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2-ARACHIDONYL GLYCEROL AND ANANDAMIDE OCCURS
THROUGH PEROXISOME PROLIFERATOR ACTIVATED
RECEPTOR γ INDEPENDENTLY OF THE CANNABINOID
RECEPTORS

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CHERYL ELIZABETH ROCKWELL

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of the requirements for the

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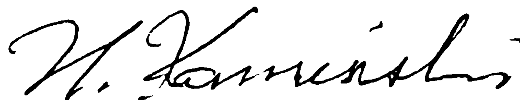

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INHIBITION OF INTERLEUKIN-2 SECRETION BY 2-ARACHIDONYL GLYCEROL AND ANANDAMIDE OCCURS THROUGH PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ INDEPENDENTLY OF THE CANNABINOID RECEPTORS

By

Cheryl Elizabeth Rockwell

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ABSTRACT

INHIBITION OF INTERLEUKIN-2 SECRETION BY 2-ARACHIDONYL GLYCEROL AND ANANDAMIDE OCCURS THROUGH PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ INDEPENDENTLY OF THE CANNABINOID RECEPTORS

By

Cheryl Elizabeth Rockwell

2-Arachidonyl glycerol (2-AG) and anandamide (AEA) are endogenous arachidonic acid derivatives which activate the cannabinoid receptors, CB1 and CB2, hence termed endocannabinoids. 2-AG and AEA modulate a variety of immunological responses, including induction of transient calcium influx in HL-60 cells, modulation of lymphocyte proliferation, and suppression of cytokine production. As is observed with the plant-derived cannabinoids, 2-AG and AEA suppress interleukin (IL)-2 production. The overall goal of these studies was to determine the mechanism of the inhibitory effects of AEA and 2-AG upon IL-2 secretion. The suppression of IL-2 by 2-AG and AEA in splenocytes derived from CB1/CB2 null mice coupled with the failure of the CB1/CB2 antagonists to block the decrease in IL-2 by AEA and 2-AG, demonstrates that the cannabinoid receptors are not involved. Interestingly, arachidonic acid causes a concentration-dependent suppression of IL-2 secretion, which was similar to that of structurally-related AEA and 2-AG. The decrease in IL-2 by AEA and 2-AG was partially reversed by pretreatment with the nonspecific cyclooxygenase (COX) inhibitor, flurbiprofen, as well as the COX-2 specific inhibitor, NS398, suggesting that COX-2 metabolites of 2-AG and AEA are responsible for the inhibitory effects upon IL-2, rather than the parent molecules. Because peroxisome proliferator activated receptor γ (PPARγ)

activation has been correlated with IL-2 suppression in T cells and a number of COX metabolites are known PPARγ agonists, the ability of 2-AG to activate PPARγ was investigated. Both 2-AG and 2-AG ether, a non-hydrolyzable analogue of 2-AG, activate PPARγ, as evidenced by differentiation of 3T3-L1 cells into adipocytes, induction of aP2 mRNA levels, and activation of a PPARγ -specific luciferase reporter in transiently transfected 3T3-L1 cells. Consequently, the putative role of PPARγ in IL-2 suppression by 2-AG and 2-AG ether was examined. IL-2 suppression by 2-AG and 2-AG ether in activated T cells was blocked by T0070907, a potent PPARγ -specific antagonist. Similarly, T0070907, also blocked AEA-mediated IL-2 suppression. Additionally, 2-AG was also found to inhibit the transcriptional activity of nuclear factor of activated T cells (NFAT) and nuclear factor κB (NFκB), transcription factors that are critical for IL-2 transcription. In addition to its effects upon IL-2, 2-AG suppresses the transcription of IL-4 and IFNγ, cytokines that are also regulated by NFAT. Moreover, the inhibition of NFAT and NFκB transcriptional activity by 2-AG was abrogated in the presence of T0070907. Collectively, the aforementioned studies provide evidence that suppression of IL-2 by COX-2 metabolites of 2-AG and AEA is mediated through activation of PPARγ independently of CB1/CB2. In addition, evidence is provided that PPARγ activation by 2-AG or its metabolite inhibits the transcriptional activity of NFAT and NFκB, which ultimately results in suppression of IL-2 by activated T cells.

DEDICATION

This dissertation is dedicated to my husband, Bryan, for his love and support throughout all the good times as well as the less-than-great times that have marked these crazy years at graduate school, and to my parents, for a lifetime of support and encouragement.

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LIST OF ABBREVIATIONS

AEA	Anandamide (arachidonyl ethanolamine)
2-AG	2-Arachidonyl glycerol
AMT	Anandamide membrane transporter
ANOVA	Analysis of variance
AP-1	Activator protein-1
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
COX	Cyclooxygenase
CP55940	(-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol
CREAE	Chronic relapsing experimental allergic encephalomyelitis
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
ETYA	Eicosatetraynoic acid
FAAH	Fatty acid amidohydrolase
FR122047	1-[[4,5-bis(4-methoxyphenyl)-2-thiazolyl]carbonyl]4-methylpiperazine
GW9662	2-Chloro-5-nitro-N-phenylbenzamide
HETE	Hydroxyeicosatetraenoic acid

IκB	Inhibitor of NFκB
IFN	Interferon
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
JWH015	(2-Methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone
JWH133	(6aR, 10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran
NAPE	N-arachidonyl phosphatidylethanolamine
LPS	Lipolysaccharide
MAFP	Methyl arachidonyl fluorophosphonate
MAP	mitogen activated protein
MS	Multiple sclerosis
NFkB	Nuclear factor of κ B
NFAT	Nuclear factor of activated T cells
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
NS398	N-[-2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
Oct	Octamer
PBS	phosphate buffered saline
PEA	Palmitoylethanolamine
PG	Prostaglandin
15d-PGJ_2	15-deoxy-$\Delta^{12,14}$ prostaglandin J_2

PHA	Phytohemagglutinin
PKC	Protein kinase C
PL	Phospholipase
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR response element
RXR	Retinoid X receptor
SC560	5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
SC58125	5-(4-fluorophenyl)-1-[4-(methylsulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazole
SR141716A	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorphenyl)-4-methyl-H-pyrazole-3 carboxyamidehydrochloride
SR144528	N-[(1S)-endo-1,3,3-trimethyl-bicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
Δ^9-THC	Delta 9-tetrahydrocannabinol
TNFα	Tumor necrosis factor α
T0070907	2-chloro-5-nitro-N-4-pyridinyl-benzamide
UCM707	N-(3-furanylmethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide
VR1	Vanilloid receptor 1

WIN55212-2	R (+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
WIN55212-3	S (-)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

LITERATURE REVIEW

I. Cannabinoid Receptors

Two cannabinoid receptors have been isolated and cloned to date, CB1 and CB2 (1-3). Both CB1 and CB2 have seven transmembrane domains and are G-protein coupled. A splice variant, CB1A, has also been discovered, although its physiological significance has yet to be determined (4). CB1 and CB2 are 44% homologous, which increases to 68% when considering only the transmembrane domains that compose the ligand binding site. Although there is considerable overlap in the ligands for CB1 and CB2, the receptors differ substantially in distribution and in activity.

