximal (AP-1p) site of the IL-2 promoter (Chen and Rothenberg, 1993). In addition, stimulation of EL-4.IL-2 cells with PMA/Io plus forskolin enhanced binding to the AP-1p site further suggesting the direct involvement of CREB/ATF proteins in IL-2 regulation through the cAMP pathway (Condie *et al.*, 1996). Collectively, these findings indicate that the cAMP pathway has a positive regulatory role during T-lymphocyte activation.

MATERIALS AND METHODS

I. Animals

Virus-free female B6C3F1 mice, 5-6 weeks of age were purchased from the National Cancer Institute. On arrival, mice were randomized and transferred to plastic cages containing a saw dust bedding (5 mice per cage). Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum*. Thymocyte studies were performed using mice between 6 and 8 weeks of age. Animal holding rooms were kept at 21-24°C and 40-60% relative humidity with a 12 hour light/dark cycle.

II. Chemicals

Cannabinol (CBN) and Δ^9-THC were both provided by the National Institute on Drug Abuse. LPS, PMA, ionomycin, forskolin, and dibutyryl cAMP were all purchased from Sigma (St. Louis, MO). H89 was purchased from Calbiochem (San Diego, CA).

III. Culture Medium

For cell proliferation, IgM AFC responses, and the ELISA, splenocytes or thymocytes were cultured in RPMI 1640 supplemented with 5% BCS (Hyclone, Logan, UT), 2 mM L-glutamine, antibiotic-antimicotic (100 units penicillin and 100 μg streptomycin) (GIBCO, Grand Island, NY), and 5 x 10^{-5} M 2-mercaptoethanol (complete RPMI medium). For EMSA and western analysis, 1% BCS was used in complete medium.

IV. Antibodies

Rabbit polyclonal antibodies for CREB-1, ATF-1, ATF-2, p50, p65, c-Rel, and IκB-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-

CREB/phospho-ATF-1 antibody was purchased from New England Biolabs, Inc. (Beverly, MA).

V. *In vitro* proliferative responses

Splenocytes were cultured in a 96 well culture plate at 1 x 10^5 cells/well in the presence of either vehicle (0.1% ethanol), CBN (1, 5, 10, 15, 20 μM), or Δ^9-THC (22 μM) and stimulated with either anti-CD3 (1.0 μg/ml; PharMingen, San Diego, CA), LPS (100 μg/ml), or PMA (80 nM) plus ionomycin (1 μM). Splenocytes were cultured at 37°C and 5% CO_2 for 48 hr (PMA/Io) or 72 hr (anti-CD3 and LPS) and pulsed with 1.0 μCi/well of [^{3}H]-thymidine (NEN, Boston, MA) for the last 15 hr of the culture period. Cells were harvested onto glass fiber filters with a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA), and tritium incorporation was measured using a Packard 460C liquid scintillation counter. Results are expressed as mean cpm ± SE of quadruplicate samples.

VI. *In vitro* IgM antibody forming cell response (AFC)

Splenocytes were cultured at 1 x 10^7 cells/ml in a 48 well Costar culture plate (Cambridge, MA) and Δ^9-THC (22 μM), CBN (1, 5, 10, 15, 20 μM), or vehicle (0.01% ethanol) were added directly to spleen cell cultures. Each well was sensitized with 6.5 x 10^6 sRBC and cultured for 5 days in a Bellco stainless steel tissue culture chamber pressurized to 6.0 psi with a gas mixture consisting of 10% O_2, 7% CO_2, and 83% N_2. The culture chamber was continously rocked on a rocking platform for the duration of the culture period. Enumeration of the AFC response was performed as previously described (Kaminski and Holsapple, 1987). Results were expressed as the mean AFC/10^6 splenocytes ± SE.

VII. *In vivo* IgM antibody forming cell response (AFC)

Mice (5 animals/group) were treated orally with either vehicle (corn oil, Sigma, St. Louis, MO), cannabinol, or Δ^9-THC for 3 consecutive days and were sensitized on day 2 with sheep erythrocytes (sRBC) (5 x 10^8 c/ml) by i.v. injection. Enumeration of the AFC response was performed on day 5 as previously described (Schatz *et al.*, 1993). Briefly, spleens were removed from the mice, made into a single cell suspension, and adjusted to 1 x 10^7 cells/ml in RPMI 1640. A 50 µl aliquot of splenocytes was added to a 12 x 75 mm heated culture tube containing 400 µl of 0.5% agar (DIFCO, Detroit, MI) dissolved in EBSS plus 0.05% DEAE-Dextran. Guinea pig complement (25 µl) and sRBC (25 µl) were added to each tube and samples were quickly vortexed. Each sample was poured onto a 100 x 15 mm petri dish and immediately covered with a 45 x 50 mm microscope cover slip. The petri dishes were placed in an incubator at 37°C for 3 hr and the number of AFCs were counted using a plaque viewer. Results are expressed as the mean AFC/10^6 splenocytes ± SE for 5 mice.

VIII. Pronase viability determination

Resuspended splenocytes (100 µl) from each AFC treatment group were added to an equal volume of pronase (Calbiochem-Behring Corp., San Diego, CA) and incubated for 10 min at 37°C. Following incubation, splenocytes were diluted with 10 ml of Isoton (Coulter, Addison, NJ) and counted on a Coulter counter. The percent viability was calculated by the following equation: (cell counts with pronase/cell counts without pronase) x 100 = viable cells.

IX. cAMP determination

Splenocytes or thymocytes were resuspended in RPMI 1640 containing 1 mg/ml fatty acid-poor bovine serum albumin and adjusted to 5 x 10^6 c/ml. Aliquots of cells (1 ml) were incubated with either vehicle (0.1% ethanol), Δ^9-THC (22 µM), or CBN (1, 5,

10, 15, 20 μM) for 10 min followed by stimulation with forskolin (50 μM) for 15 min at 37°C in 5% CO_2. Adenylate cyclase activity was terminated by addition of extraction buffer (1 ml 1N HCl/100 ml EtOH) and cells were sonicated for 20 sec on ice to release the intracellular cAMP into solution. Samples were centrifuged at 1600 x g for 15 min to pellet the cellular debris and the supernatant was retained. Levels of cAMP in reconstituted cell lysates were determined using a cAMP RIA assay kit (Diagnostic Products Inc., Los Angeles, CA). Tritium incorporation was measured using a Packard 460C liquid scintillation counter. The concentration of cAMP in each sample was determined by comparison with a standard curve. Results are expressed as mean cpm ± SE of triplicate samples.

X. Analysis of PKA activity

Splenocytes were washed in phosphate buffered saline and lysed in ice cold lysis buffer (0.25 M Sucrose, 5 mM Tris Base, 5mM EGTA, 1 mM PMSF, 0.1 mM DTT, 0.1% Trition X100, 10 μg/ml leupeptin, 10 mg/ml aprotinin) followed by gentle sonication (i.e., twice at 50 Hz for 5 sec). Lysates were centrifuged at 270 x g for 5 min, and aliquots of the cell extract (100 μl) were incubated with appropriate concentrations of CBN or Δ^9-THC for 5 min in triplicate for use in the PKA assay (GIBCO BRL, Grand Island, NY). The assay reaction mixture contained 10 μl of cell extract, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP (20 μCi/ml γ-[32P]ATP, 0.25 mg/ml BSA, 50 μM Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; PKA substrate). The background enzyme activity for each treatment group was determined using 1 μM PKI-(6-22)-amide and total PKA activity was assessed using 10 μM cAMP. Samples were incubated at 37°C for 10 min and spotted on phosphocellulose discs (20 μl of sample) followed by two phosphoric acid washes (1% v/v) and two washes with distilled water. The amount of ^{32}P was quantified by scintillation counting.

XI. Cellular Lysis Methods

A. Whole Cell

Thymocytes (1 x 10^6 c/ml) were stimulated with PMA/Io (80 nM/1 μM) for the indicated time points (0-120 min). For the studies with CBN, thymocytes were pretreated with CBN (1, 5, 10, 15, 20 μM) for 15 min and stimulated with PMA/Io (80 nM/1 μM) for 30 min. At the end of the culture period, thymocytes were lysed with 500 μl of RIPA buffer (1x phosphate buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1mM DTT, 1 mM PMSF, and 1 μg/ml of aprotinin and leupeptin. Following addition of RIPA buffer, cells were homogenized with a dounce homogenizer (30 strokes) and incubated on ice for 30 min. Samples were then centrifuged at 17500 x g for 20 min and the supernatant retained as whole cell protein.

B. Nuclear

Splenocytes and thymocytes were stimulated with forskolin (50 μM) or PMA/Io (80 nM/ 1 μM) in the presence and absence of CBN (20 μM). Following treatment, cells were lysed with HB buffer (10 mM HEPES and 1.5 mM $MgCl_2$, 1mM DTT, 1mM PMSF, and 1 μg/ml of aprotinin and leupeptin) and the nuclei were pelleted by centrifugation at 6700 x g for 5 min. Nuclei were lysed on ice for 30 min in hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) supplemented with 1mM DTT, 1 mM PMSF, and 1 μg/ml of aprotinin and leupeptin. Following nuclear lysis the samples were centrifuged at 17500 x g for 15 min, and the supernatant was retained.

C. Cytosolic

Treated thymocytes were lysed with 500 μl of HB buffer (same as above in nuclear lysis protocol) and incubated on ice for 15 min. The nuclei were pelleted by centrifugation at 6700 x g for 5 min and the supernatant was retained. Glycerol (10%)

was added to the supernatant and samples were centrifuged at 100,000 x g for 1 hr at 4°C. The resulting supernatant contained cytosolic proteins.

XII. Protein Determination

Determination of protein values for samples isolated using all lysis methods was performed using the bicinchoninic acid method (Sigma, St. Louis, MO). A standard curve was prepared using increasing concentrations of BSA (0, 5, 10, 15, 20, and 25 μg) in a total volume of 100 μl. Unknown protein samples (10 μl) were also prepared into a final volume of 100 μl. The protein assay reagent (bicinchoninic acid plus copper (II) sulfate) was added (2.0 ml/sample) and samples were incubated for 30 min at 37°C. The absorbance was determined at 562 nm using a Beckman DU-600 spectrophotometer (Fullerton, CA).

XIII. Electrophoretic mobility shift assay (EMSA)

Double stranded DNA oligomers containing either the CRE (TGACGTCA) or the κB (GGGGACTTTCC) consensus sequence were end-labeled with [γ-^{32}P]dATP (NEN, Boston, MA) using T4 kinase (Pharmacia Biotech, Piscataway, NJ). Nuclear extract (5 μg) was incubated in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1mM DTT, 1 mM PMSF, and 1 μg/ml of aprotinin and leupeptin) with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled probe for 10 min on ice. DNA binding activity was separated from free probe using a 4% acrylamide gel (National Diagnostics, Atlanta, GA) in 1x TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried and autoradiographed for analysis. For supershift analysis, antibodies for ATF-1, ATF-2, p50, p65, and c-Rel were added following the incubation of nuclear proteins with radiolabeled probe. CREB-1 antibody was incubated with the nuclear proteins prior to addition of the radiolabeled CRE probe.

XIV. Western analysis

Isolated proteins from treated thymocytes (25 μg) were incubated with 4x loading dye (62.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol) and heated for 10 min at 95 °C. Samples were then resolved on a 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose in transfer buffer (24 mM Tris, 191 mM Glycine, 20% methanol). The nitrocellulose was blocked for 1 hr with 5% milk-TBST (Tris Buffered Saline plus Tween 20) and probed with the indicated primary antibody. For the IκB-α studies, the nitrocellulose was incubated with either IκB-α (200 ng) or IκB-α (200 ng) plus p65 antibody (200 ng) for 2 hr at room temperature. Analysis of phospho-CREB was determined using 30 ng of phospho-CREB/ATF-1 specific antibody. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for phospho-CREB, p65, and IκB-α protein detection using the ECL chemiluminescent system (Amersham, Arlington Heights, IL).

XV. EMSA-Western

Nuclear proteins (8 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and either the ^{32}P-labeled κB probe (30,000 cpm) or the cold κB probe (10 pMoles) in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Following electrophoresis, the ^{32}P-labeled samples were dried and subjected to autoradiography, and the protein complexes bound to cold κB probe were transferred overnight at 4°C to nitrocellulose in transfer buffer (0.4% SDS, 48 mM Tris, 39 mM Glycine, 20% methanol). Following transfer, nitrocellulose was blocked with 1% milk-TBST for 1 hr followed by incubation with either p65 (200 ng) or c-Rel (400 ng) antibody for 2 hr. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system (Amersham, Arlington Heights, IL).

XVI. Quantitative RT-PCR

A. Preparation of Internal Standard for RT-PCR

A recombinant mRNA (rcRNA) was constructed using a rat β-globin sequence as the spacer gene for the IL-2 internal standard (IS). The IS contained specific PCR primer sequences for mouse IL-2 and was added to RNA samples in a dilution series. The primer sequences from 5' to 3' for IL-2 are: forward primer = TGCTCCTTGTCAACAGCG, and reverse primer = TCATCATCGAATTGGCACTC. The design of the IS primers from 5' to 3' is: IS forward primer = T7 promoter (TAATACGACTCACTATAGG), IL-2 forward primer (stated above), and rat β-globin forward primer (GGTGCTTGGAGACAGAGGTC); and IS reverse primer = $(dT)_{18}$, IL-2 reverse primer (stated above), and rat β-globin reverse primer (TCCTGTCAACAATCCACAGG). PCR conditions for making the internal standard were performed using 100 ng of rat genomic DNA as previously described (Vanden Heuvel *et al.*, 1993). Purification of PCR products was performed using the Wizard PCR Prep DNA purification system (Promega, Madison, WI) and was followed by transcription of the products into RNA using Promega's Gemini II *In Vitro* Transcription System. The IS was treated with RNase-free DNase to remove the DNA template.

B. Quantitative competitive reverse transcriptase-polymerase chain reaction

RNA was isolated using TriReagent (Sigma, St. Louis, MO) as outlined by Chomczynski and Mackey (Chomczynski and Mackey, 1995). Competitive RT-PCR was performed as previously described (Gilliland *et al.*, 1990) except that rcRNA was used as the internal standard instead of genomic DNA. Aliquots of internal standard ranging from 5×10^2 to 1×10^6 molecules were used to generate a standard curve for IL-2. Total RNA from treated thymocytes (100 ng) and known amounts of IS rcRNA were reverse-transcribed into cDNA using $oligo(dT)_{15}$ as primers. A PCR reaction mixture containing PCR buffer, 4 mM MgCl2, 6 pmol of IL-2 forward and reverse primers, and 2.5 units of

Taq DNA polymerase was added to the cDNA products. Samples were then heated to 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec followed by an additional extension step at 72°C for 5 min. This process was repeated for 32 cycles to amplify the IL-2 DNA product. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. The IL-2 primers produce an amplified product of 391 bp from the sample RNA and a 474 bp product from the IS. Quantitation was performed using the Gel Doc 1000 (BioRad, Hercules, CA) to determine the amount of IL-2 present in the cellular RNA. The ratio of the volume of the IS rcRNA to IL-2 RNA bands is plotted against the concentration of IS rcRNA (molecules) added to each reaction. The point at which the ratio of IS to IL-2 RNA equals one is designated the "cross-over" point. This point indicates the amount of IL-2 molecules present in the cellular RNA sample.

XVII. ELISA

Thymocytes were cultured in triplicate (1 x 10^6 c/ml) in 12-well culture plates for 24 hr. Supernatants were collected post-stimulation and quantified for IL-2 using the sandwich ELISA method. The IL-2 standard (mouse recombinant IL-2), purified rat anti-mouse IL-2 antibody, and biotinylated anti-mouse IL-2 antibody were purchased from PharMingen (San Diego, CA). Immunolon IV Removawell microtiter strip wells (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 50 µl of purified rat anti-mouse IL-2 antibody (1.0 µg/ml) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were blocked with 300 µl of BSA (3% v/v) in 0.01 M PBS containing 0.1% (v/v) Tween 20 (BSA-PBST) at 37 °C for 30 min. Wells were washed 4 times with PBST followed by addition of IL-2 standard or sample (50 µl) and incubated at 37°C for 1 hr. The standard curve was generated using recombinant mouse IL-2. After the incubation period, the plate was washed 4 times with PBST and once with distilled water. The biotinylated anti-mouse IL-2 (1.5 µl/ml) was diluted in 3% BSA-PBST, added to each

well (50 µl), and incubated at room temperature for 1 hr. The plate was washed 6 times using the PBST solution and once with distilled water followed by addition of 50 µl streptavidin-horseradish peroxidase (1.5 µg/ml) for 1 hr at room temperature. Lastly, samples were washed 8 times and the bound peroxidase conjugate was detected by addition of substrate solution (100 µl/well) containing 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/ml tetramethylbenzidine (Fluka Chemical Corp., Ronkonkoma, NY), and 1% H_2O_2. The reaction was terminated with an equal volume of 6N H_2SO_4, and absorbance values were obtained at 450 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). IL-2 concentrations were quantitated using Delta Soft 3 software (BioMetallics, Princeton, NJ).