A. Cellular signaling of cannabinoid receptors

Agonist-induced activation of both CB1 and CB2 inhibits adenylate cyclase in a variety of different model systems (1, 5-8). Inhibition of adenylate cyclase by both CB1 and CB2 is pertussis toxin-sensitive, indicating that both receptors associate with $G_{i/o}$ proteins (6, 7, 9, 10). Conversely, CB1 activation has also been shown to stimulate adenylate cyclase in pertussis toxin-treated cells, suggesting that in the absence of $G_{i/o}$ proteins, CB1 can associate with G_s (11). It has been hypothesized that CB1 and CB2 can couple to multiple isoforms of adenylate cyclase and that the result of cannabinoid receptor activation is dependent upon which adenylate cyclase isoforms are present (12).

Activation of CB1 and CB2 has also been correlated with ion channel regulation. Modulation of cAMP levels by CB1 has been shown to activate A-type potassium channels in rat hippocampal cells (13). Similarly, exogenously expressed CB1 in AtT-20

pituitary cells induces inwardly rectifying potassium channels in a pertussis toxin-sensitive manner (14, 15). In addition to potassium channels, CB1 activation has also been associated with modulation of calcium channel activity. Cannabinoid-mediated inhibition of L-type calcium channels is blocked with CB1 antagonist pretreatment and has been correlated with vasorelaxation of feline cerebral arterial rings (16). It has also been demonstrated that CB1 activation inhibits N-type calcium channels through $G_{i/o}$ (17-21). Likewise, CB1 activation has also been associated with a pertussis toxin-sensitive inhibition of Q-type calcium channels in AtT-20 pituitary cells expressing recombinant CB1 as well as P/Q-type calcium channels in rat cortical or cerebellar brain slices (15, 22).

In addition to its ability to inhibit voltage-gated calcium channels, CB1 activation also induces a rapid, transient increase in intracellular calcium in certain cell types. Cannabinoid-evoked transient calcium influx in NG108-15 neuroblastoma/glioma cells, is sensitive to pertussis toxin and inhibition of CB1 and phospholipase C (PLC), suggesting that activation of PLC by $G_{i/o}$ (β/γ subunit) induces the release of inositol-1,4,5-triphosphate (IP_3) that subsequently results in calcium influx (23-25). Likewise, cannabinoids augment depolarization-induced calcium influx in cultured cerebellular granule cells, which is also sensitive to pertussis toxin and inhibition of CB1 and PLC (26). Similar to CB1 activity in NG108-15 cells, CB2 activation has been correlated with the induction of transient calcium influx in HL-60 cells, which is blocked with a CB2 antagonist (27). Furthermore, pretreatment with both CB1 and CB2 antagonists attenuates calcium influx by cannabinol in primary splenocytes, suggesting that CB1 and CB2 also modulate calcium channels in this cell type (28). Interestingly, Δ^9-THC

induces a robust increase in intracellular calcium in primary splenocytes, which is also inhibited with both CB1 and CB2 antagonists, but is also observed in CB1/CB2 null mice (29) (unpublished observations). The aforementioned results suggest that another receptor may exist which is also activated by Δ^9-THC and inhibited by CB1 and CB2 antagonists. Consequently, studies characterizing the effects of CB1 and CB2 solely through the use of cannabinoid receptor antagonists may need to be reevaluated.

CB1 and CB2 have also been implicated in the activation of mitogen activated protein (MAP) kinase in a number of different cell types (30-32). Cannabinoid-mediated MAP kinase activation is inhibited by pertussis toxin and a CB1 antagonist in C6 glioma cells and primary astrocytes (33, 34). In U373 MG astrocytoma cells, phosphatidylinositol 3-kinase (PI3K) appears to be involved with CB1-mediated MAP kinase activation as protein kinase B is phosphorylated upon CB1 activation and PI3K inhibitors block MAP kinase signaling by CB1 (35). The activation of MAP kinase by CB1 has been correlated with the expression of a number of immediate early genes, such as krox-24, c-fos, and c-jun (36-40). Similarly, CB2 activation has also been associated with MAP kinase activation and an upregulation of krox-24 (41). Conversely, cannabinoids have also been shown to inhibit MAP kinase activation and immediate early genes under certain conditions, such as cellular activation (42). In addition to their effects upon calcium channel regulation and MAP kinase signaling, one or both cannabinoid receptors have also been linked to the activation of a number of other second messengers, such as cyclic guanosine monophosphate (cGMP), focal adhesion kinase, ceramide, and NO (43-46).

B. Expression of CB1

CB1 receptors are expressed in a number of different areas within the central nervous system (CNS) but have also been detected in a variety of peripheral tissues, including immune cells at low levels, sympathetic ganglia, reproductive tissues, gastrointestinal tissues, urinary bladder, heart, adrenal gland, lung, spleen, and the pituitary gland (47-53). Within the brain, CB1 is highly expressed in the cerebral cortex, hippocampus, amygdala, lateral caudate-putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, basal ganglia, and the cerebellum (54-58). The distribution pattern of CB1 in neuronal tissues is thought to be consistent with the ability of cannabinoids to diminish locomotor activity, produce catalepsy, as well as to impair cognition and memory (59). The expression of CB1 in the basal ganglia may have clinical applications for the treatment of diseases which are caused by the degeneration of basal ganglia neurons, such as Parkinson's disease and Huntington's disease (60). Additionally, CB1 has also been detected in the neuronal tissues of pain pathways in the spinal cord and brain, which may be responsible for cannabinoid-mediated analgesia (61). Conversely, there are relatively few CB1 receptors in the brainstem, which may explain the lack of toxicity associated with high doses of Δ^9-THC and other cannabinoids (62).

Several studies have demonstrated that CB1 is presynaptically localized on both inhibitory and excitatory neuronal fibers, suggesting a modulatory role in neurotransmission (58, 63-68). Consistent with the aforementioned findings, CB1 activation has been correlated with inhibition of GABA release from hippocampal interneurons and suppression of glutamate secretion from cerebellar basket cells (69, 70).

In addition to its role in short-term neurotransmission, CB1 activation has also been shown to inhibit long-term potentiation in rat hippocampal slices (71).

C. Physiological effects of CB1 activation

A variety of different physiological effects have been attributed to CB1 in a number of different systems, including the cardiovascular, gastrointestinal, reproductive, and the CNS. Within the CNS, CB1 activation results in decreased locomotion, analgesia, hypothermia, immobility, prolonged sleep duration, and hyperphagia (72-80). Additionally, activation of CB1 has been demonstrated to reduce spasticity in chronic relapsing experimental allergic encephalomyelitis (CREAE), an animal model of multiple sclerosis (MS), which has contributed to the interest in the clinical use of cannabinoids for the treatment of MS. In the cardiovascular system, CB1 activation causes bradycardia and vasorelaxation, which is not observed in CB1 null mice (81-85). Interestingly, CB1 may also play a role in reproduction as CB1 is highly expressed in the uterus and cannabinoids have been correlated with inhibition of embryo implantation and increased numbers of stillbirths (86-90). Within the gastrointestinal system, there has been considerable clinical interest in the ability of CB1 activation to prevent emesis (91, 92). Indeed, Δ^9-THC is prescribed in the U.S. and other countries for this purpose.