XVIII. Densitometry

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

XVIX. Statistical analysis

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

EXPERIMENTAL RESULTS

The identification of CB1 and CB2 cannabinoid receptors on immunocompetent cells has provided a putative mechanism of action for cannabinoids; however, the molecular mechanisms of cannabinoid-induced immune suppression have not been characterized. Furthermore, the preference of cannabinol for the CB2 receptor coupled with its minimal activity in the CNS makes cannabinol an intriguing plant-derived cannabinoid compound. The hypothesis of this research project was: Cannabinol disrupts the cAMP signaling cascade in thymocytes thereby decreasing the activation of CREB/ATF and NF-κB/c-Rel transcription factors. The DNA binding of these proteins within the promoter region of IL-2, a T-cell derived lymphokine, is subsequently inhibited and ultimately alters immunocompetence. The specific aims of this research were: 1) to examine the effects of cannabinol on immune function, cAMP-mediated signal transduction, and IL-2 expression; 2) to identify specific CREB/ATF and NF-κB/c-Rel proteins modulated by cannabinol in activated thymocytes; 3) to characterize the inhibition of CREB and NF-κB transcription factor activation by cannabinol, and 4) to determine the role of cAMP and PKA in the regulation of IL-2, CREB, and NF-κB following T-cell activation.

The results for studies fulfilling these objectives are described in six separate sections below. The effects of cannabinol on immune function endpoints are described in section I. Inhibition of the cAMP signaling pathway by cannabinol, including effects on adenylate cylcase, PKA, and CRE binding activity in splenocytes and thymocytes is presented in section II. Section III describes the cannabinol-induced inhibition of IL-2 mRNA and protein secretion in thymocytes. In section IV, the effects of cannabinol on CREB/ATF and NF-κB/c-Rel DNA binding activity following PMA/Io activation of thymocytes are presented. The identification of specific CRE and κB transcription factors modulated by cannabinol is also provided in section IV. The studies in section V

address the mechanism by which cannabinol modulates the activation of CREB and NF-κB proteins. Lastly, results characterizing the role of cAMP and PKA in the regulation of CREB, NF-κB, and IL-2 are shown in section VI.

I. Effects of cannabinol on immune function

A. Proliferative responses

To determine the potential immunomodulatory activity of cannabinol, mitogen-induced proliferation of mouse splenocytes was measured. As presented in Table 1, cannabinol inhibited anti-CD3 induced proliferation by 30% and 74% at 15 and 20 μM concentrations, respectively. Cannabinol also produced a dose dependent inhibition of LPS-induced proliferation at all concentrations (1, 5, 10, 15, 20 μM) tested (Table 1). It is notable that the magnitude of inhibition by Δ^9-THC (22 μM), a positive control, and cannabinol (20 μM) were very comparable. Cannabinol was also found to alter antigen receptor independent lymphocyte activation as shown by a marked inhibition of PMA/Io-induced proliferation (Table 1). Interestingly, as previously reported with Δ^9-THC, of these three proliferative stimuli tested, the PMA/Io response appeared to be the most sensitive to inhibition by cannabinol (Kaminski *et al.*, 1994; Schatz *et al.*, 1993).

B. IgM Antibody forming cell response

The effects of cannabinol on humoral immunity were investigated by employing the hemolytic plaque assay. The IgM T-dependent AFC humoral response has been previously found to be highly sensitive to inhibition by Δ^9-THC, CP-55,940, and HU-210 (Kaminski *et al.*, 1992; Schatz *et al.*, 1993); therefore, a similar experimental approach was utilized to evaluate the activity of cannabinol. In the present studies, cannabinol exhibited a dose dependent inhibition of the *in vitro* T-dependent AFC response at 10, 15, and 20 μM concentrations as compared to the vehicle control (0.01% ethanol) (Table 2). No effect on cell viability was observed at any of the cannabinol concentrations tested.

TABLE 1

Effect of cannabinol on mitogen-stimulated proliferation

Naive (NA) mouse spleens were isolated and made into a single cell suspension. Splenocytes were cultured in a 96-well plate at 1 x 10^5 cells/well in the presence of either vehicle (VH; 0.1% EtOH), CBN, or Δ9-THC and stimulated with either PMA (80 nM) and Io (1 μM), anti-CD3 (0.5 μg/ml), or LPS (100 μg/ml). Splenocytes were pulsed with [^{3}H]-thymidine for the last 15 hr of the culture period. Data are expressed as the mean ± SE for quadruplicate samples. *$p < 0.05$ as determined by Dunnett's t-test with comparison to the vehicle group.

	Mitogenic Stimuli					
Treatment	anti-CD3	% Control	LPS	% Control	PMA/Io	% Control
No mitogen	1203±74*	7	1015± 96*	3	1386± 166*	2
NA	18368±777	102	34544±1277	94	90018±2864	101
VH	17933± 1009	100	36902±1609	100	89175±3429	100
THC 22 μM	8062±448*	45	8964± 646*	24	44585±3653*	50
CBN 1 μM	20020±487	112	29968± 219*	81	76303± 862*	86
CBN 5 μM	22677± 1304*	126	25411± 339*	69	63870±1549*	72
CBN 10 μM	20793±1245	116	21389±1251*	58	41339±1935*	46
CBN 15 μM	12470± 900*	70	19153±1393*	52	37721±2506*	42
CBN 20 μM	4683± 789*	26	14001± 904*	38	16615± 760*	19

TABLE 2

Effect of cannabinol on the *in vitro* IgM AFC response to sRBC

Spleens from naive B6C3F1 mice were isolated and made into a single cell suspension. Splenocytes (1 x 10^7 cells/ml) were added to a 48 well culture plate and treated with either vehicle (VH; 0.1% EtOH), CBN, or Δ9-THC. The cultures were sensitized with sRBC (1 x 10^9 cells/ml). On day 5, the number of antibody forming cells (AFC) were determined. The results are expressed as the mean ± SE for quadruplicate samples. *$p<0.05$ as determined by Dunnett's t-test with comparison to the vehicle group.

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

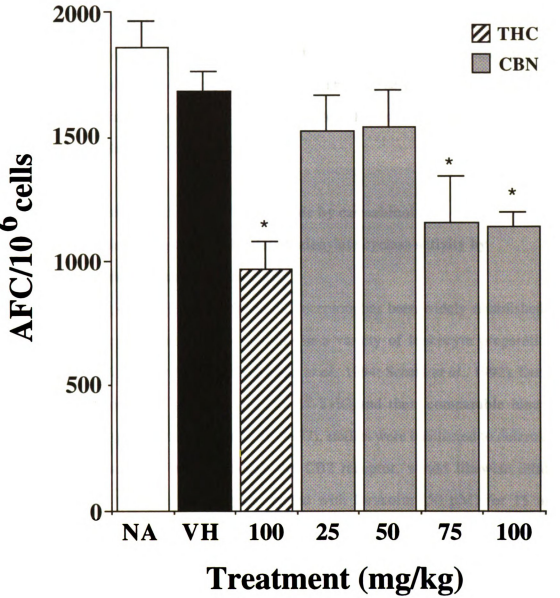


Figure 8. Effect of cannabinol on the *in vivo* IgM AFC response to sRBC. Female B6C3F1 mice were treated orally for 3 consecutive days with either vehicle (VH; corn oil), Δ^9-THC, or CBN at the indicated doses. Mice were sensitized with sRBC (5 x 10^8 c/ml) on day 2 and the IgM AFC response was measured on day 5. The results are expressed as the mean $\pm$ SE for five animals per treatment group. *$p<0.05$ as determined by Dunnett's t-test with comparison to the vehicle group.

The effects of cannabinol administration on the *in vivo* IgM AFC response were also examined. For these studies, mice were treated with cannabinol (25, 50, 75, and 100 mg/kg) for 3 consecutive days and sensitized with sRBC on day 2. As shown in figure 8, cannabinol inhibited the IgM AFC response at 75 mg/kg and 100 mg/kg.

In summary, the analysis of immune function endpoints demonstrated that cannabinol exhibits immunosuppressive activity. The degree of immune suppression produced by cannabinol was comparable to that of Δ^9-THC and was consistent with the preference of cannabinol for the CB2 receptor.

II. Inhibition of the cAMP signaling cascade by cannabinol

A. Inhibition of forskolin-stimulated adenylate cyclase activity by cannabinol in murine leukocytes

The binding of Δ^9-THC to cannabinoid receptors has been widely established to negatively regulate adenylate cyclase activity in a variety of leukocyte preparations (Condie *et al.*, 1996; Jeon *et al.*, 1996; Kaminski *et al.*, 1994; Schatz *et al.*, 1992). Due to the similarity in structure of cannabinol to Δ^9-THC and their comparable binding affinities in mouse splenocytes (Schatz *et al.*, 1997), studies were conducted to determine if cannabinol, presumably acting through the CB2 receptor, would likewise inhibit adenylate cyclase. Mouse splenocytes treated with forskolin (50 μM) for 15 min exhibited stimulation of adenylate cyclase as demonstrated by approximately a 4-fold increase in intracellular cAMP as compared to the unstimulated naive and vehicle treated cells (Figure 9). Pretreatment of splenocytes with cannabinol prior to forskolin stimulation decreased intracellular cAMP by 25% at 15 and 20 μM. The magnitude of inhibition by 20 μM cannabinol was again comparable to 22 μM Δ^9-THC, the positive control. The effects of cannabinol on adenylate cyclase activity in thymocytes were also investigated because past studies have shown that T-cells are markedly sensitive to inhibition by cannabinoid compounds (Condie *et al.*, 1996; Kaminski *et al.*, 1992; Schatz

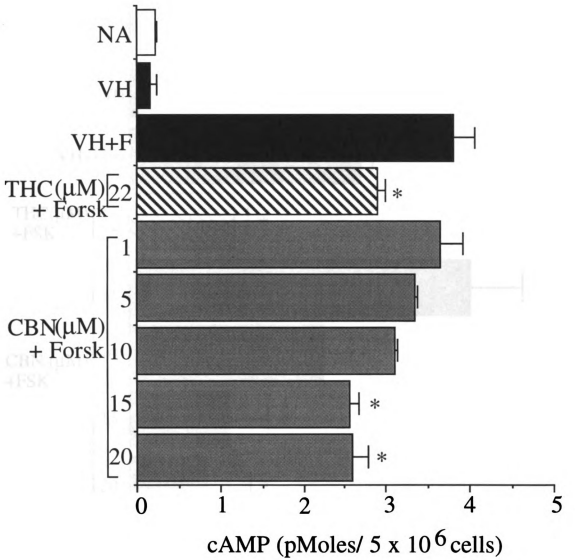


Figure 9. Inhibition of forskolin-stimulated adenylate cyclase activity by cannabinol in mouse splenocytes. Spleens were isolated and made into a single cell suspension of 5 x 10^6 cells/ml. Splenocytes were treated with either vehicle (VH; 0.1% ethanol), CBN, or Δ^9-THC for 10 min followed by a 15 min forskolin stimulation (50 μM). Intracellular cAMP concentrations are expressed as the mean ± SE for triplicate samples. *$p < 0.05$ as determined by Dunnett's t-test with comparison to the forskolin-stimulated vehicle group. One of three representative experiments is shown.

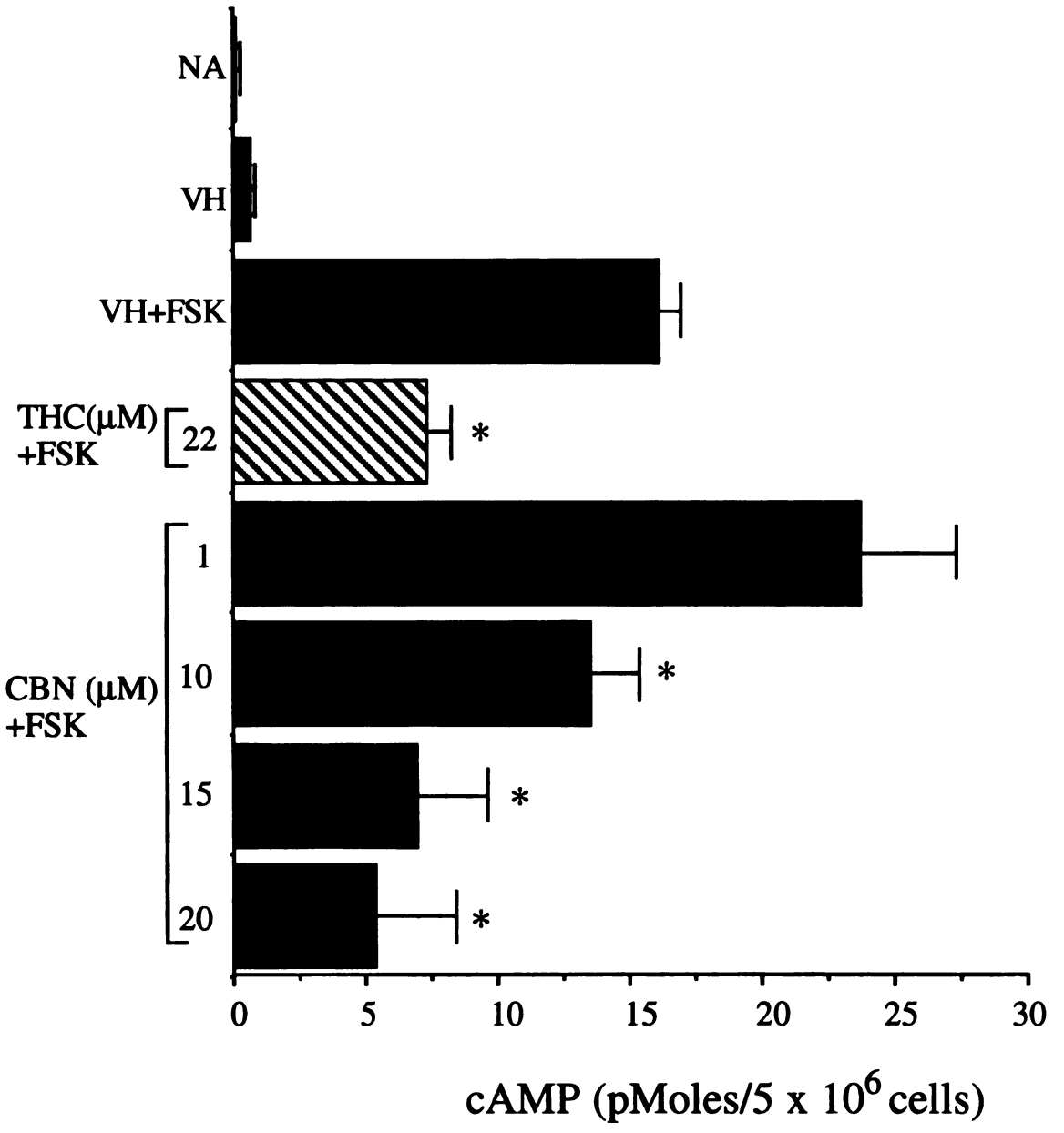


Figure 10. Cannabinol-mediated inhibition of forskolin-stimulated adenylate cyclase activity in mouse thymocytes. Thymocytes were freshly isolated, adjusted to 5 x 10^6 cells/ml, and incubated with either vehicle (VH; 0.1% ethanol), CBN, or Δ^9-THC for 10 min followed by a 15 min stimulation with forskolin (50 μM). Intracellular cAMP concentrations are expressed as the mean ± SE for triplicate samples. *$p < 0.05$ as determined by Dunnett's t-test with comparison to the forskolin-stimulated vehicle group. One of three representative experiments is shown.

et al., 1993). Consistent with this observation, cannabinol dose-dependently inhibited forskolin-stimulated adenylate cyclase activity in mouse thymocytes (Figure 10). Interestingly, the increase in adenylate cyclase activity by forskolin was significantlygreater in thymocytes than in splenocyte preparations. Scherer and coworkers have recently demonstrated a similar difference in intracellular cAMP levels following forskolin stimulation of thymocytes suggesting that cAMP may play a critical role in T-cell differentiation (Scherer *et al.*, 1995). Moreover, the magnitude of adenylate cyclase inhibition by cannabinol is significantly greater in thymocytes than in splenocytes which further supports the sensitivity of T-cells to cannabinoids.

B. Effect of cannabinol on PKA activity

PKA is immediately downstream from adenylate cyclase, and increases in intracellular cAMP result in the dissociation and activation of the kinase catalytic subunit. The inhibition of adenylate cyclase by Δ^9-THC in the EL-4 cell line consequently leads to a reduction in PKA activity (Condie *et al.*, 1996). Considering the inhibition of adenylate cyclase by cannabinol, splenocyte PKA activity was evaluated in the presence of cannabinol. As shown in figure 11, cannabinol produced a concentration-dependent inhibition of PKA activity at all concentrations (1, 5, 10, 15, 20 μM) tested. Again, the magnitude of PKA inhibition between cannabinol and Δ^9-THC was comparable at 20 μM and 22 μM concentrations, respectively. It is notable that experiments have been performed in the presence of exogenous cAMP and no direct inhibition of PKA activity was observed with Δ^9-THC (Koh *et al.*, 1997). These studies indicate that the inhibition of PKA activity by cannabinol is mediated through an inhibition of cAMP formation rather than through direct modulation of the protein kinase.

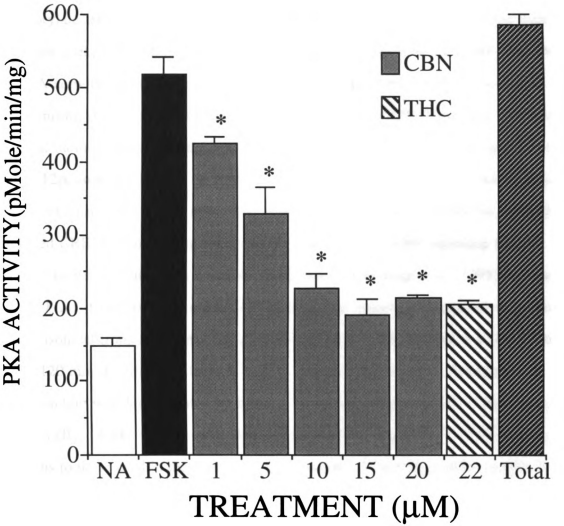


Figure 11. Inhibition of PKA activity in forskolin-stimulated splenocytes by cannabinol. Cell extracts were preincubated with CBN or Δ^9-THC for 5 min and then placed in reaction mixture containing substrate and γ-^{32}P in the presence or absence of forskolin (FSK; 50 μM) for 10 min. The results are expressed as the mean ± SE for triplicate samples. *$p<0.05$ as determined by Dunnett's t-test as compared to the forskolin control group. One of two representative experiments is shown.

C. Inhibition of transcription factor binding to a CRE motif by cannabinol

Upon activation of PKA, the catalytic subunits translocate to the nucleus to phosphorylate target proteins including the CREB/ATF family of transcription factors (Hagiwara *et al.*, 1993; Meinkoth *et al.*, 1993). Due to the inhibition of cAMP formation and PKA activity by cannabinol, the binding of PKA activated transcription factors to DNA binding motifs was investigated. These studies demonstrated that forskolin treatment alone induced binding to the CRE in spleen cells at 15, 30, 60 and 90 min (Figure 12A compare lane 2, no forskolin stimulation to lanes 3, 5, 7, and 9) which returned to basal level at 120 min. The observed time course is typical for forskolin-stimulated CRE protein binding following activation of the cAMP signaling cascade as measured by gel shift assays in a variety of cell types (Armstrong *et al.*, 1995; Hagiwara *et al.*, 1992). Conversely, protein/CRE binding was markedly decreased in nuclear proteins isolated from cannabinol (20 μM) treated splenocytes as evident at 15, 30, 60, 90, and 120 min (Figure 12A; lanes 4, 6, 8, 10, and 12 respectively). The specificity of protein binding was demonstrated by addition of excess unlabeled CRE oligonucleotide (Figure 12B). CRE binding was also investigated in thymocytes under identical conditions to those used in the splenocyte preparations. A distinct protein complex was induced by forskolin treatment of thymocytes at 15, 30, 60, 90, and 120 min (Figure 13A; lanes 3, 5, 7, 9, and 11) with maximum binding detected at 90 min. The kinetics of CRE binding appear to be slightly delayed in the thymocytes as compared to splenocytes in that protein binding remains induced at 120 min in thymocytes whereas CRE binding activity returned to basal levels in splenocytes by 120 min. Similarly, stimulation of thymocytes with forskolin in the presence of cannabinol (20 μM) resulted in a marked inhibition of CRE binding at all time points assayed (Figure 13A; lanes 4, 6, 8, 10, and 12). Protein binding to the CRE consensus motif was also specific as determined by cold competitor studies (Figure 13B). In general, the diminution of CRE binding by

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

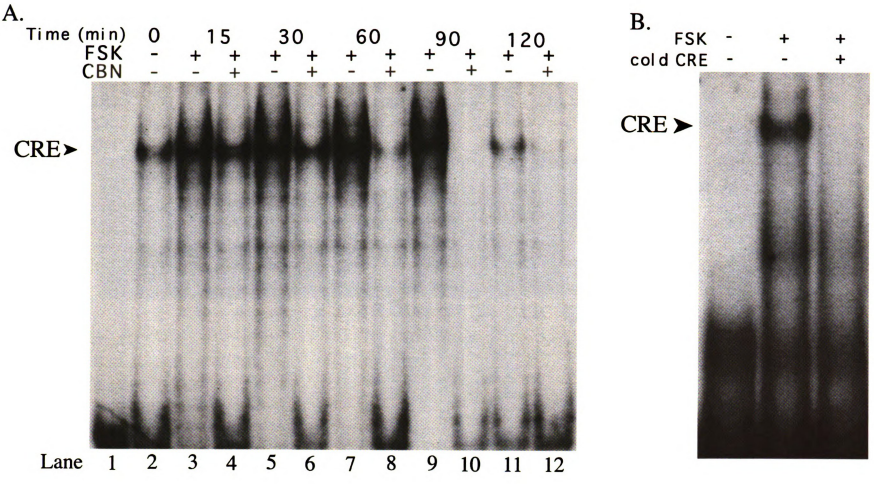
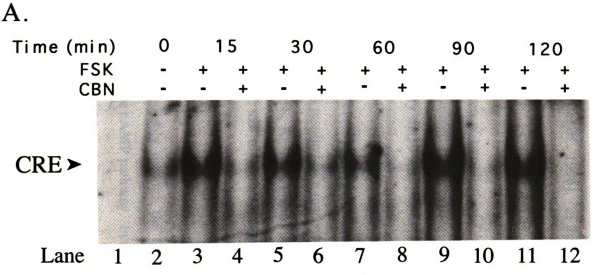

A.
Time (min) 0 15 30 60 90 120
FSK - + + + + + + + + + + +
CBN - - + - + - + - + - + +
CRE
Lane 1 2 3 4 5 6 7 8 9 10 11 12
B.
FSK - + +
cold CRE - - +
CRE

Figure 12

Figure 13. Inhibition of forskolin-induced binding to a CRE motif in mouse thymocytes by cannabinol. **A)** Nuclear proteins (3 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lanes 3, 5, 7, 9, and 11 represent forskolin stimulated thymocytes while lanes 4, 6, 8, 10, and 12 indicate forskolin stimulated/CBN treated thymocytes. **B)** Cold competitor studies were performed by adding 1 pmol of unlabeled CRE to the nuclear protein isolated from the 90 min forskolin sample. Results are representative of three separate experiments.


A.
Time (min) 0 15 30 60 90 120
FSK - + + + + + + + + + + +
CBN - - + - + - + - + - +
CRE
Lane 1 2 3 4 5 6 7 8 9 10 11 12

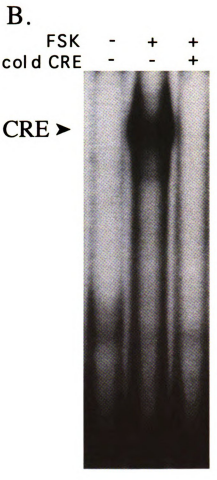

B.
FSK - + +
cold CRE - - +
CRE

Figure 13

cannabinol is indicative of a marked decrease in the activation of CREB/ATF family of transcription factors.

D. Inhibition of transcription factor binding to a κB motif by cannabinol

PKA is also involved in the activation of NF-κB/c-Rel transcription factors as demonstrated by the induction of κB binding following stimulation with cAMP elevating agents such as LPS, forskolin, and IL-1 (Muroi and Suzuk, 1993; Shirakawa *et al.*, 1989; Shirakawa and Mizel, 1989). Additionally, we have recently reported the regulation of NF-κB/c- Rel transcription factors by the cAMP signaling cascade in the macrophage cell line, RAW 264.7 (Jeon *et al.*, 1996). In light of this, the DNA binding activity of NF-κB/c-Rel proteins was examined in primary spleen cells and thymocytes. Incubation of nuclear proteins from forskolin-stimulated splenocytes with a ^{32}P-labeled κB oligomer resulted in the formation of two distinct DNA binding complexes (Figure 14A). More importantly, cells stimulated in the presence of cannabinol exhibited an attenuation of NF-κB binding activity at 30 and 60 min (Figure 14A; lanes 6 and 8 respectively). Studies in thymocytes revealed two major κB complexes and a minor upper complex in forskolin-stimulated nuclear proteins (Figure 15A). Similarly, stimulation of cells in the presence of cannabinol resulted in a marked inhibition of κB binding at 60, 90, and 120 min (Figure 15A; lanes 8, 10, 12). The formation of all protein complexes was inhibited by excess unlabeled κB oligonucleotide in both cell preparations (Figure 14B splenocytes; Figure 15B thymocytes).

This series of experiments demonstrated that cannabinol inhibits adenylate cyclase activity presumably through the CB2 receptor which leads to downstream signaling changes in the cAMP pathway. In addition, forskolin stimulation was shown to induce κB protein binding that is sensitive to inhibition by cannabinol. As T-cells are sensitive to inhibition by cannabinoid compounds, thymocytes were chosen as the

Figure 14. Inhibition of NF-κB/c-Rel binding to a κB motif in forskolin-stimulated splenocytes by cannabinol. **A)** Nuclear proteins (3 μg) from treated and untreated spleen cells were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated splenocytes. Lanes 3, 5, and 7 represent forskolin stimulated splenocytes while lanes 4, 6, and 8 indicate forskolin stimulated/CBN treated spleen cells. **B)** Cold competitor studies were done by adding 1 pmol of unlabeled κB to the nuclear protein isolated from the 60 min forskolin sample. One of three representative experiments is shown.

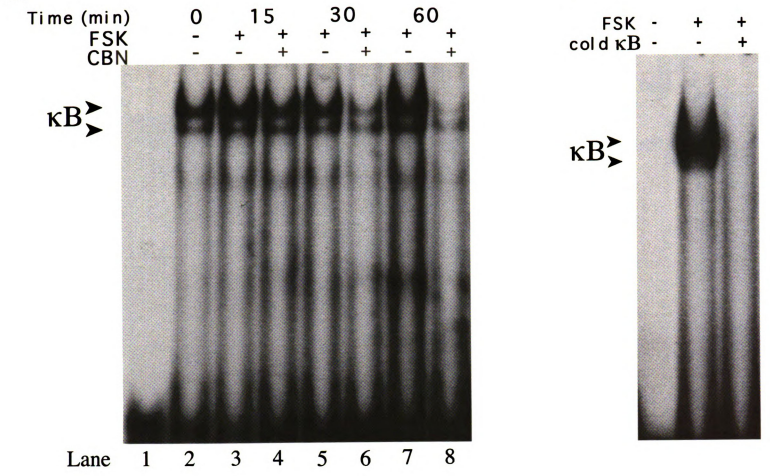


Figure 14

Figure 15. Inhibition of NF-κB/c-Rel binding to a κB motif in forskolin-stimulated thymocytes by cannabinol. **A)** Nuclear proteins (3 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates unstimulated thymocytes. Lanes 3, 5, 7, 9, and 11 represent forskolin stimulated thymocytes while lanes 4, 6, 8, 10, and 12 indicate forskolin stimulated/CBN treated thymocytes. **B)** Cold competitor studies were done by adding 1 pmol of unlabeled κB to the nuclear protein isolated from the 90 min forskolin sample. One of three representative experiments is shown.

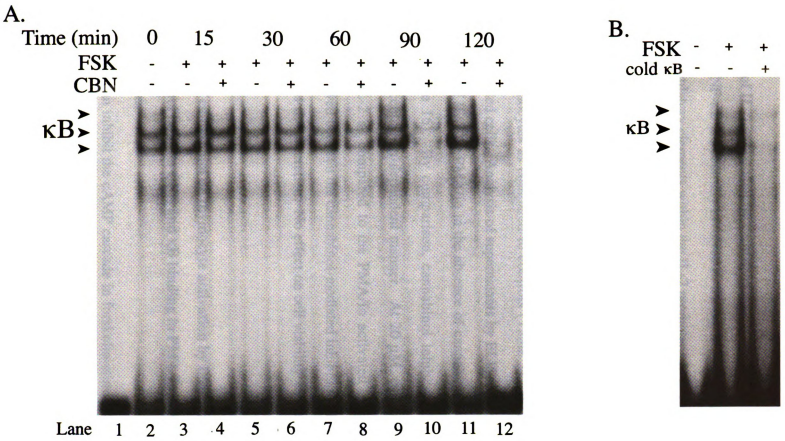

A.
Time (min) 0 15 30 60 90 120
FSK - + + + + + + + + + + +
CBN - - + - + - + - + - +
κB
Lane 1 2 3 4 5 6 7 8 9 10 11 12
B.
FSK - + +
cold κB - - +
κB

Figure 15

primary cell model for all subsequent experiments. The purpose of these studies was to further investigate the mechanism of T-cell suppression by cannabinol.

III. Inhibition of IL-2 expression by cannabinol

CREB and NF-κB are centrally involved in the regulation of IL-2 gene expression through several regulatory elements within the IL-2 promoter. In light of the cannabinol-mediated inhibition of CRE and κB DNA binding (Figures 13 and 15), the effects of cannabinol on IL-2 production in primary T-cells were examined. For these studies, thymocytes were treated with cannabinol prior to activation with PMA/Io for 24 hr. A significant reduction in IL-2 steady state mRNA expression was observed in cannabinol (20 μM) treated thymocytes as compared to controls (Table 3). IL-2 mRNA was not detected in unstimulated cells. Analysis of supernatants by ELISA demonstrated that the maximal induction of IL-2 by PMA/Io in the absence of cannabinol was approximately 16.1 units/ml (Figure 16). By comparison, cannabinol inhibited IL-2 secretion by thymocytes in a concentration-dependent manner. At 20 μM, cannabinol produced a 55% reduction in IL-2 as compared to the PMA/Io activated control. A positive correlation was observed between the cannabinol-mediated inhibition of IL-2 steady state mRNA expression and IL-2 secretion. No effect on cell viability was observed in any of the treatment groups.

IV. The effects of cannabinol on thymocyte activation by PMA/Ionomycin

A. Cannabinol inhibits CRE and κB binding in PMA/Io activated thymocytes

Cannabinol can inhibit the cAMP cascade in forskolin-stimulated thymocytes as evidenced by a decrease in intracellular cAMP and protein binding to a CRE motif (figures 10 and 13). Although forskolin is useful for assessing alterations of the cAMP pathway, it is not a relevant T-cell activation stimulus. Antigen stimulation induces

TABLE 3

Inhibition of IL-2 gene expression by cannabinol in PMA/Io activated thymocytes

Thymocytes (1 x 10^6 c/ml) were activated with PMA/Io (80 nM/1 μM) in the presence or absence of CBN (20 μM) for 24 hr. Total RNA was isolated and the molecules of IL-2 mRNA were quantified using competitive reverse transcriptase-polymerase chain reaction (RT-PCR). The results of two separate experiments are shown.