Additionally, CB1 activation has been correlated with certain immunological effects, although CB1 is typically expressed at much lower levels than CB2 in immune cells. CB1 activation inhibits nitric oxide (NO) in primary murine astrocytes as well as in rat microglial cells and feline macrophages stimulated with interferon (IFN) γ/lipopolysaccharide (LPS) (45, 93, 94). In a mouse peritonitis model, CB1 has been

shown to suppress neutrophil migration to the peritoneal cavity, which has been attributed to a delay in neutrophil chemokine production (95). Additionally, agonist-activated CB1 is associated with inhibition of interleukin (IL) -12 and tumor necrosis factor (TNF) α and induction of the immunosuppressive cytokine, IL-10, in LPS-treated mice (96, 97). Conversely, CB1 activation has also been correlated with immunostimulatory effects, such as induction of IL-6 in mouse astrocytes (96).

D. Expression of CB2

While CB1 is highly expressed in the CNS, CB2 is predominantly expressed in immune cells. In addition to immune cells, CB2 has also been detected in astrocytes, C6 glioma cells, rat oligodendrocytes, rat retina, embryonic rat liver, rat placenta, and rat uterus (98-101). Interestingly, CB2 is upregulated in neuritic plaque-associated glia in Alzheimer's disease as well as in the lumbar spinal cord in peripheral nerve injury (102, 103). Within the immune system, CB2 has been detected in B cells, natural killer cells, monocytes, macrophages, neutrophils, cytotoxic T cells, helper T cells, mast cells, dendritic cells, and microglial cells (53, 104-110). In addition, CB2 is differentially expressed in macrophages and microglia relative to activation state. CB2 is upregulated in IFNγ-primed macrophages and microglia and thioglycollate-treated macrophages, but down-regulated in LPS-treated macrophages and microglia (107). Additionally, CB2 is downregulated in LPS-treated splenocytes and upregulated in CD40-stimulated splenocytes (106, 111). Interestingly, CB2 is also upregulated in the peripheral blood mononuclear cells of marijuana smokers (112).

E. Physiological effects of CB2 activation

CB2 activation has been correlated with a number of immune effects in a variety of different models. Agonist-induced CB2 has been shown to enhance the proliferation of CD40-stimulated B cells, while conversely inducing apoptosis in thymocytes and splenocytes (113, 114). In primary murine astrocytes and feline macrophages, CB2 activation has been associated with inhibition of LPS-induced NO release (45, 94). Activated CB2 has also been shown to inhibit macrophage-induced activation of helper T cells, which is likely due to diminished antigen processing by the macrophages (115, 116). Furthermore, CB2 activation has been correlated with induction of cell migration in a number of different immune cell types, including microglia, myeloid precursor cells, differentiated HL-60 cells, and human peripheral blood monocytes (117-120). Migration of HL-60 cells may be related to the induction of the chemokines, monocyte chemotactic protein-1 (MCP-1) and IL-8, by CB2 (121). In addition to its effects upon the migration of myeloid precursor cells, CB2 also blocks neutrophilic differentiation in this cell type (119, 122). Modulation of cytokine release has also been reported to occur through CB2 activation, including induction of the immunosuppressive cytokine, transforming growth factor β (TGF-β), in human peripheral blood lymphocytes, and suppression of TNFα in human mononuclear cells (123, 124). Diminished overall antitumor immunity has also been attributed to CB2, resulting in larger tumors in cannabinoid-treated animals which is blocked with a CB2 antagonist (125). In addition to effects upon the immune system, CB2 activation has also been associated with antinociceptive effects to thermal stimuli as well as to formalin (126, 127).

F. Cannabinoid receptor specific agonists, antagonists, and knockout mice

Synthetic antagonists have been developed for both CB1 and CB2. SR141716A, AM251, and AM281 are CB1 antagonists, which are widely used (128-130). Likewise, SR144528 and AM630 are the most widely used CB2 antagonists (131, 132). SR141716A and SR144528 were developed first, while AM251, AM281, and AM630 were developed more recently. There are also a number of CB1 antagonists which are not commercially available (133). In addition to the antagonists, CB1- and CB2-specific agonists have also been developed. Arachidonyl-2'-chloroethylamide (ACEA) and arachidonyl cyclopropylamide (ACPA) are potent CB1-specific agonists, whereas JWH133 is a potent CB2-specific agonist (134, 135). JWH015 is less potent than JWH133, but is also CB2-selective agonist that is widely used (136). Perhaps the most powerful tools that have been developed for the identification of CB1/CB2-mediated activity are transgenic animals, including CB1 heterozygous knockout mice, CB1 homozygous knockout mice, CB2 homozygous knockout mice, and CB1/CB2 homozygous double knockout mice (84, 116, 137, 138).

II. Endocannabinoids

A. Identification of the endogenous cannabinoids

With the discovery of the cannabinoid receptors, a search for the endogenous ligands ensued. Isolated from porcine brain, anandamide (AEA) was the first endocannabinoid discovered (139). Shortly thereafter, a second endogenous ligand was isolated by two separate laboratories simultaneously (140, 141). 2-Arachidonyl glycerol (2-AG) was isolated from canine gut by Mechoulam's group, who also demonstrated that

2-AG possessed cannabimimetic activity, and from rat brain by Sugiura's laboratory, who determined that 2-AG is extremely sensitive to hydrolysis. Both AEA and 2-AG are arachidonic acid derivatives, and are structurally distinct from plant-derived and synthetic cannabinoids (Figure 1). Prior to its identification as a cannabinoid receptor agonist, 2-AG was regarded as a degradation product of inositol phospholipids and as a potential source of arachidonic acid under certain conditions, but was not considered to have biological activity of its own (142).

B. AEA biosynthesis and physiological concentrations

Two different pathways have been proposed for the synthesis of AEA (Figure 2). The first pathway, initially described by Deutsch and Chin in 1993, is the direct condensation of arachidonic acid and ethanolamine (143). Although other laboratories have confirmed that AEA can be formed from free arachidonic acid and ethanolamine, the physiological relevance of this has been called into question due to the exceptionally high concentrations of ethanolamine required (144-150). The direct condensation of free arachidonic acid and ethanolamine is now believed to occur through the enzyme, fatty acid amidohydrolase (FAAH), which is also considered to be the principal enzyme responsible for the catabolism of AEA (146, 148). While FAAH generally hydrolyzes AEA into arachidonic acid and ethanolamine, it is also thought to catalyze the reverse reaction, resulting in AEA synthesis. The evidence for the role of FAAH in AEA synthesis comes from the ability of FAAH inhibitors to block the condensation of arachidonic acid and ethanolamine (150).