	Molecules/100 ng RNA	
Treatment	Experiment 1	Experiment 2
NA	N.D.	N.D.
PMA/Io	5.5×10^4	8.6×10^4
PMA/Io +CBN	2.5×10^4	4.6×10^4

N.D.: not detected

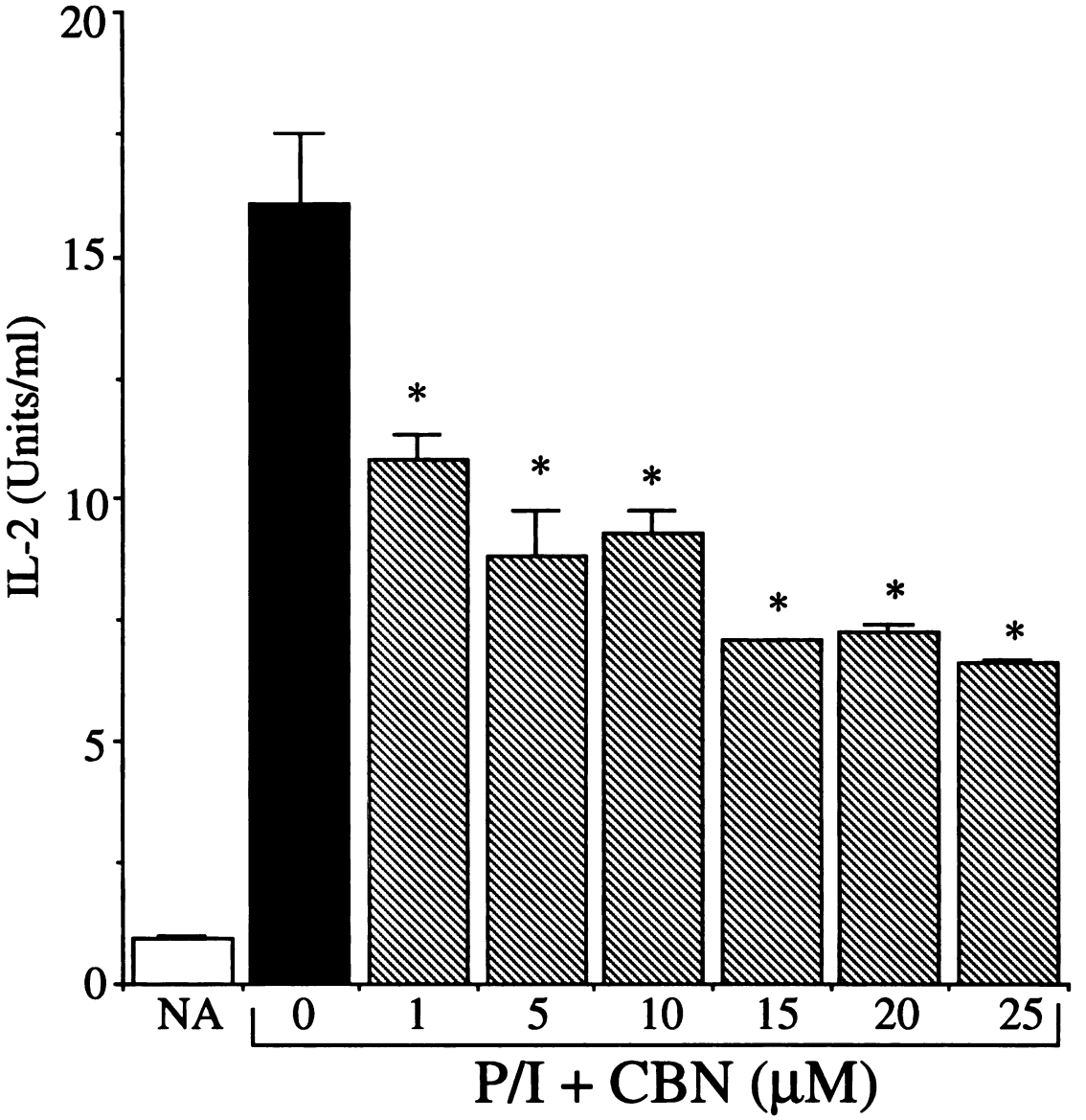


Figure 16. Inhibition of IL-2 protein secretion by cannabinol in thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (1, 5, 10, 15, 20, 25 μM) for 15 min followed by PMA/Io activation (80 nM/1 μM) for 24 hr at 37°C. IL-2 levels were determined by ELISA. Data are expressed as the mean ± SE for triplicate cultures. *p< 0.05 with comparison to the PMA/Io group.

multiple signaling pathways, including the cAMP cascade, that are essential for the complete activation of T-cells. PMA/Io is one stimulus that simulates antigen activation of T-cells as it mimics signaling induced through the TCR. Consequently, PMA/Io was employed in order to further investigate the effects of cannabinol in activated thymocytes. The EMSA was utilized to characterize the effect of cannabinol on CRE and κB binding activity following PMA/Io activation of thymocytes. Nuclear proteins were prepared from thymocytes activated with PMA/Io (80 nM/1μM) for 60 min in the presence or absence of cannabinol (20 μM). PMA/Io treatment induced the formation of two CRE binding complexes, a major complex (lower band) and a minor complex (upper band) (Figure 17). Cannabinol treatment produced a marked decrease in the DNA binding of the major CRE complex induced by PMA/Io.

Using identical culture conditions as for the CRE EMSA studies, the effect of cannabinol on NF-κB/c-Rel DNA binding was examined in PMA/Io activated thymocytes. Two distinct κB binding complexes were detected in naive thymocytes. PMA/Io strongly induced only the upper κB binding complex (Figure 18) which was significantly inhibited in the presence of cannabinol. Interestingly, we also observed that the percentage of bovine calf serum in the medium exhibited some influence on the ability of cannabinol to inhibit NF-κB binding activity. Specifically, the inhibition of NF-κB binding by cannabinol was marked in the presence of 1% serum while no inhibition was observed when cells were cultured in 5% serum (Figure 19).

B. Identification of the specific CRE binding proteins regulated by cannabinol

The CREB/ATF family of transcription factors is composed of several different proteins which can form homodimers or heterodimers to regulate gene expression. In order to determine the specific components of the CRE complexes induced by PMA/Io and inhibited by cannabinol, supershift analysis was performed. In these experiments

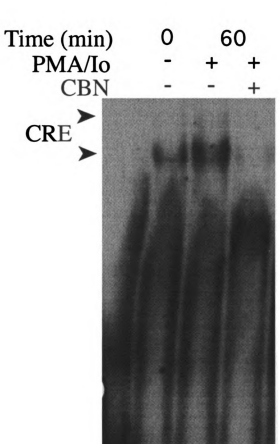


Figure 17. Cannabinol inhibits protein binding to a CRE motif in PMA/Io activated mouse thymocytes. Nuclear proteins (5 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates naive thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1 x 10^6 c/ml) activated for 60 min with PMA/Io (80nM/1 μM). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μM) for 15 min followed by PMA/Io (80 nM/1 μM) for 60 min. One of three representative experiments is shown.

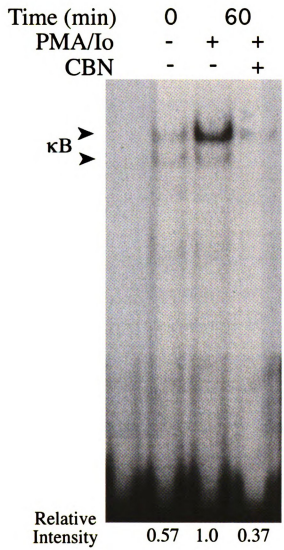


Figure 18. Inhibition of PMA/Io-induced protein binding to a κB motif in mouse thymocytes by cannabinol. Nuclear proteins (5 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates naive thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1 x 10^6 c/ml) activated for 60 min with PMA/Io (80 nM/1 μM). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μM) for 15 min followed by PMA/Io (80 nM/1 μM) for 60 min. One of three representative experiments is shown.

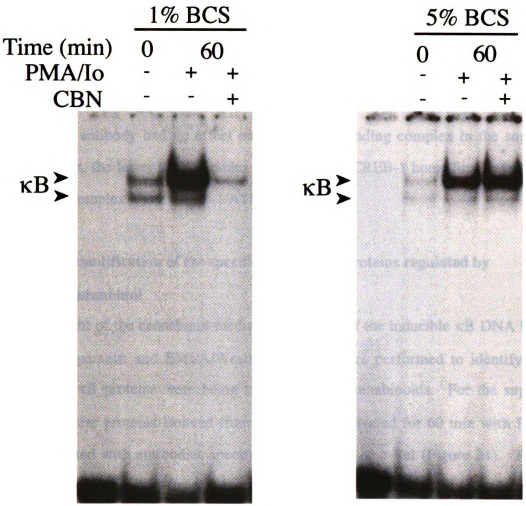


Figure 19. Effect of bovine calf serum on the cannabinol-mediated inhibition of NF-κB DNA binding activity in PMA/Io activated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (20 μM) for 15 min and activated with PMA/Io (80 nM/1 μM) for 60 min in complete medium supplemented with either 1% or 5% bovine calf serum (BCS). Nuclear proteins (5 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. One of two representative experiments is shown.

nuclear proteins were isolated from thymocytes 60 min after PMA/Io treatment. As shown in figure 20, CREB-1 was identified in both the upper and lower binding complexes as evidenced by the loss of CRE binding activity in the presence of anti-CREB-1. Anti-CREB-1 recognizes epitopes within the DNA binding domain of CREB-1 to block DNA binding. ATF-2 was identified only in the minor upper CRE complex. Anti-ATF-1 antibody had no effect on either CRE binding complex in the supershift assays. Thus, the lower CRE complex consisted of a CREB-1 homodimer whereas the upper CRE complex was a CREB-1/ATF-2 heterodimer.

C. Identification of the specific κB binding proteins regulated by cannabinol

In light of the cannabinol-mediated inhibition of the inducible κB DNA binding complex, supershift and EMSA/Western analysis were performed to identify which specific NF-κB proteins were being modulated by cannabinoids. For the supershift studies, nuclear proteins isolated from thymocytes activated for 60 min with PMA/Io were incubated with antibodies specific for p50, p65, or c-Rel (Figure 21). The p50 antibody produced a shift (lane 3) that was predominantly from the lower κB complex. By comparison, anti-p65 and anti-c-Rel appeared to primarily shift the upper κB complex. Due to the difficulty in determining which κB binding complexes were being supershifted, EMSA/Western was conducted to confirm the identity of the DNA binding proteins. In these experiments, the protein/κB complexes were subjected to Western analysis using either p65 or c-Rel antibody and compared to the EMSA. EMSA/Western analysis identified both p65 and c-Rel proteins as components of the upper κB complex which verified the supershift results (Figure 22). Therefore, the lower κB complex was identified as a p50 homodimer whereas the inducible (upper) κB complex consisted of a p65/c-Rel heterodimer. These findings also demonstrated that cannabinol primarily inhibits the DNA binding of p65 and c-Rel in PMA/Io activated thymocytes (Figure 22).

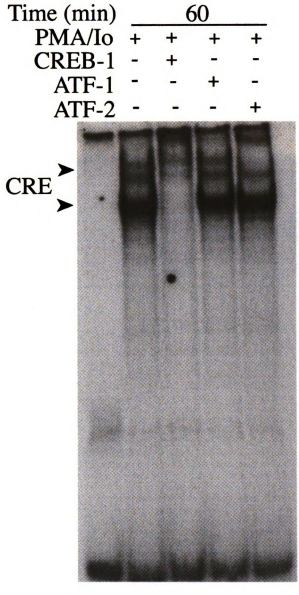


Figure 20. Identification of specific CRE transcription factors modulated by cannabinol following PMA/Io activation of thymocytes. EMSA was performed using nuclear proteins isolated from thymocytes activated with PMA/Io (80 nM/1 μM) for 60 min and a ^{32}P-labeled CRE probe. CREB-1 antibody (1 μg) was incubated with nuclear proteins at 4°C for 45 min prior to addition of the CRE probe. ATF-1 or ATF-2 antibody (1 μg) was incubated with the protein/CRE complex at 4°C for 45 min. Lane 1 represents free probe and lane 2 indicates PMA/Io activated thymocytes. Lanes 3, 4, and 5 contain CREB-1, ATF-1, and ATF-2 antibody respectively. One of three representative experiments is shown.

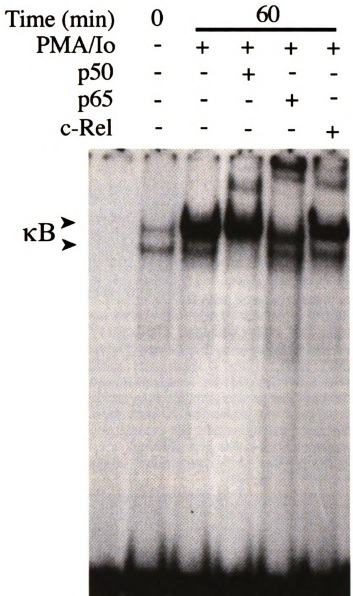


Figure 21. Identification of specific κB transcription factors modulated by cannabinol following PMA/Io activation of thymocytes. EMSA was performed using nuclear proteins isolated from thymocytes stimulated with PMA/Io (80 nM/ 1 μM) for 60 min and a ^{32}P-labeled κB probe. p50, p65, or c-Rel antibody (1 μg) was incubated with the protein/kB complex for 30 min at room temperature. Lane 1 represents free probe, lane 2 indicates basal binding activity, and lane 3 indicates PMA/Io activated thymocytes. Lanes 4, 5, and 6 contain p50, p65, and c-Rel antibody respectively. One of three representative experiments is shown.

Figure 22. Identification of the components of the upper κB binding complex induced by PMA/Io in thymocytes. **A)** Nuclear proteins (8 μg) from treated and untreated thymocytes were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel. Lane 1 represents free probe and lane 2 indicates naive thymocytes. Lane 3 represents nuclear proteins isolated from thymocytes (1 x 10^6 c/ml) activated for 60 min with PMA/Io (80nM/1 μM). Lane 4 represents nuclear proteins isolated from thymocytes treated with CBN (20 μM) for 15 min followed by PMA/Io (80 nM/1 μM) for 60 min. **B)** Identical nuclear protein samples were incubated with 0.5 μg of poly (dI-dC) and 10 pMoles of cold κB probe for 10 min on ice and separated on a 4% acrylamide gel. Following electrophoresis, the protein complexes were transferred to nitrocellulose and incubated with either p65 (200 ng) or c-Rel (400 ng) antibody for 2 hr. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

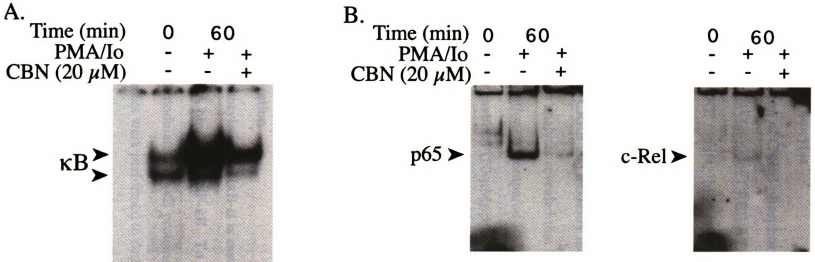

A.
Time (min) 0 60
PMA/Io - + +
CBN (20 μM) - - +
κB
B.
Time (min) 0 60
PMA/Io - + +
CBN (20 μM) - - +
p65
0 60
- + +
- - +
c-Rel

Figure 22

V. The effects of cannabinol on CREB and NF-κB activation in PMA/Io activated thymocytes

A. Inhibition of CREB phosphorylation by cannabinol

The phosphorylation of CREB/ATF proteins facilitates protein dimerization and DNA binding to CRE motifs. In light of the inhibition of CRE binding by cannabinol described above (Figures 13 and 17), the effect of cannabinol on forskolin and PMA/Io induced phosphorylation of CREB and ATF-1 nuclear proteins was examined. Stimulation of thymocytes with either forskolin or PMA/Io for 60 min induced the phosphorylation of CREB and modestly increased the phosphorylation of ATF-1 (Figure 23). Densitometric analysis revealed that PMA/Io produced a 2-fold greater increase in CREB phosphorylation as compared to forskolin. Conversely, thymocytes that were pretreated with cannabinol (20 μM) prior to stimulation exhibited a marked decrease in the phosphorylation status of CREB and ATF-1 (Figure 23). Interestingly, this decrease in phosphorylation correlated with the inhibition in CRE binding activity. Moreover, the modest amount of phosphorylated ATF-1 in thymocytes is most likely the reason why ATF-1 was not detected in the supershift studies.