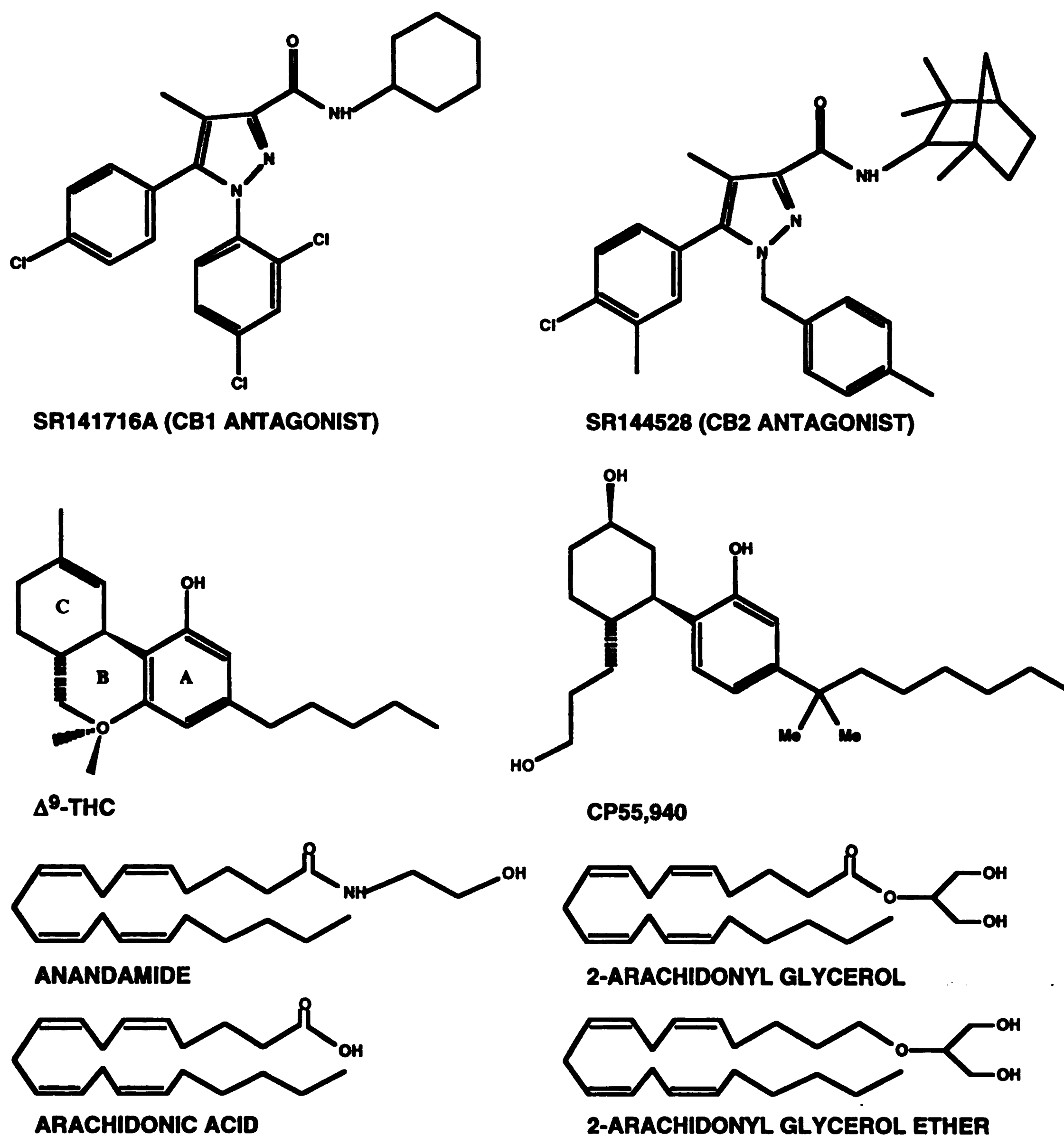


Figure 1. The structures of various cannabinoids. The putative endogenous cannabinoids, AEA, 2-AG, and 2-AG ether, are structurally related to arachidonic acid. Δ^9-THC is a plant-derived cannabinoid. SR141716A and SR144528 are antagonists of CB1 and CB2, respectively. CP55,940 is a synthetic agonist of both CB1 and CB2.

Due to the extraordinarily high concentrations of substrate needed for the condensation pathway, it is considered to be a minor mode of AEA biosynthesis. The bulk of AEA synthesis is believed to occur through a transacylase/phosphodiesterase pathway (Figure 2). According to the proposed pathway, the addition of an arachidonyl group from a donor phospholipid (thought to be phosphatidylcholine) onto phosphatidylethanolamine occurs through a transacylase to produce N-arachidonyl phosphatidylethanolamine (NAPE). NAPE is then hydrolyzed by phospholipase D (PLD), producing AEA and phosphatidic acid (151, 152). One troubling aspect of this pathway is that it is dependent upon the arachidonyl group being esterified in the sn-1 position of the donor phospholipid, which rarely occurs. As a result, it is tempting to postulate that there may be other pathways that also contribute to AEA synthesis. Nonetheless, there is evidence to suggest that this pathway is at least partially responsible for AEA production in N18TG2 neuroblastoma cells, J774 macrophages as well as in the rat brain and testis (153-156). Additionally, it has also been demonstrated that both the transacylase and PLD are calcium dependent, which corresponds to ionomycin-stimulated AEA release from N18TG2 and J774 cells (155-157). Calcium influx is not the only stimulus for AEA synthesis as simultaneous stimulation of NMDA and nicotinic receptors has also been shown to result in increased AEA levels in cortical neurons (158). Within the immune system, AEA has been detected in U937 lymphoma cells, macrophages, and dendritic cells (108, 154, 159). Furthermore, it has been demonstrated that AEA is induced in LPS-treated macrophages (160).

While the physiological concentrations of AEA vary somewhat between tissue types, AEA has been detected in mouse brain (10-15 pmol/g tissue), human brain (25-150