B. The effect of cannabinol on IκB-α degradation and p65 cellular localization

The phosphorylation of IκB-α is essential for ubiquitination and required for the activation and DNA binding of NF-κB. To gain further insights into the mechanism by which cannabinol modulates NF-κB proteins, we examined the effects of cannabinol on IκB-α. Initial studies were performed to determine the time course of IκB-α degradation in thymocytes. Thymocytes were activated with PMA/Io for 15, 30, 60, 45, 90, and 120 min and whole cell lysates were analyzed for IκB-α protein levels. As expected, PMA/Io produced a rapid degradation of IκB-α during the first 60 min which was followed by an increase in IκB-α at 90 and 120 min (Figure 24). Because maximal degradation of IκB-α

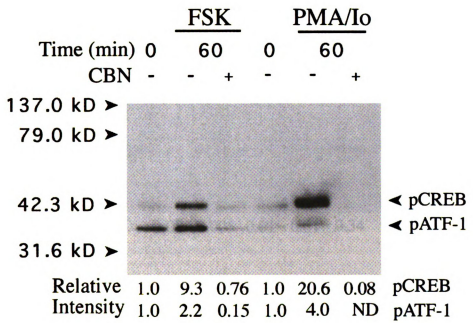


Figure 23. Phosphorylation of CREB and ATF-1 is inhibited by cannabinol in stimulated thymocytes. Nuclear proteins (25 µg) from treated thymocytes were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody which recognizes the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system. CREB-1 is 43 kD and ATF-1 is 38 kD.

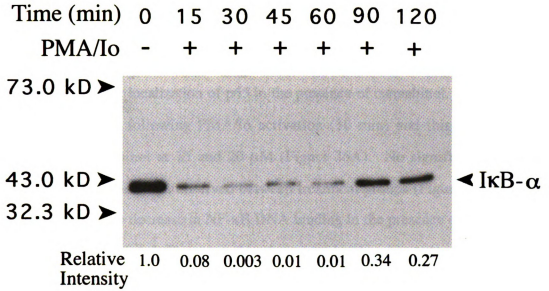


Figure 24. Time course of IκB-α degradation following PMA/Io activation of thymocytes. Thymocytes (1 x 10^6 c/ml) were treated with PMA/Io for 0-120 min and whole cell lysates (25 μg) were resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated with 200 ng of a rabbit polyclonal antibody for IκB-α for 2 hr. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for detection using the ECL system. IκB-α is a 38 kD protein.

was detected 30 min after PMA/Io activation, this time point was chosen to examine the effects of cannabinol on IκB-α. As shown in figure 25, cannabinol prevented the degradation of IκB-α in a concentration dependent manner presumably through an inhibition of IκB-α phosphorylation.

The level of p65 protein was also examined at 30 min in the presence of cannabinol to investigate possible direct effects of cannabinol on p65 expression. p65 protein levels were relatively unchanged in the presence of increasing concentrations of cannabinol (Figure 25) suggesting that the decrease of NF-κB DNA binding activity by CBN occurs at the level of IκB-α and not p65. This was further demonstrated by examining the cellular localization of p65 in the presence of cannabinol. Nuclear levels of p65 were induced following PMA/Io activation (30 min) and this induction was suppressed by cannabinol at 15 and 20 μM (Figure 26A). No significant change in cytosolic p65 expression was observed between treatment groups (Figure 26B). These results suggest that the decrease in NF-κB DNA binding in the presence of cannabinol is due to an inhibition of IκB-α phosphorylation and subsequent degradation which precludes NF-κB translocation into the nucleus.

VI. The role of cAMP and PKA in the cannabinol-mediated inhibition of CREB, NF-κB, and IL-2 in activated thymocytes

A. Effect of DBcAMP on the inhibition of CREB, NF-κB, and IL-2 by cannabinol

Membrane permeable analogs of cAMP are often employed as a biological probe to experimentally modulate the cAMP pathway. Dibutyryl cAMP (DBcAMP) was used in the present studies to determine the involvement of the cAMP cascade in the cannabinol-mediated inhibition of CREB, NF-κB, and IL-2 in activated T-cells. Treatment of thymocytes with DBcAMP (60 min) induced CREB phosphorylation at 10 and 100 μM with the greatest magnitude of phosphorylation being detected at 100 μM

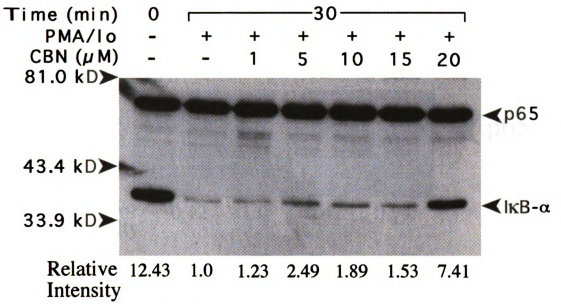


Figure 25. Inhibition of IκB-α degradation by cannabinol. Whole cell lysates were prepared from thymocytes (1 x 10^6 c/ml) pretreated with CBN (1, 5, 10, 15, 20 μM) for 15 min followed by PMA/Io (80 nM/1 μM) for 30 min. In each experiment, whole cell lysates (25 μg) were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated for 2 hr with 200 ng of a rabbit polyclonal antibody for IκB-α. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection. The whole cell lysates (25 μg) were also examined for changes in p65 protein expression using 200 ng of p65 antibody and the anti-rabbit Ig horseradish peroxidase secondary antibody.

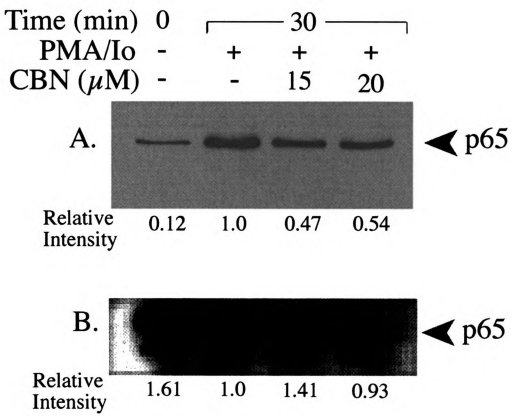


Figure 26. Cellular localization of p65 in the presence of cannabinol. A) Nuclear proteins were isolated from thymocytes (1 x 10^6 c/ml) pretreated with CBN (15 or 20 μM) for 15 min followed by PMA/Io (80 nM/1 μM) for 30 min. **B)** Cytosolic proteins isolated from thymocytes treated as described in part A. Proteins (25 μg) were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated for 2 hr with 200 ng of p65 antibody. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection.

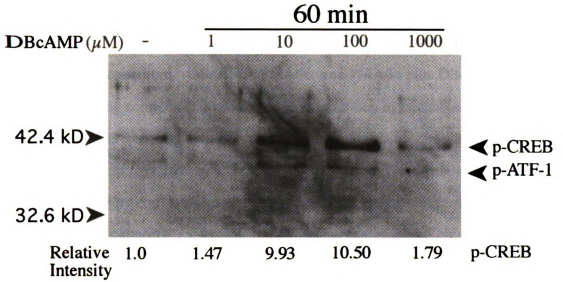


Figure 27. DBcAMP induces the phosphorylation of CREB. Thymocytes were treated with DBcAMP (1, 10, 100, 1000 μM) for 60 min. Nuclear proteins (25 μg) were isolated and resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody directed toward the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

DBcAMP (Figure 27). In light of this, studies were performed to examine whether the inhibition of CREB phosphorylation and CRE binding by cannabinol in activated thymocytes could be reversed with DBcAMP. Thymocytes were treated with either DBcAMP (100 μM), PMA/Io (80 nM/1 μM), or PMA/Io plus DBcAMP for 60 min in the presence or absence of cannabinol (5, 10, 15 μM). As shown in figure 28, CREB phosphorylation was induced by DBcAMP or PMA/Io alone. Densitometric analysis revealed that PMA/Io plus DBcAMP produced an additive effect on CREB phosphorylation. Interestingly, the inhibition of CREB phosphorylation by cannabinol in PMA/Io activated thymocytes could not be reversed by concomitant treatment with DBcAMP (Figure 28). CRE binding activity was also investigated in thymocytes under identical culture conditions. DBcAMP, PMA/Io, and PMA/Io plus DBcAMP induced a major CRE binding complex that was sensitive to inhibition by cannabinol. However, the decrease in CRE binding activity produced by cannabinol in PMA/Io activated thymocytes was not reversed by DBcAMP co-stimulation (Figure 29). The profile of CRE binding activity correlated strongly with the CREB phosphorylation results.

Previous experiments demonstrating the modulation of NF-κB binding by cannabinol in forskolin-stimulated thymocytes also suggested that NF-κB/c-Rel proteins are regulated, in part, by the cAMP signaling pathway. Based on this, the ability of DBcAMP (100 μM) to reverse the cannabinol-mediated inhibition of κB DNA binding in activated thymocytes was examined. As shown in figure 30, thymocyte activation by PMA/Io or PMA/Io plus DBcAMP induced one of the two constitutive (upper) κB DNA binding complexes. By comparison, DBcAMP alone failed to increase binding to the κB motif in thymocytes. This is in contrast to the induction of NF-κB binding activity observed after forskolin stimulation (Figure 15). Additionally, DBcAMP was unable to reverse the inhibition of κB binding activity by cannabinol in PMA/Io activated thymocytes (Figure 30).

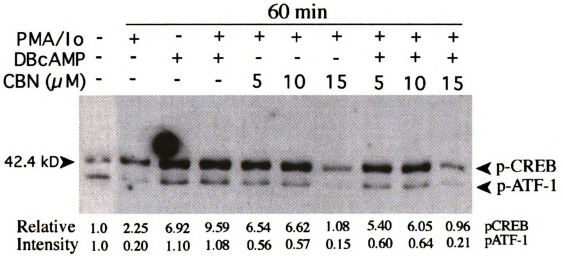


Figure 28. Inhibition of CREB phosphorylation by cannabinol in activated thymocytes is not reversed by DBcAMP. Thymocytes were pretreated with CBN (5, 10, 15 μM) for 15 min followed by stimulation with either PMA/Io or PMA/Io plus DBcAMP (100 μM). Nuclear proteins (25 μg) were isolated and resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody directed toward the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

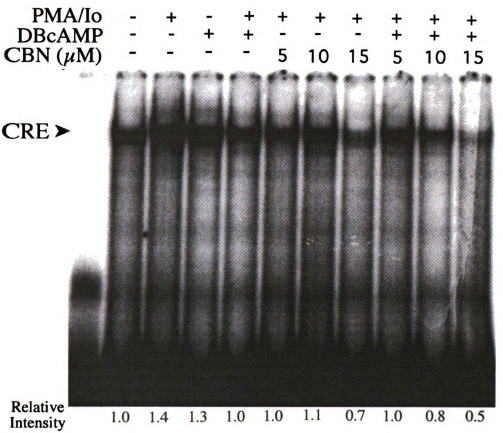


Figure 29. Effect of DBcAMP on the inhibition of CRE binding by cannabinol in activated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (5, 10, 15 μM) and stimulated with PMA/Io, DBcAMP (100 μM), or PMA/Io plus DBcAMP for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

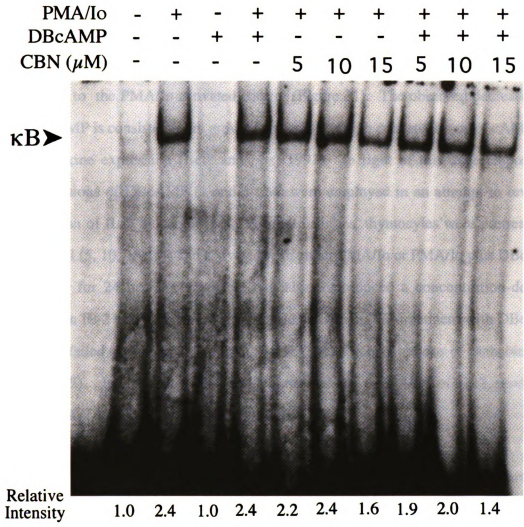


Figure 30. Effect of DBcAMP on the inhibition of κB binding by cannabinol in activated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (5, 10, 15 μM) and stimulated with PMA/Io, DBcAMP (100 μM), or PMA/Io plus DBcAMP for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

Based on the inhibitory effects cannabinol exerts on the cAMP cascade, the suppression of thymocyte IL-2 expression by cannabinol implicated a positive role for cAMP in the regulation of IL-2. Therefore, studies were also performed to determine whether DBcAMP could reverse the cannabinol-induced inhibition of IL-2 in activated T-cells. Thymocytes were treated simultaneously with DBcAMP (1, 10, 100, 1000 μM) and PMA/Io for 24 hr, and supernatants were analyzed for IL-2 protein by ELISA. DBcAMP produced a marked inhibition of IL-2 secretion at 10, 100, and 1000 μM as compared to the PMA/Io activated control (Figure 31). The observed decrease in IL-2 by DBcAMP is consistent with previous reports that high concentrations of cAMP inhibit Th1 cytokine expression (Betz and Fox, 1991). In light of this dose response, low concentrations of DBcAMP (1 and 5 μM) were employed in an attempt to reverse the suppression of IL-2 by cannabinol. For these studies, thymocytes were pretreated with cannabinol (5, 10, 15 μM) and activated with either PMA/Io or PMA/Io plus DBcAMP (1 or 5 μM) for 24 hr. As expected, cannabinol produced a concentration-dependent decrease in IL-2 secretion following PMA/Io activation. Co-treatment with DBcAMP (1 or 5 μM) failed to abolish the inhibition of IL-2 at all concentrations of cannabinol tested (Figure 32). Interestingly, DBcAMP enhanced the inhibition of IL-2 produced by cannabinol at 10 and 15 μM in activated thymocytes.

B. Effect of forskolin on the inhibition of CREB and NF-κB by cannabinol

As already discussed, forskolin (FSK) directly activates adenylate cyclase to elevate endogenous intracellular cAMP levels. To ensure that the inability of DBcAMP to reverse the cannabinol-mediated inhibition of CREB and NF-κB in activated thymocytes was not unique to cAMP analogs, similar experiments were performed with forskolin. Stimulation of thymocytes with forskolin, PMA/Io, or PMA/Io plus forskolin induced CREB phosphorylation and CRE binding activity. In contrast, concomitant stimulation with forskolin and PMA/Io failed to reverse the inhibition of CRE binding

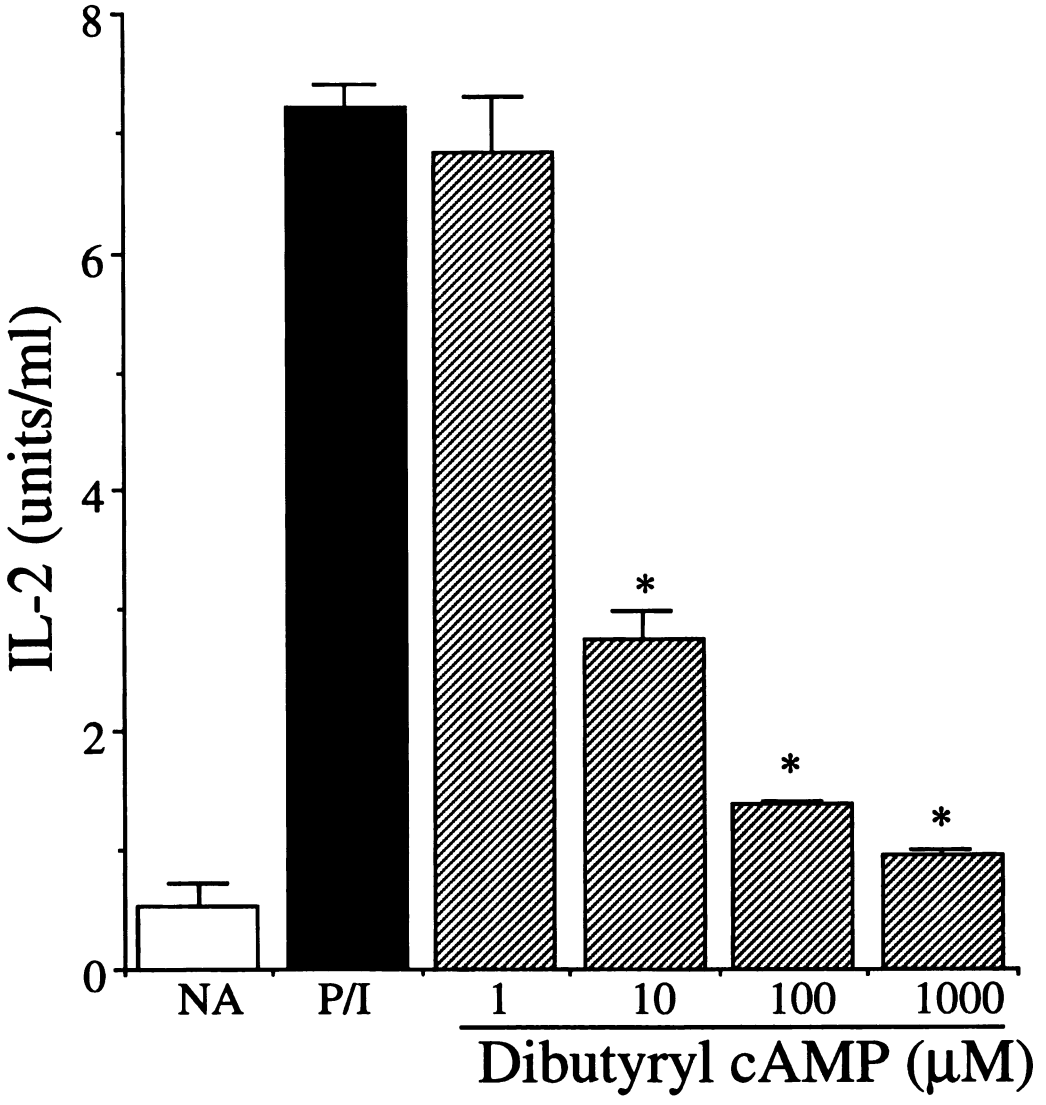


Figure 31. Inhibition of IL-2 protein secretion by DBcAMP in thymocytes. Thymocytes (1 x 10^6 c/ml) were concurrently treated with DBcAMP (1, 10, 100, 1000 μM) and PMA/Io (80 nM/1 μM) for 24 hr at 37°C. IL-2 levels in the supernatant were determined by ELISA. Data are expressed as the mean ± SE for triplicate cultures. *p< 0.05 with comparison to the PMA/Io group.

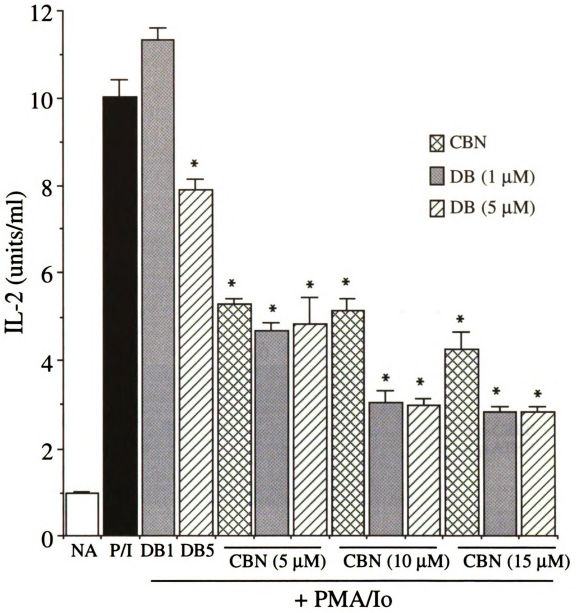


Figure 32. DBcAMP fails to reverse cannabinol-mediated inhibition of IL-2 protein secretion in PMA/Io activated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (5, 10, 15 μM) for 15 min followed by stimulation with PMA/Io (80 nM/1 μM), DBcAMP (1 or 5 μM), or PMA/Io plus DBcAMP (1 or 5 μM) for 24 hr at 37 °C. IL-2 levels in the supernatant were determined by ELISA. Data are expressed as the mean ± SE for triplicate samples. *$p < 0.05$ with comparison to the PMA/Io group.

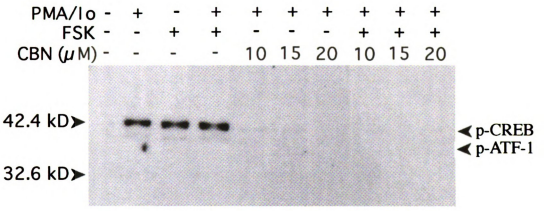


Figure 33. Forskolin failed to reverse the inhibition of CREB phosphorylation by cannabinol in activated thymocytes. Thymocytes were pretreated with CBN (10, 15, 20 μM) for 15 min followed by stimulation with either PMA/Io or PMA/Io plus FSK (50 μM). Nuclear proteins (25 μg) were isolated and resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody directed toward the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

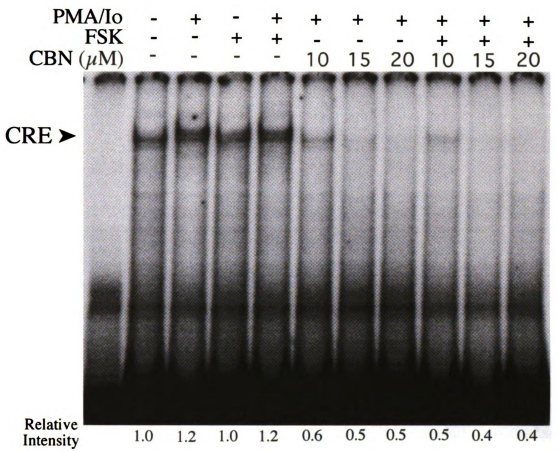


Figure 34. Inhibition of CRE binding activity by cannabinol is not reversed by forskolin. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (10, 15, 20 μM) and stimulated with PMA/Io, FSK (50 μM), or PMA/Io plus FSK for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

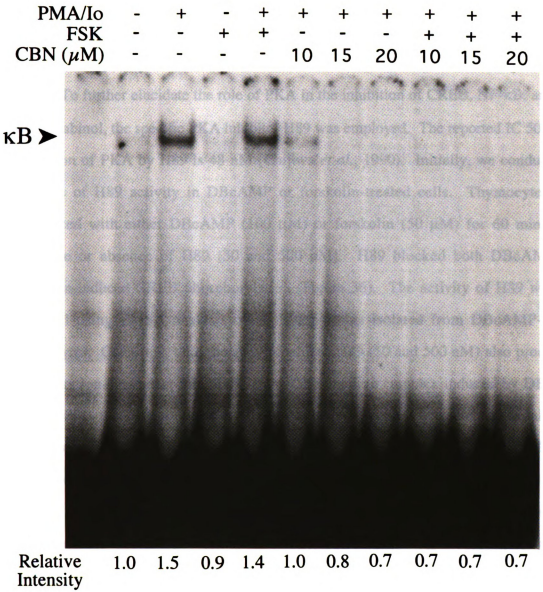


Figure 35. Inhibition of κB binding activity by cannabinol is not reversed by forskolin. Thymocytes (1 x 10^6 c/ml) were pretreated with CBN (10, 15, 20 μM) and stimulated with PMA/Io, FSK (50 μM), or PMA/Io plus FSK for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

and CREB phosphorylation by cannabinol (Figures 34 and 33, respectively). Similarly, forskolin co-stimulation was unable to reverse the decrease in NF-κB binding activity produced by cannabinol in activated thymocytes (Figure 35).

C. Effect of H89 on the regulation of CREB, NF-κB, and IL-2 in activated thymocytes

To further elucidate the role of PKA in the inhibition of CREB, NF-κB, and IL-2 by cannabinol, the specific PKA inhibitor H89 was employed. The reported IC 50 for the inhibition of PKA by H89 is 48 nM (Chijiwa *et al.*, 1990). Initially, we conducted an analysis of H89 activity in DBcAMP or forskolin-treated cells. Thymocytes were stimulated with either DBcAMP (100 μM) or forskolin (50 μM) for 60 min in the presence or absence of H89 (50 and 500 nM). H89 blocked both DBcAMP and forskolin-induced CREB phosphorylation (Figure 36). The activity of H89 was also assessed using EMSA analysis of nuclear proteins isolated from DBcAMP-treated thymocytes. Consistent with the above findings, H89 (50 and 500 nM) also produced a concentration-dependent inhibition of the CRE binding complex induced by DBcAMP (Figure 37). Collectively these results demonstrated that H89 was effective in inhibiting PKA function. To examine the involvement of PKA in the regulation of CREB in activated T-cells, thymocytes were pretreated with increasing concentrations of H89 (10-500 nM) for 30 min prior to activation with PMA/Io. H89 produced a 30% inhibition of PMA/Io-induced CREB phosphorylation at 50 nM with relatively little effect detected at other H89 concentrations (Figure 38). Similarly, H89 (50 nM) produced a modest reduction of protein/DNA binding to a CRE motif in PMA/Io activated thymocytes whereas minimal effects were detected in all other H89 treatment groups (Figure 39).

Identical EMSA studies were performed for κB DNA binding activity to determine the role of PKA in NF-κB regulation following T-cell activation. Interestingly, pretreatment of thymocytes with H89 (10-500 nM) produced no effect on the PMA/Io

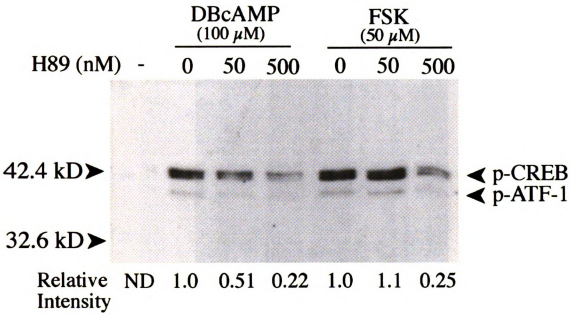


Figure 36. Phosphorylation of CREB is inhibited by H89. Thymocytes (1 x 10^6 c/ml) were pretreated with H89 (50 or 500 nM) for 30 min and stimulated with either DBcAMP (100 μM) or FSK (50 μM) for 60 min. Nuclear proteins (25 μg) were isolated and resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody directed toward the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

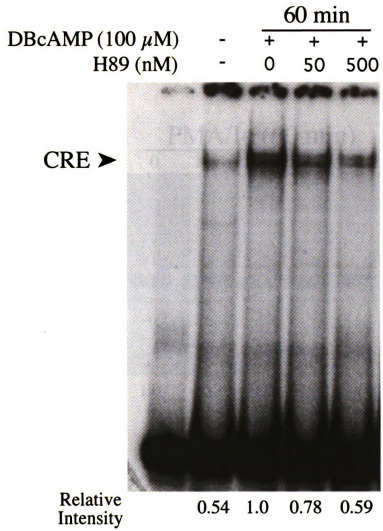


Figure 37. Inhibition of CRE binding by H89 in DBcAMP treated thymocytes. Thymocytes were incubated with H89 (50 or 500 nM) for 30 min and treated with DBcAMP (100 μM) for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

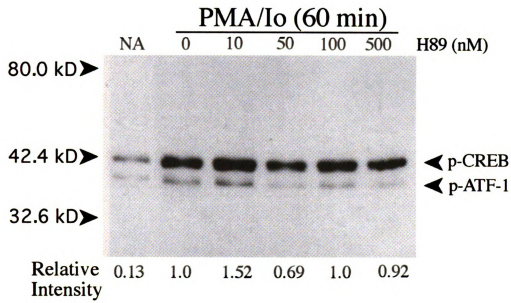


Figure 38. H89 has a modest effect on PMA/Io-induced CREB phosphorylation in thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated (30 min) with H89 (10, 50, 100, 500 nM) prior to activation with PMA/Io for 60 min. Nuclear proteins (25 µg) were isolated and resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and incubated for 2 hr with 30 ng of a rabbit polyclonal antibody directed toward the phosphorylated Ser-133 residue on CREB and ATF-1. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL system.

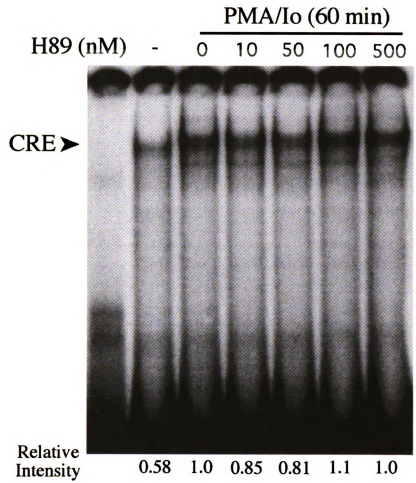


Figure 39. Effect of H89 on CRE binding activity in PMA/Io activated thymocytes. Thymocytes were pretreated with H89 (10, 50, 100, 500 nM) for 30 min followed by PMA/Io for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled CRE probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

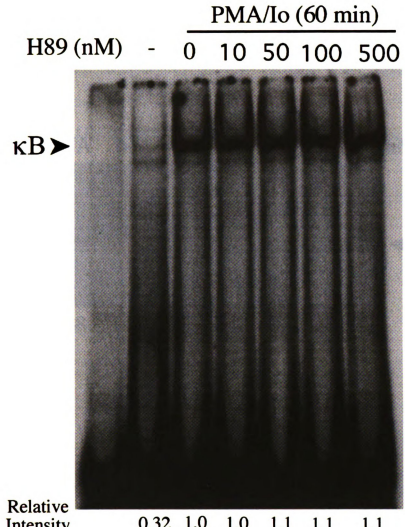


Figure 40. H89 does not inhibit binding to a κB motif in PMA/Io activated thymocytes. Thymocytes were pretreated with H89 (10, 50, 100, 500 nM) for 30 min followed by PMA/Io for 60 min. Nuclear proteins (5 μg) were incubated with 0.5 μg of poly (dI-dC) and the ^{32}P-labeled κB probe in binding buffer on ice for 10 min followed by separation on a 4% acrylamide gel.

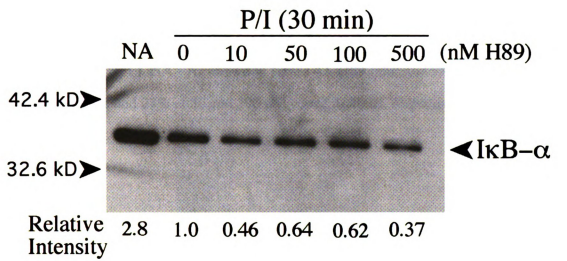


Figure 41. Effect of H89 on IκB-α degradation in activated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated for 30 min with H89 (10, 50, 100, 500 nM) followed by PMA/Io for 30 min. Whole cell lysates (25 μg) were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose and incubated with 200 ng of a rabbit polyclonal antibody for IκB-α for 2 hr. An anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for detection using the ECL system. IκB-α is a 38 kD protein.

induced protein binding to a κB motif (Figure 40). Consistent with these findings, H89 did not prevent the IκB-α degradation induced by PMA/Io and appeared to enhance the degradation at all concentrations of H89 tested (Figure 41).

Although a central role for CREB in T-cell activation and IL-2 expression has recently been established, the specific involvement of PKA remains unclear. As a result, H89 was employed to assess the participation of PKA in the cannabinol-mediated inhibition of IL-2 expression. Thymocytes were pretreated for 30 min with H89 (10-500 nM) followed by PMA/Io activation for 24 hr. Supernatants were collected and analyzed for IL-2 activity by ELISA. As shown in figure 42, H89 inhibited PMA/Io-induced IL-2 secretion by 17% and 27% at 50 and 100 nM concentrations, respectively.