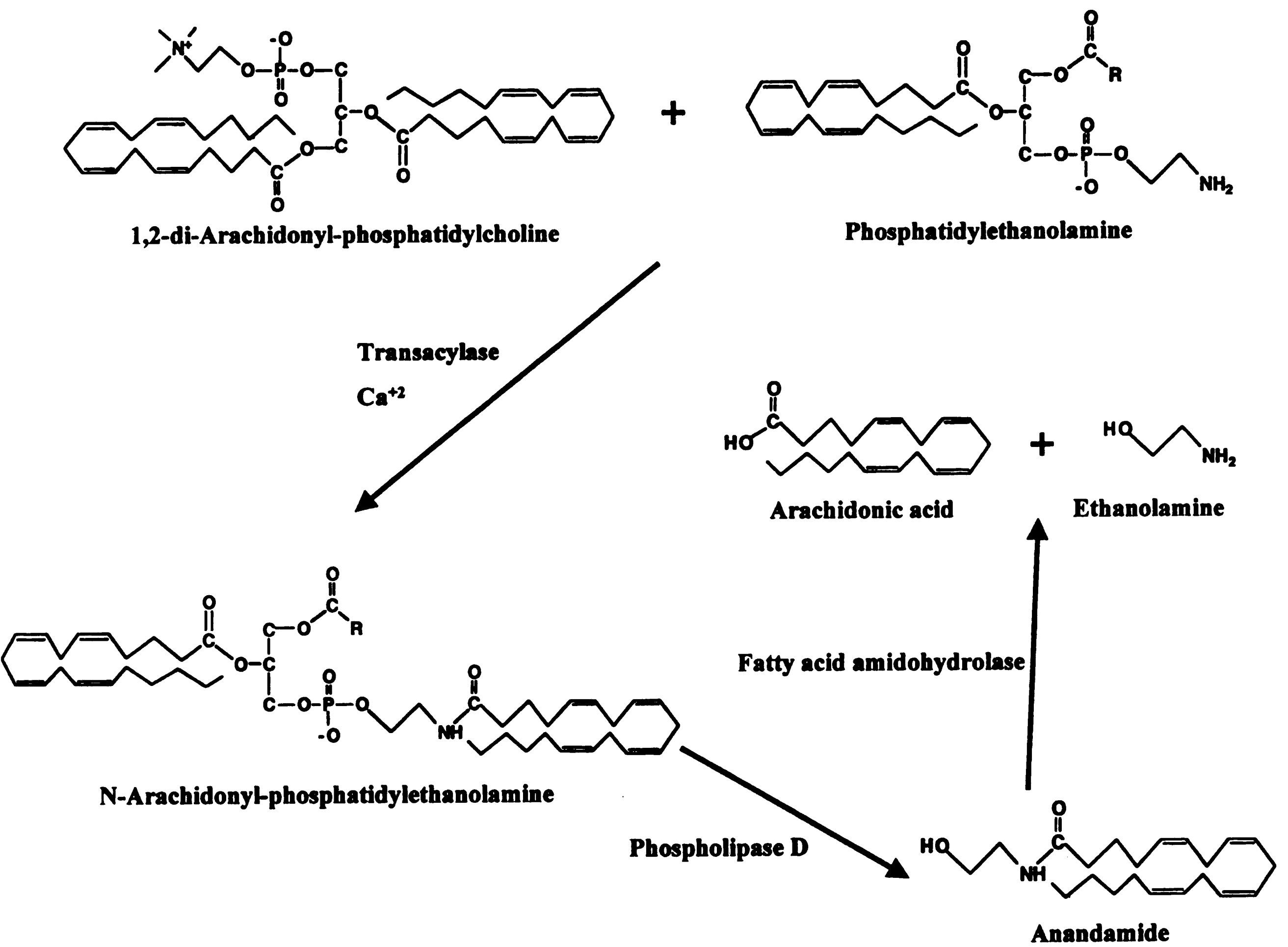


Figure 2. Schematic representation of AEA biosynthesis and catabolism.

pmol/g tissue), rat liver (20-77 pmol/g tissue), rat kidney (64-164 pmol/g tissue), human spleen (15 pmol/g tissue), rat spleen (6 pmol/g tissue), and rat thymus (40-137 pmol/g tissue) (161, 162). While AEA levels are relatively low in most tissues, high levels of AEA have been detected in mouse uterus (2200-21000 pmol/g tissue), leading to the supposition that AEA plays a role in reproduction (88). AEA levels have also been detected in rat plasma (144 nM), human sera (4 nM), as well as in human sera collected from patients who suffer from endotoxic shock (18 nM) (163, 164). Interestingly, several laboratories have demonstrated that various cell types sequester AEA, such that intracellular concentrations far exceed that of extracellular concentrations (165-167). Intracellular concentrations of AEA have been detected to be as high as 2 μM in Hep2 human laryngeal carcinoma cells (166). Furthermore, intracellular concentrations of AEA have been determined to be 1000 times higher than extracellular concentrations in cerebellular granule cells (167).

C. Metabolism of AEA

The catabolism of AEA is believed to occur largely through its hydrolysis into arachidonic acid and ethanolamine by fatty acid amidohydrolase (FAAH) (Figure 2) (143). The purification and sequencing of FAAH revealed a putative transmembrane domain, which correlates with previous studies demonstrating that FAAH is membrane-associated. FAAH appears to be widely expressed with the highest levels found in the liver, brain, and testis and relatively low levels found in the spleen. In addition to FAAH, another catabolic enzyme has been identified which hydrolyzes AEA (168, 169). While the optimal pH for FAAH activity is 8.5-10, this recently identified enzyme catalyzes

AEA hydrolysis most efficiently at a pH level of 5. The acid amidase was originally detected in human megakaryoblastic leukemia cells (CMK), but has since been found in a variety of tissues with the highest levels detected in lung and spleen (169).

In addition to hydrolysis by FAAH, the catabolism of AEA is also thought to be dependent upon the putative AEA membrane transporter (AMT). The evidence for transporter-mediated uptake of AEA consists of observations that AEA accumulation is temperature-dependent, saturable, selective for AEA and other closely related lipid substrates, and can be blocked by specific inhibitors (152, 170-175). The carrier-mediated transport does not require intracellular ATP or an ion gradient and accordingly, is assumed to occur through facilitated diffusion (171). Because the AMT has not yet been cloned despite considerable efforts, there has been a continuing controversy over its existence.

In addition to hydrolysis by FAAH and the acid amidase, it has also been demonstrated that AEA undergoes oxidative metabolism similar to that of arachidonic acid. The first report of the enzymatic oxygenation of AEA was in 1997 when Yu et al. demonstrated that AEA could be metabolized by cyclooxygenase-2 (COX-2) to produce prostaglandin (PG) –ethanolamides (EA) (176). The aforementioned study also demonstrated that AEA could neither bind to nor be metabolized by COX-1. The main product detected by Yu et al. was PGE_2-EA, but PGD_2-EA and other COX products were also tentatively identified. The production of PGD_2-EA was later confirmed when it was also detected in activated RAW 264.7 macrophages treated with AEA, demonstrating that the oxygenation and subsequent isomerization of AEA by COX-2 and PGD synthase, respectively, also occurs in immune cells (177). Likewise, AEA is also oxygenated by

COX-2 to produce $PGF_{2\alpha}$-EA in HCA-7 cells, which also produce PGE_2-EA and PGD_2-EA (177). The aforementioned studies also strongly suggest that AEA can be metabolized by thromboxane synthase or prostacyclin synthase subsequent to COX-2 oxygenation, resulting in the production of thromboxane A_2 (TxA_2) -EA or prostacyclin-EA, respectively. Because the metabolism of PGH_2-EA by thromboxane synthase is relatively inefficient, however, the production of TxA_2-EA is not likely to be physiologically relevant. Interestingly, PGE_2-EA binds to all four receptors for PGE_2 (EP_1-EP_4) and appears to mediate physiological effects similar to those produced by PGE_2, although these findings are in conflict with the observations of another laboratory (178, 179). It has also been determined that PGE_2-EA, PGA_2-EA, and PGB_2-EA all exhibit poor affinity for CB1 (180). Notably, recent studies have demonstrated that PGE_2-EA, PGD_2-EA and $PGF_{2\alpha}$-EA, are produced in vivo in mice treated with AEA (181). The levels of PGE_2-EA and PGD_2-EA were significantly higher in FAAH null mice, demonstrating that COX-2 plays a greater role in AEA metabolism in the absence of FAAH (181).