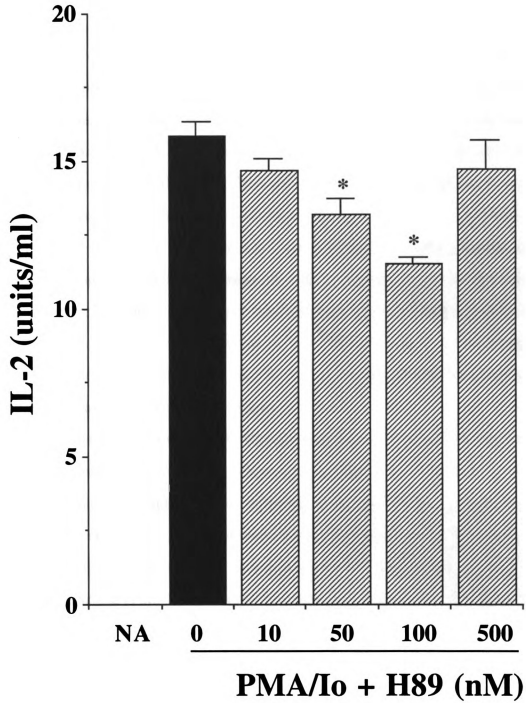


Figure 42. Effect of H89 on IL-2 protein secretion in PMA/Io stimulated thymocytes. Thymocytes (1 x 10^6 c/ml) were pretreated with H89 (10, 50, 100, 500 nM) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM) for 24 hr at 37 °C. IL-2 levels in the supernatant were determined by ELISA. Data are expressed as the mean ± SE for triplicate samples. *$p < 0.05$ with comparison to the PMA/Io group.

DISCUSSION

Cannabinoid compounds produce a diverse range of physiological effects in the CNS and peripheral organ systems. Due to the lipophilic structure of cannabinoids, the original mechanism for these effects was attributed to intercalation and disruption of the plasma membrane lipid bilayer. However, the isolation and cloning of CB1 and CB2 cannabinoid receptors in the early 1990's provided a putative mechanism of action for cannabinoid compounds (Matsuda *et al.*, 1990; Munro *et al.*, 1993). CB1 and CB2 are novel G-protein coupled receptors, and ligand binding to either receptor subtype negatively regulates adenylate cyclase through a pertussis toxin-sensitive protein. Previous studies in mouse splenocytes have provided evidence for the involvement of CB1 in the immunosuppressive effects of Δ^9-THC (Kaminski *et al.*, 1992; Schatz *et al.*, 1992). By comparison, the role of the CB2 receptor in cannabinoid-mediated immune suppression was relatively uncharacterized.

A systematic evaluation of cell-type receptor distribution has not yet been performed; however, previous studies have identified RNA transcripts for both CB1 and CB2 in a number of lymphoid tissue preparations (Kaminski *et al.*, 1992; Munro *et al.*, 1993; Schatz *et al.*, 1997), purified leukocytes (Bouaboula *et al.*, 1993; Facci *et al.*, 1995), and immune system-derived cell lines (Condie *et al.*, 1996; Facci *et al.*, 1995; Jeon *et al.*, 1996; Schatz *et al.*, 1997). Northern analysis and quantitative RT-PCR of mRNA determined a greater expression of CB2 than CB1 in mouse spleen and expression of only CB2 in thymus (Schatz *et al.*, 1997). Furthermore, competition binding analysis in mouse splenocytes demonstrated that cannabinol exhibited modestly greater binding affinity than Δ^9-THC (Schatz *et al.*, 1997) which is similar to previous results in CB2 transfected cells (Felder *et al.*, 1995; Munro *et al.*, 1993). Taken together, these findings suggested that CB2 was the predominant cannabinoid receptor expressed on primary mouse leukocytes.

Although Δ^9-THC and the synthetic bicyclic cannabinoid CP-55,940 are two of the most widely utilized cannabinoids experimentally, they are incapable of distinguishing between CB1 and CB2. In contrast, cannabinol, which is similar in structure to Δ^9-THC, is one of the first cannabinoid receptor ligands identified which exhibits higher binding affinity for CB2 than CB1 (Felder *et al.*, 1995; Munro *et al.*, 1993; Schatz *et al.*, 1997). In light of this property, we utilized cannabinol in the present studies as a biological probe to examine the functional role of CB2 on immune modulation by cannabinoids in primary mouse splenocytes and thymocytes. Direct addition of cannabinol to mouse spleen cell cultures produced a significant inhibition of proliferative responses to anti-CD3, LPS, and PMA plus ionomycin. Cannabinol also inhibited the *in vivo* and *in vitro* T-cell dependent IgM antibody forming cell response to sRBC. It is important to emphasize that cannabinol produced no effect on cell viability at the concentrations utilized *in vitro* even after 5 days of culture. Interestingly, cannabinol exhibited a similar profile of immunomodulatory activity in the B6C3F1 mouse as previously described for Δ^9-THC (Schatz *et al.*, 1993). The suppression of functional immune responses by cannabinol suggests an involvement of the CB2 receptor in immune modulation by cannabinoid compounds.

Based on the fact that cannabinoid receptors negatively regulate adenylate cyclase activity, one of the major focuses of this research project was to evaluate the status of the cAMP signaling pathway in splenocyte and thymocyte preparations in the presence of cannabinol. For these studies, forskolin was used to activate the cAMP cascade through the direct stimulation of adenylate cyclase activity. Forskolin stimulation of either splenocytes or thymocytes in the presence of cannabinol resulted in a significant inhibition of intracellular cAMP levels indicating the functional expression of CB2 receptors in both of these murine cell preparations. In addition to the fact that thymocytes express virtually no CB1 mRNA, the thymocyte studies are particularly interesting for two reasons: (1) intracellular cAMP levels were approximately 4-fold greater in

thymocytes than splenocytes following forskolin stimulation; and (2) the magnitude of adenylate cyclase inhibition by cannabinol was significantly greater in thymocytes. A similar difference in intracellular cAMP levels has been shown in thymocytes and peripheral T-cells following forskolin stimulation suggesting an important role for cAMP in T-cell development and differentiation (Scherer *et al.*, 1995).

The effect of cannabinol on downstream components of the cAMP signaling cascade; specifically, the activation of PKA and the induction of PKA-regulated transcription factors was further examined. These studies showed that cannabinol produced a marked inhibition of PKA activity in forskolin-stimulated splenocytes. It is notable that the inhibition of PKA activity was not due to a direct effect of cannabinoids on the kinase. Rather, PKA inhibition occurs indirectly through a decrease in cAMP formation as demonstrated by the ability of exogenous cAMP to activate PKA in the presence of cannabinoids (Koh *et al.*, 1997). Interestingly, although the reduction in adenylate cyclase activity by cannabinol was moderate (approximately 30% decrease at 20 μM), changes at the level of PKA were more profound as evidenced by a greater than 50% decrease in kinase activity at the same cannabinol concentration. This difference is most likely due to the amplification of the signal as it is transduced from the plasma membrane to the nucleus.

The effect of cannabinol on the terminal event of the cAMP cascade, the binding of CREB/ATF transcription factors to a CRE motif, was evaluated by EMSA. Forskolin treatment alone (0-120 min) readily induced a CRE binding complex in both splenocytes and thymocytes which was markedly inhibited by cannabinol at every time point tested. The kinetics of DNA binding and the sensitivity to inhibition by cannabinol correlate with previous findings demonstrating that cAMP analogs reverse the cannabinoid-mediated inhibition of the IgM AFC response only within the first 60 min after antigen sensitization (Kaminski *et al.*, 1994). The regulation of NF-κB/c-Rel transcription factors has been shown to be partially under the control of PKA in leukocytes (Muroi and Suzuk,

1993; Shirakawa *et al.*, 1989; Shirakawa and Mizel, 1989). Cannabinol was also found to inhibit the NF-κB/c-Rel DNA binding complexes in primary mouse splenocytes and thymocytes following forskolin stimulation. The decrease in NF-κB binding by cannabinoids is further supported by additional studies from our laboratory. A direct association has been identified between the inhibition of cAMP signaling, a decrease in NF-κB/c-Rel DNA binding, and the inhibition of iNOS in macrophages treated with Δ^9-THC (Coffey *et al.*, 1996; Jeon *et al.*, 1996). Taken together, the cannabinol-induced inhibition of CRE and κB DNA binding complexes within 60 min of stimulation supports the hypothesis that cannabinoids inhibit an early leukocyte activation event.

The role of cAMP signaling in immune regulation is not well defined, however numerous studies suggest a positive/stimulatory role for cAMP in mediating certain leukocyte cellular responses. Evidence supporting this premise includes a rapid and transient increase in intracellular cAMP following mitogenic stimulation of splenocytes (Hadden *et al.*, 1972; Kaminski *et al.*, 1994; Russell, 1978; Smith *et al.*, 1971) and enhancement of proliferative and T-cell dependent AFC responses by cAMP analogs (Kaminski *et al.*, 1994; Koh *et al.*, 1995). Additionally, inhibition of adenylate cyclase activity by cannabinoids is closely correlated with the suppression of certain cell-mediated and humoral immune responses (Kaminski *et al.*, 1994). A cause and effect relationship between the inhibition of intracellular cAMP and decreased immune function is further supported by the ability of exogenous cAMP or glucagon, a hormone which elevates cAMP levels, to reverse the inhibition of immune function by cannabinoids (Kaminski *et al.*, 1994; Koh *et al.*, 1996). Studies investigating the inhibitory effects of Δ^9-THC on humoral immune responses have shown that only immunoglobulin production to T-cell dependent antigens (i.e., sheep erythrocytes) is suppressed by cannabinoids (Schatz *et al.*, 1993) suggesting that helper T-cells are a sensitive target for inhibition by cannabinoid compounds. Additional evidence supporting the sensitivity of helper T-cells to cannabinoids includes Δ^9-THC-mediated disruption of cAMP signal

transduction and IL-2 production in the murine T-cell line EL-4.IL-2 (Condie *et al.*, 1996). Collectively, these findings suggest that alterations in cAMP signaling may lead to T-lymphocyte dysfunction.

It is notable that not all immune responses appear to be sensitive to inhibition by cannabinoid compounds; however, this differential sensitivity does not appear to be due to a lack of cannabinoid receptor expression in certain subpopulations of cells. Cannabinoid receptor expression has been detected in all three major leukocyte cell types present in the spleen; B-cells, T-cells, and macrophages (Bouaboula *et al.*, 1993; Condie *et al.*, 1996; Jeon *et al.*, 1996; Munro *et al.*, 1993; Schatz *et al.*, 1997). A more likely explanation for the differential sensitivity of immune responses to cannabinoids pertains to whether the cAMP signaling cascade is critical to a specific effector function. For example, iNOS expression is positively regulated by cAMP in macrophages (Alonso *et al.*, 1995; Jeon *et al.*, 1996; Koide *et al.*, 1993; Mullet *et al.*, 1997), and Δ^9-THC suppresses iNOS transcription in these cells (Coffey *et al.*, 1996; Jeon *et al.*, 1996). Conversely, B-cells do not appear to be as dependent on cAMP signals for immunoglobulin secretion. This is based on the fact that IgM secretion in response to T-cell independent antigens (i.e., LPS or DNP-Ficoll) is refractory to inhibition by Δ^9-THC (Schatz *et al.*, 1993) despite a marked decrease in B-cell adenylate cyclase activity (Schatz *et al.*, 1997). Interestingly, the current studies demonstrate that cannabinol inhibits LPS-induced proliferation by B-cells which may reflect the critical role PKA plays in cell-cycle control (Grieco *et al.*, 1996). Proliferating-cell nuclear antigen (PCNA), an auxiliary factor of DNA polymerase δ, is also central to proliferation and cell cycle progression (Bravo *et al.*, 1987; Madsen and Celis, 1985). The regulation of PCNA gene expression is mediated by tandem CRE sequences in the PCNA promoter, and IL-2 stimulation of T-cells induces CREB/ATF protein binding to these CRE motifs (Feuerstein *et al.*, 1995; Huang *et al.*, 1994). Thus, the inhibition of CRE binding

complexes by cannabinol suggests that a reduction in PCNA expression may also be involved in the inhibition of lymphoproliferation by cannabinoids.

In light of the T-cell sensitivity to inhibition by cannabinoids and the sole expression of CB2, thymocytes were chosen as the experimental model for all subsequent studies of this research project. The focus on thymocytes provided a primary T-cell model to further characterize the mechanism of cannabinoid-mediated suppression of T-cells. Although the forskolin studies contributed significant insight into the effects of cannabinol on the cAMP cascade in mouse thymocytes, the effects of cannabinol in the presence of a relevant T-cell activation signal had not been examined. PMA/Io is often used to activate T-cells as it mimics signaling through the T-cell antigen receptor. Previous studies have shown a rapid and transient increase in intracellular cAMP levels following PMA/Io activation of splenocytes suggesting that PMA/Io can activate the cAMP pathway in leukocytes (Kaminski *et al.*, 1994). Furthermore, phorbol ester activation of PKC was reported to enhance adenylate cyclase activity indicating cross-talk between the cAMP and PKC signaling pathways (Yoshimasa *et al.*, 1987). The present results demonstrate that PMA/Io activation of thymocytes induced a predominant CRE complex consisting of a CREB-1 homodimer. The binding of the CREB-1 homodimer was markedly inhibited by cannabinol indicating the potential activation of the cAMP pathway by PMA/Io. The detection of CREB-1 in this CRE complex is consistent with recent findings that CREB-1 is a major component of the CRE complexes induced following T-cell activation through the antigen receptor or by co-treatment with Con A plus TPA (Feuerstein *et al.*, 1996; Wollberg *et al.*, 1994).

PKA is the most extensively characterized kinase by which CREB/ATF proteins are regulated, and PKA phosphorylation of CREB at Ser-133 induces the expression of cAMP responsive genes. In light of the cannabinol-mediated inhibition of forskolin-stimulated PKA activity, changes in the phosphorylation status of CREB were investigated in the presence of cannabinol. Cannabinol produced a marked inhibition of

CREB phosphorylation in thymocytes treated with either forskolin or PMA/Io. The cannabinol-mediated decrease in phospho-CREB following forskolin stimulation demonstrates the regulation of CREB by PKA and provides a mechanism for the suppression of CRE DNA binding complexes. Based on the established cross-talk between PKA and PKC pathways, the induction of CREB phosphorylation by PMA/Io and the inhibition by cannabinol implicates PKA phosphorylation of CREB in activated T-cells. However, several other kinases have been shown to phosphorylate CREB at Ser-133 including casein kinase, PKC, CaM kinase II and IV, and the RSK family of kinases (Gonzalez *et al.*, 1991; Gonzalez *et al.*, 1989; Means *et al.*, 1997; Tamai *et al.*, 1997). It is also notable that recent studies have suggested that CREB phosphorylation following T-cell activation occurs by a cAMP-independent mechanism (Barton *et al.*, 1996; Hsueh *et al.*, 1997).