Similarly, it has also been demonstrated that AEA can be oxygenated by lipoxygenase (LOX) and cytochrome P450 in addition to COX-2. Initially, it was demonstrated that AEA is a substrate for porcine leukocyte 12-LOX, soybean 15-LOX, as well as rat brain 12-LOX (182, 183). Subsequent studies determined that AEA is metabolized by lipoxygenase-rich cells, including human platelets and polymorphonuclear cells, resulting in the production of 12(S)-hydroxyeicosatetraenoic acid ethanolamide (HETE-EA) and 15(S)-HETE-EA (184). While it was determined that 12(S)-HETE-EA has affinity for CB1 and CB2 comparable to that of AEA, 15(S)-HETE-

EA has no detectable affinity for CB2 and affinity which is four to sixfold lower than that of AEA for CB1 (183, 184). Consequently, lipoxygenase metabolism of AEA has been used to synthesize CB1- and CB2-selective agonists (185). Additionally, studies have demonstrated that AEA can be oxygenated by cytochrome P450 in murine brain and liver. Over twenty P450 metabolites have been identified, which may be due to metabolism by a number of different P450 subtypes, including, CYP1A, CYP3A, and CYP4A, as well as nonenzymatic conversions following P450 metabolism (186, 187).

D. AEA binding affinity for CB1/CB2 and cellular signaling

While it has been determined that AEA binds to both CB1 and CB2, the affinity of AEA for the cannabinoid receptors varies considerably among different assays and experimental conditions, such that there are a broad range of K_i values reported (0.14-543 nM and 33-1940 nM for CB1 and CB2, respectively) (3, 109, 134, 140, 188-193). Because AEA is metabolically and chemically unstable, one reason for the discrepancies between the reported K_i values for AEA is enzymatic hydrolysis and nonenzymatic conversions. For instance, many of the early studies were conducted in the absence of phenylmethylsulfonyl fluoride, which inhibits FAAH, and as a consequence report higher K_i values.

The efficacy of AEA for CB1 and CB2 has also been somewhat disputed. AEA was initially characterized as a full agonist of CB1, as determined from inhibition of cAMP accumulation (190). Later studies described AEA as a partial agonist for CB1 when compared to synthetic cannabinoids as determined by stimulation of GTPγS binding (194-196). A possible explanation for the aforementioned observations is that

these studies were performed in neuronal tissues with a mixed G-protein population of which G_o is dominant. Furthermore, G_o shows a more rapid rate of GDP-GTP exchange than G_i, such that the majority of the signal observed is likely to represent G_o. Because it has been demonstrated that AEA is a partial agonist in the activation of G_o, AEA is only partially efficacious in GTPγS assays in neuronal cell preparations (197, 198). Furthermore, the instability of AEA may also confound the interpretation of experimental results in studies which do not account for metabolism. In addition to $G_{i/o}$, CB1 can also couple to G_s under certain circumstances resulting in increased cAMP levels. Similar to G_o, AEA is only a partial agonist in the activation of CB1 coupled to G_s (199). Likewise, AEA is a partial agonist in the inhibition of cAMP accumulation through CB2 (200). Interestingly, the same study demonstrated that AEA was a full agonist in stimulating GTPγS binding in cell lines expressing high levels of CB2, whereas AEA was a partial agonist in cell lines expressing lower levels of CB2.

In addition to its effects upon cAMP and GTPγS, it has also been demonstrated that AEA modulates ion channels, particularly calcium channels, in a variety of different cell types. Similar to the effects of AEA upon GTPγS binding, the level of CB1 expression also affects the efficacy of AEA in the inhibition of calcium influx. AEA is a partial agonist in N18TG2 neuroblastoma cells, but is fully efficacious in AtT20 cells, which are stably transfected with CB1 and therefore express a higher number of CB1 receptors than N18TG2 cells (15, 20). In arterial smooth muscle cells, it has been demonstrated that AEA inhibits L-type calcium currents via CB1 activation (16). It has also been demonstrated that AEA both inhibits and enhances NMDA-receptor mediated calcium responses, depending upon the cell type and level of cellular activation (26, 201).

The inhibition of NMDA-stimulated calcium influx by AEA can be blocked with a CB1 antagonist, suggesting the involvement of CB1. The enhancement of NMDA-induced calcium influx by AEA is likely due to PLC activation, which causes a downstream release of calcium from intracellular stores (26, 201). Additionally, AEA also induces a transient calcium elevation in calf pulmonary endothelial cells, which is blocked with a CB2 antagonist (202).

Similar to its effects upon calcium influx, it has been demonstrated that AEA both enhances and inhibits potassium channels. While AEA enhances the response of G-protein coupled inwardly rectifying potassium channels, AEA also inhibits potassium current in synaptosomes isolated from rat hippocampus (15, 203, 204). Additionally, methanandamide, an analogue of AEA, inhibits post-synaptic potassium currents in hippocampal neurons, an effect that is likely mediated by CB1 (205). Independent of CB1, AEA inhibits the potassium channel, TASK-1, which functions to set resting membrane potential (206).

In addition to effects upon cAMP and ion channels, AEA modulates other signal transduction pathways, such as MAP kinases. Upregulation of MAP kinase by AEA appears to be mediated by CB2 in 32D/EPO hematopoietic cells, but at least partially by CB1 in ECV304 human endothelial cells and hippocampal neurons (31, 207, 208). Activation of MAP kinase by AEA correlated with enhanced proliferation in 32D/EPO hematopoietic blood cells, whereas it associated with arachidonic acid release and eicosanoid biosynthesis in WI-38 fibroblast cells (207, 209). Conversely, AEA also inhibits ERK activation in a CB1-dependent manner in neuronal progenitor cells, which may cause the AEA-mediated inhibition of cellular differentiation observed in this cell

type (210). Interestingly, AEA-mediated MAP kinase activation via CB1 has been shown to result in decreased prolactin receptor and nerve growth factor receptor expression and consequently inhibited proliferation of human breast cancer cells (211). Likewise, AEA treatment of PC-12 pheochromocytoma cells also results in decreased proliferation, albeit through induction of apoptosis rather than downregulation of growth factor receptors (212). The aforementioned studies have led to some discussion about the utility of AEA as a chemotherapeutic for cancer. Such discussion may be premature, however, in light of recently published studies demonstrating that methanandamide treatment results in augmented tumor growth in a murine model of lung cancer (213).

E. Other targets of AEA

In addition to its activity through CB1 and CB2, AEA also acts as a full agonist through the vanilloid receptor, VR1 (214, 215). Activation of VR1 by AEA modulates a variety of different responses, perhaps most notably, induction of neuropeptide release from sensory neurons as well as vasorelaxation and hyperpolarizaton of arterial smooth muscle tissue (215-218). Additionally, AEA-mediated VR1 activation has been correlated with apoptosis in a number of different cell types (219-221). Interestingly, enzymatic oxygenation of AEA may play a role in AEA-mediated VR1 activation as a lipoxygenase metabolite of AEA induces VR1-mediated contraction in guinea pig bronchus (222). Similarly, it has been demonstrated that a cytochrome P450 metabolite of AEA activates TRPV4, a receptor that is structurally related to VR1 (223).