The studies with H89 indicate a modest involvement of PKA in CREB phosphorylation in PMA/Io activated thymocytes. Furthermore, DBcAMP failed to reverse the cannabinol-mediated inhibition of CREB phosphorylation and IL-2 following PMA/Io activation of thymocytes. These findings suggest that an inhibition of PKA by cannabinol can not fully account for the significant decrease in phospho-CREB following thymocyte activation in the presence of cannabinol. These results are supported by recent work demonstrating that H89 was unable to inhibit the phosphorylation of CREB induced by phorbol ester plus calcium ionophore in EL-4 cells (Hsueh *et al.*, 1997). Moreover, CREB phosphorylation following phorbol ester and CD28 costimulation of EL-4 cells was associated with MAPKK activity rather than PKC, PKA, or p70s6k (Hsueh *et al.*, 1997). The responsiveness of CREB to cAMP, calcium, and mitogenic stimuli implicates CREB as a convergence point for multiple pathways in activated thymocytes, and more importantly, offers the possibility that cannabinol may modulate kinases other than PKA. Of these kinases, CaM kinase IV (CaMKIV) is critically relevant to T-cell activation studies. CaM kinase IV has been shown to phosphorylate Ser-133 of CREB in activated

T-cells indicating that CaMKIV functions as a CREB kinase in T lymphocytes (Gringhius *et al.*, 1998; Means *et al.*, 1997). This premise was strengthened further by recent transgenic studies using a kinase inactive form of CaMKIV specifically expressed in thymocytes. Anderson and coworkers reported a significant inhibition of CREB phosphorylation and IL-2 production when CaMKIV transgenic thymocytes were activated with PMA/Io (Anderson *et al.*, 1997). Their results were strikingly similar to the thymocyte-specific expression of a dominant negative CREB (Barton *et al.*, 1996) suggesting a mechanistic relationship between CREB and CaMKIV in early T-cell activation events. Interestingly, kinetic studies for Δ^9-THC demonstrated that maximal inhibition of the T-cell dependent IgM antibody forming cell response occurred within 30 min of drug exposure suggesting that cannabinoids inhibit an early T-cell activation event (Schatz *et al.*, 1993). In addition, Δ^9-THC has been shown to decrease intracellular calcium flux in Con A stimulated thymocytes (Yebra *et al.*, 1992). Based on the above evidence, CaMKIV appears to be one potential target of cannabinol-mediated inhibition in T-cells. An inhibition of CaMKIV by cannabinol may partially explain the effects of H89 and DBcAMP on CREB phosphorylation and IL-2 production in activated thymocytes. The MAP kinase cascade is also central to T-cell activation, and recent studies have shown an inhibition of MAP kinase activity by cannabinol in PMA/Io activated splenocytes (Faubert and Kaminski, 1999). Thus, the modulation of MAPK by cannabinol may also contribute to the inhibition of CREB phosphorylation and IL-2 secretion following PMA/Io activation of thymocytes.

According to densitometric analysis, cannabinol inhibited CREB phosphorylation below the basal level of phosphorylation detected in naive thymocytes. The magnitude of this effect may be partially explained by the protein phosphatase regulation of CREB; specifically the dephosphorylation of Ser-133 by PP-1. PP-1 activity is regulated by the I-1 inhibitor, and I-1 requires PKA phosphorylation in order to effectively inhibit PP-1 (Hagiwara *et al.*, 1992). The inhibition of PKA by cannabinol may indirectly decrease I-

1 regulation of PP-1 resulting in an enhancement of phosphatase activity. Therefore, an inhibition of kinase-induced phosphorylation coupled with an increase in PP-1 activity likely accounts for the marked decrease in phospho-CREB produced by cannabinol.

As previously noted, several studies have suggested that an elevation of intracellular cAMP leads to the activation of NF-κB (Muroi and Suzuk, 1993; Shirakawa *et al.*, 1989; Shirakawa and Mizel, 1989). The κB DNA binding results with forskolin and cannabinol would implicate an involvement of the cAMP pathway in NF-κB regulation. PMA/Io activation of thymocytes induced an upper κB binding complex that was sensitive to inhibition by cannabinol. The specific NF-κB/c-Rel proteins induced by PMA/Io and modulated by cannabinol were identified as a p65/c-Rel heterodimer. Interestingly, the cAMP cascade has been found to regulate c-Rel in T-cells as evidenced by increased c-Rel DNA binding after the activation of PKA (Lahdenpohja *et al.*, 1996). An increase in p65 binding activity has also been reported following phosphorylation by a PKA catalytic subunit found associated with the cytosolic NF-κB-IκB complex (Zhong *et al.*, 1997; Zhong *et al.*, 1998). This PKAc is inactive when bound to the NF-κB-IκB complex and becomes activated upon degradation of IκB-α; therefore, this regulation of PKA has been classified as a cAMP-independent mechanism. The phosphorylation of p65 by PKAc has also been shown to potently increase the transactivating activity of NF-κB (Zhong *et al.*, 1998). Moreover, the p65/c-Rel heterodimer is a transcriptional activator (Hansen *et al.*, 1994) and suggests that the inhibition of p65/c-Rel DNA binding by cannabinol decreases the expression of critical genes regulated by p65/c-Rel dimers.

The activation and subsequent nuclear translocation of NF-κB requires phosphorylation of the cytosolic IκB-α inhibitor which was initially thought to be mediated by several kinases including PKA and PKC. Recently, a large cytoplasmic IκB kinase complex has been characterized, and two IκB kinases (IKKα and IKKβ) that can phosphorylate IκB-α in response to activating stimuli have been identified as part of this larger IκB regulatory complex (DiDonato *et al.*, 1997). Although the regulation of the

IκB kinase complex is still unclear, it has been speculated that this complex may integrate signals from a variety of NF-κB activation pathways (May and Ghosh, 1998). In fact, upstream signals from NIK and MEKK have been reported to activate IKKα and IKKβ, respectively (Ling *et al.*, 1998; Nakano *et al.*, 1998). Based on the present results, the inhibition of NF-κB activation and DNA binding by cannabinol appears to be mediated through a reduction in IκB-α degradation. We believe that an inhibition of phosphorylation is the primary mechanism by which cannabinol interferes with the degradation of IκB-α. This initial decrease in phosphorylation retains NF-κB in the cytosol and prevents the degradation of IκB-α. As a result, the PKAc associated with the NF-κB-IκB complex remains inactive and unable to phosphorylate p65 thereby inhibiting its DNA binding and activation of target gene expression.

The H89 studies revealed no involvement of PKA in the regulation of NF-κB in thymocytes as evidenced by the lack of an effect on NF-κB binding and IκB-α degradation. This was further substantiated by the inability of DBcAMP or forskolin to reverse the cannabinol-induced inhibition of NF-κB binding activity. These findings strongly suggest that the mechanism of NF-κB inhibition by cannabinol in PMA/Io activated thymocytes occurs through signaling pathways other than cAMP. With the recent identification of the IκB kinase regulatory complex, the modulation of NF-κB by cannabinol may be mediated at several levels. The inhibition of IKKα and/or IKKβ kinase activity by cannabinol is one possible mechanism consistent with the interpretation that cannabinol decreases the phosphorylation of IκB-α. Alternatively, cannabinol may interfere with upstream regulatory signals involved in the activation of the IκB kinases. For example, an inhibition of NIK or MEKK activity by cannabinol would result in the decreased phosphorylation of IκB-α by IKKα and IKKβ. These conclusions are based on the premise that cannabinol alters the phosphorylation status of IκB-α; however, the degradation of IκB-α is a multi-step process. A similar profile of IκB-α protein expression would be observed in the presence of cannabinol if the drug: (1) interfered

with the ubiquitination of IκB-α; (2) inhibited the activity of the 26S proteosome; or (3) activated a phosphatase that dephosphorylated IκB-α. These possibilities await further investigation and can not be excluded at this time.

The minimal essential promoter region of the IL-2 gene contains binding sites for several inducible transcription factors including NF-AT, AP-1 and NF-κB; however, no specific CRE binding sites are present in the IL-2 promoter region. Despite the lack of a CRE in the IL-2 promoter, several recent reports have described a critical role for the CREB/ATF proteins in IL-2 regulation following T-cell activation (Barton *et al.*, 1996; Butscher *et al.*, 1998; Hsueh *et al.*, 1997). An essential role for CREB in IL-2 regulation was initially demonstrated using a dominant negative form of CREB which revealed a drastic inhibition of IL-2 production in PMA/Io activated thymocytes (Barton *et al.*, 1996). This effect was attributed to a decrease in the CREB-dependent expression of fos and jun proteins in the transgenic thymocytes. Alternatively, a direct role for CREB at the IL-2 promoter has also been proposed. For example, supershift studies in thymocytes have identified CREB as part of the protein complex binding to the AP-1 proximal (AP-1p) site of the IL-2 promoter (Chen and Rothenberg, 1993). It is important to emphasize that this particular AP-1 site is critical for AP-1 induction of the IL-2 gene (Jain *et al.*, 1992b). In addition, treatment of EL-4.IL-2 cells with PMA/Io plus forskolin enhanced binding to the AP-1p site further suggesting the direct involvement of CREB/ATF transcription factors in IL-2 regulation (Condie *et al.*, 1996). In accordance with this, CREB has also been shown to bind to the CD28RE site within the IL-2 promoter which is further supported by the observation that activation of a CD28RE-TRE CAT reporter construct was inhibited by dominant-negative CREB expression vectors (Butscher *et al.*, 1998). In light of this, the findings with cannabinol strongly suggest that inhibition of CREB binding in PMA/Io activated thymocytes is involved in the cannabinol-mediated suppression of IL-2 in these cells. NF-κB/c-Rel transcription factors also play an important role in regulating IL-2 expression by binding to the κB sequence within the IL-

2 promoter. Several recent reports have established that members of the NF-κB family can bind to the CD28RE of the IL-2 promoter demonstrating further regulation of IL-2 by these proteins (Butscher *et al.*, 1998; Ghosh *et al.*, 1993; Lai *et al.*, 1995). p50, p65, and c-Rel have all been identified in the CD28RE binding complex and the p65/c-Rel heterodimer was specifically shown to be a potent activator of the CD28RE (Ghosh *et al.*, 1993; Lai *et al.*, 1995). In light of this evidence, the inhibition of p65/c-Rel binding to the κB motif produced by cannabinol may be modulating IL-2 levels through both the κB and CD28RE sites of the IL-2 promoter. In addition to CREB and NF-κB, the induction of NF-AT and AP-1 is also essential for IL-2 expression. Recent studies have demonstrated the inhibition of NF-AT and AP-1 DNA binding complexes by cannabinoids in mouse splenocytes and the EL-4 T-cell line (Faubert and Kaminski, 1999; Yea *et al.*, 1999). Therefore, it is likely that the coordinated inhibition of CREB, NF-κB, NF-AT, and AP-1 results in the significant suppression of IL-2 expression by cannabinol in thymocytes.

Although the cAMP signaling cascade has been the most extensively studied signal transduction pathway coupled to both CB1 and CB2 receptors, additional signaling mechanisms for cannabinoid receptors have been reported. For example, the modulation of MAP kinase by cannabinoids has been shown in unstimulated CHO cells tranfected with either CB1 or CB2 (Bouaboula *et al.*, 1995; Bouaboula *et al.*, 1996). Consistent with the predominant expression of CB1 in the CNS, the CB1 receptor can inhibit both N-type and Q-type calcium channels as well as stimulate potassium influx (Felder *et al.*, 1995; Mackie and Hille, 1992). Interestingly, transfection of the At20 pituitary cell line with either CB1 or CB2 demonstrated that modulation of ion channels by cannabinoid receptors is unique to the CB1 subtype (Felder *et al.*, 1995). With the exception of cAMP, the identification of other signaling pathways coupled to the CB2 receptor in lymphocytes has not been extensively studied. The inability of membrane permeable cAMP analogs to reverse the inhibition produced by cannabinol suggests that modulation

of the cAMP cascade is not solely responsible for the effects of cannabinol on CREB, NF-κB, and IL-2 in activated thymocytes. However, the interpretation of these results is limited by the burst-attenuation kinetics of the cAMP pathway. The continual stimulation of thymocytes with DBcAMP or forskolin (60 min) does not mimic the rapid burst of intracellular cAMP that occurs following activation and may not represent endogenous cAMP activity. Despite this limitation, the results with H89 and DBcAMP suggest that the CB2 receptor may couple to additional signaling pathways in activated T-cells. Based on the complex regulation of IL-2, CREB, and NF-κB, two signaling pathways potentially coupled to CB2 are Ca^{2+}/CaMKIV and the MAPK cascade. Compelling evidence exists to correlate CREB phosphorylation and IL-2 expression with CaMKIV activity in thymocytes (Anderson *et al.*, 1997). As previously discussed, the modulation of MAPK activity by cannabinoids has been observed (Bouaboula *et al.*, 1995; Bouaboula *et al.*, 1996; Faubert and Kaminski, 1999). However, it is presently unknown whether the cannabinol-mediated inhibition of MAPK results from upstream modulation of Ras, MEKK, and/or MEK by the CB2 receptor. Alternatively, it is also possible that cannabinol produces direct effects on CaMKIV and the MAP kinases. Previous studies have reported CB1 receptor-independent effects of cannabinoids. For example, CP-55,940 induced the release of arachidonic acid in both CB1 transfected and untransfected CHO cells (Felder *et al.*, 1992). In addition, both stereoisomers of Δ^9-THC were shown to increase arachidonic acid levels in the guinea pig cortex (Reichman *et al.*, 1988). As a result, additional studies are necessary to examine CB2-mediated and/or CB2-independent effects of cannabinol on the regulation of IL-2, CREB, and NF-κB. The development of SR141716A, a CB1 receptor antagonist, has furthered the understanding of CB1-mediated effects of cannabinoids in the CNS (Rinaldi-Carmona *et al.*, 1994). Recently, a CB2-specific antagonist, SR144528, has been identified (Rinaldi-Carmona *et al.*, 1998). Therefore, future studies with CB2 receptor antagonists should provide significant insight into CB2-mediated versus CB2-independent effects of cannabinol.

In summary, this work has demonstrated that cannabinol inhibits the cAMP signaling cascade in mouse splenocytes and thymocytes which implicates a role for the CB2 receptor in immune modulation by cannabinoid compounds (Figure 43). Previous studies have determined that T-cells are sensitive to inhibition by Δ^9-THC (Schatz *et al.*, 1993); therefore, the present studies examined molecular mechanisms whereby cannabinoids suppress T-cell activation. Specifically, cannabinol inhibits the binding of a CREB-1 homodimer and a p65/c-Rel heterodimer to a CRE and κB DNA motif, respectively, in activated thymocytes. CREB/ATF and NF-κB/c-Rel transcription factors are critically involved in the regulation of IL-2. The decrease in CRE and κB DNA binding by cannabinol provides a potential explanation for the suppression of IL-2 expression by cannabinoid compounds following T-cell activation. Additionally, cannabinol inhibited the phosphorylation of CREB and prevented the degradation of IκB-α suggesting that cannabinol inhibited CREB and NF-κB activation through an inhibition of phosphorylation. Although the cAMP cascade is the best characterized signaling pathway coupled to cannabinoid receptors, DBcAMP was unable to reverse the cannabinol-mediated inhibition of CREB, NF-κB, and IL-2 in PMA/Io activated thymocytes. The major contributions of this research are as follows: (1) these results have established that cannabinol, a plant-derived cannabinoid with minimal CNS activity, is an immunosuppressive compound; (2) these findings provide significant insight into the molecular mechanisms of immunosuppression by cannabinoid compounds; and (3) these studies also suggest that signaling pathways other than the cAMP cascade significantly contribute to the modulation of CREB, NF-κB, and IL-2 by cannabinol in mouse thymocytes (Figure 43).

Despite the potential medicinal applications of cannabinoids, they have not been widely used as therapeutic agents. This primarily stems from their undesirable CNS effects. The unique tissue distribution of the CB2 receptor enables the selective targeting of the immune system. The modulation of CREB/ATF and NF-κB/c-Rel

Figure 43. Proposed mechanism of cannabinol-mediated inhibition of T-cells. The binding of cannabinol to the CB2 receptor inhibits the cAMP signaling cascade resulting in an inhibition of CREB phosphorylation and CRE DNA binding activity. Cannabinol also prevents the degradation of IκB-α which inhibits the nuclear translocation and DNA binding of a p65/c-Rel heterodimer. The inhibition produced by cannabinol in thymocytes is denoted by an X. The cannabinol-mediated inhibition of CREB and NF-κB transcription factors partially explains the decrease in IL-2 by cannabinoid compounds. It is likely that additional signaling pathways are modulated by cannabinol and/or the CB2 receptor as indicated by the (----) arrows.

CBN
CB2
Io
PMA
TCR
α
β
γ
ε
δ
ζ
ζ
Adenylate cyclase
ATP
cAMP
PKA
PKC
Ras
MEKK
MEK
MAPK
IκB kinase complex
IKKβ
IKKα
IκB-α
p65
c-Rel
Ca2+
Calcineurin
NF-AT
CaMKIV
CREB
AP-1
IL-2
CRE
CRE
CD28RE
AP-1p
κB
NF-AT

transcription factors by cannabinol is exciting in light of the fact that several cytokines, including inflammatory mediators, are regulated by CREB and NF-κB proteins. Therefore, CB2-selective compounds may have therapeutic potential as anti-inflammatory agents, and cannabinol may be a prototype for cannabinoid based immune modulators.

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