There are a number of studies to suggest that AEA binds to and/or activates a number of other receptors, in addition to CB1, CB2, and VR1. There is evidence to

suggest that AEA binds to 5-hydroxytryptamine receptors, either in the ligand binding domain or at an allosteric site, and muscarinic receptors, particularly M1 and M4 (224-227). Additionally, there is also evidence to suggest the existence of a G-protein coupled receptor which mediates vasorelaxation in vascular endothelium and is activated by both AEA and the nonpsychotropic cannabinoid, "abnormal cannabidiol." The putative endothelial cannabinoid receptor is thought to phosphorylate ERK MAP kinase and is antagonized by the plant-derived cannabinoid, cannabidiol, as well as its synthetic analogue, O-1918, and low micromolar concentrations of SR141716A (84, 228, 229).

F. 2-AG biosynthesis and physiological concentrations

There are multiple modes of biosynthesis proposed for 2-AG. The best characterized pathway involves the rapid hydrolysis of phosphatidylinositol bisphosphate by PLC to produce diacylglycerol (DAG), which is then subsequently cleaved by DAG lipase to produce 2-AG (Figure 3) (71, 142, 230). There is evidence to suggest that the aforementioned pathway produces 2-AG in a variety of different cell types. Sugiura et al. have suggested that the combined actions of PLA_1 and PLC upon phosphatidylinositol bisphosphate could also result in 2-AG production (Figure 4) (230). Additionally, there is also evidence for a third mechanism of 2-AG biosynthesis, which involves a phosphatase that hydrolyzes lysophosphatidic acid to yield 2-AG (Figure 4). Both arachidonic acid-containing lysophosphatidic acid as well as the corresponding phosphatase activity have been detected in rat brain (230). The existence of still another biosynthesis pathway for 2-AG has been suggested, which involves the hydrolysis of arachidonic acid-containing phosphatidic acid by phosphatidic acid phosphohydrolase

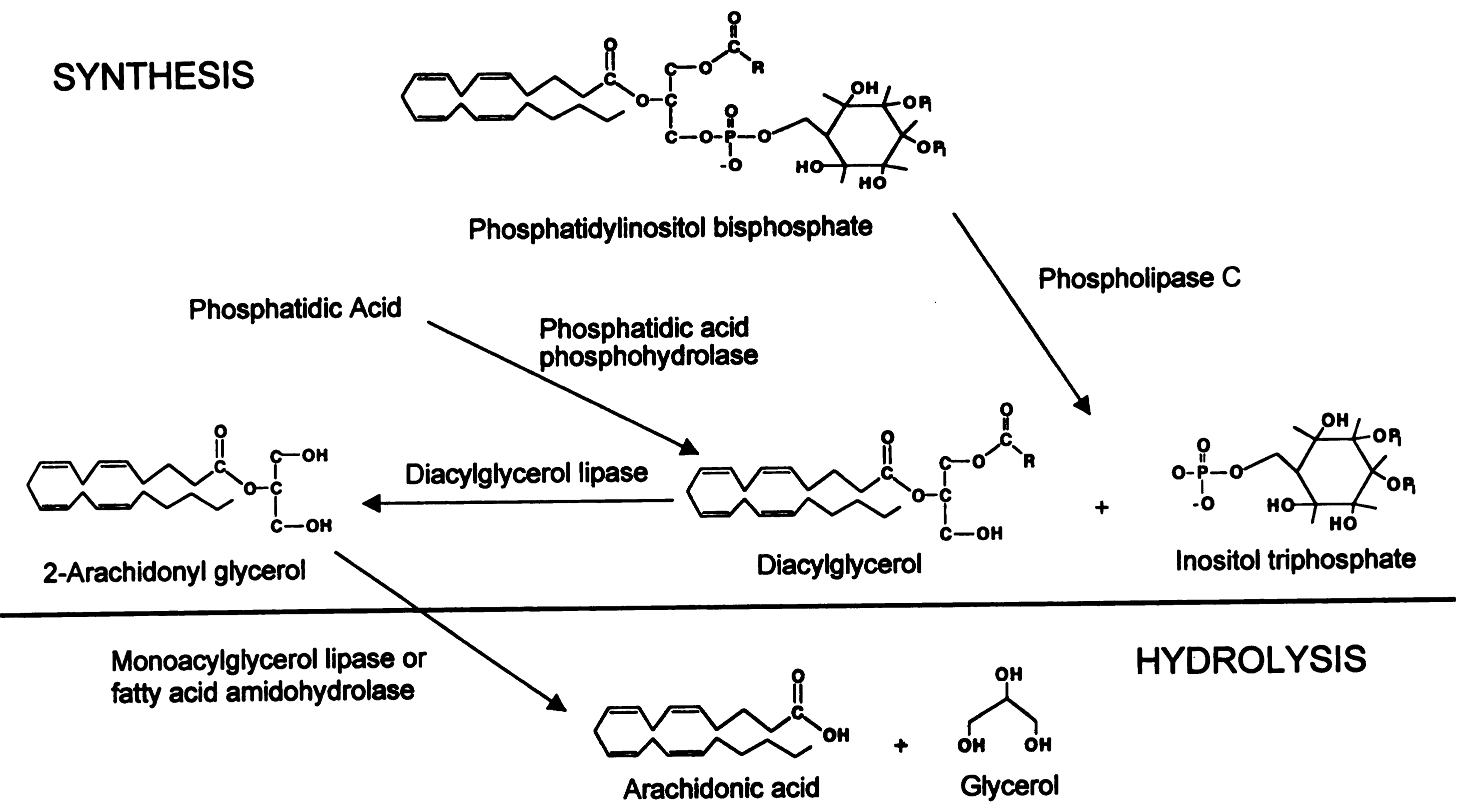


Figure 3. Schematic representation of 2-AG biosynthesis and hydrolysis.

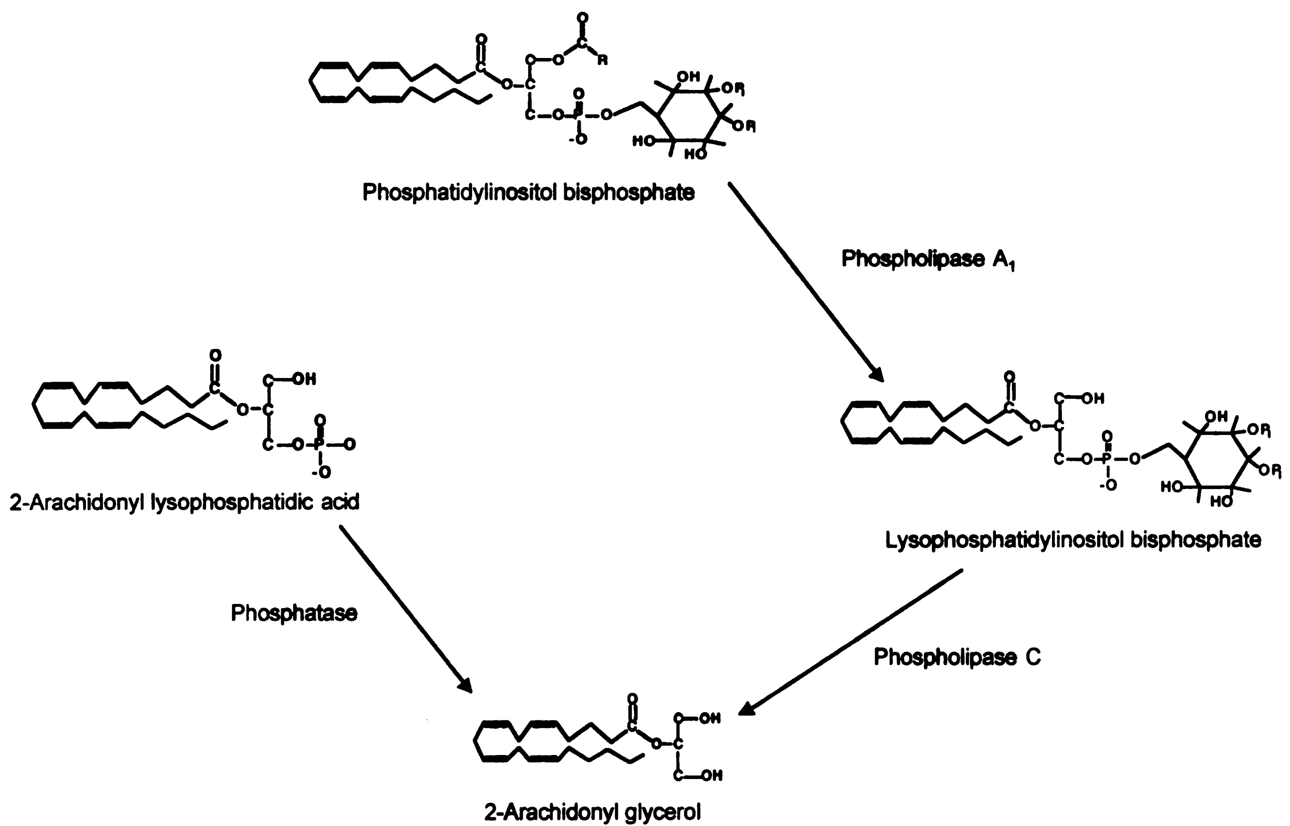


Figure 4. Schematic representation of alternative modes of 2-AG biosynthesis.

into DAG, which is then subsequently cleaved by DAG lipase (Figure 3) (231, 232). While the biological significance of the multiple proposed routes of 2-AG biosynthesis remains largely undetermined, there is evidence to suggest that the PLC/DAG lipase pathway is active in cortical neurons and platelets (71, 142). Likewise, the phosphatidic acid pathway appears to operate in mouse neuroblastoma cells and rat microglial cells (231, 232).

While the upstream events that result in the activation of PLC, phosphatidic acid phosphohydrolase, and DAG lipase have not been well characterized, increased intracellular calcium and glutamate receptor activation result in elevated 2-AG synthesis. Likewise, the activation of $P2X_7$ purinergic receptors has been implicated in 2-AG biosynthesis in murine astrocytes and microglial cells (233, 234) . Activation of immune cells, such as macrophages and dendritic cells, with LPS or platelet activating factor also results in increased 2-AG levels (108, 235, 236).

Physiological concentrations of 2-AG are generally higher than those of AEA in most cell types and can exceed the levels of AEA by as much as nearly 3 orders of magnitude. 2-AG levels have been detected in rat brain (3-65 nmol/g tissue), human brain (65 nmol/g tissue), rat liver (1.15 nmol/g tissue), rat spleen (1.17 nmol/g tissue), rat lung (0.78 nmol/g tissue), and rat kidney (0.98 nmol/g tissue) (71, 159, 237). Additionally, 2-AG has been detected in rat plasma (12 nM), human sera (10 nM), and human sera from patients with endotoxic shock (30 nM) (164). The accuracy of the reported 2-AG levels is not entirely clear due to a number of confounding factors. 2-AG is highly unstable, both chemically and metabolically, which results in the rapid isomerization and hydrolysis of 2-AG under a variety of different experimental

conditions. Furthermore, it has been reported that the half-life of 2-AG under culture conditions ranges from 2 to 10 minutes, depending upon the presence and concentration of serum or albumin (238). Moreover, it has also been reported that a number of the detection techniques commonly used are inefficient in the detection of both 2-AG and AEA (164).

G. Metabolism of 2-AG

2-AG hydrolysis occurs through at least two different pathways (Figure 3). In addition to AEA, FAAH can also hydrolyze 2-AG into arachidonic acid and glycerol (239). FAAH-mediated hydrolysis of 2-AG has been demonstrated in several different cell types but is not likely to be the primary mode of 2-AG hydrolysis. While AEA has significantly greater binding affinity for CB1 in FAAH null mice, there is no difference in the binding affinity of 2-AG for CB1 between FAAH null and wild-type control animals (240). Furthermore, the silencing of monoacylglycerol (MAG) lipase through RNA interference results in marked increases in 2-AG accumulation, suggesting that MAG lipase is the primary mechanism of 2-AG hydrolysis (241). Like FAAH, MAG lipase has been cloned and it has been determined that the two enzymes are not homologous (242, 243). Additionally, there is evidence to suggest that 2-AG may also be transported into some cell types through facilitated diffusion by either the putative AMT or a similar mechanism (244).

In addition to hydrolysis, 2-AG is also undergoes oxidative metabolism. As observed with AEA, 2-AG is metabolized directly by COX-2, but is not a substrate for COX-1 (245). The products of 2-AG metabolism by COX-2 include, 12-

hydroxyheptadecatrienoic acid glyceryl ester (G), PGE_2-G, PGD_2-G, $PGF_{2\alpha}$-G, thromboxane (Tx) B_2-G, a hydrolysis product of TxA_2-G, as well as 6-keto-$PGF_{1\alpha}$-G, a hydrolysis product of prostacyclin-G (177). The efficiency of COX-2 and the corresponding PG synthases to metabolize 2-AG is comparable to their ability to metabolize arachidonic acid. In contrast, metabolism of PGH_2-G by thromboxane synthase is markedly less efficient than the free acid, which suggests that the formation of TxA_2-G may not be physiologically relevant. Furthermore, platelets, which express only COX-1, are the predominant source for TxA_2 in vivo.

Additionally, 2-AG binds to and is oxidized by leukocyte 12-LOX and 15-LOX but is not efficiently metabolized by human 5-LOX or platelet 12-LOX (177, 246). The metabolites generated from 2-AG metabolism by LOX have been identified as 12(S)-HETE-G and 15-HETE-G. Although the physiological activity of the LOX metabolites is largely unknown, 15-HETE-G, which is the result of 2-AG metabolism by 15-LOX, has been found to be a ligand for peroxisome proliferator activated receptor α (247). It is unknown whether 2-AG, like AEA and arachidonic acid, can be metabolized by cytochrome P450 enzymes.

H. 2-AG binding affinity for CB1/CB2 and cellular signaling

Initial radioligand binding experiments with 2-AG demonstrated that 2-AG has lower affinity for both CB1 (K_i = 472 nM) and CB2 (K_i = 1400 nM) than AEA (140). Subsequent experiments demonstrated that the affinity of 2-AG for the cannabinoid receptors is likely higher than was initially reported. The more recent studies to examine 2-AG binding to CB1 and CB2 report K_i values of 58 and 145 nM for CB1 and CB2,

respectively (248). Despite the comparable affinity for CB1 and CB2, 2-AG exhibits greater activity in a variety of different cell systems than AEA, which is due to greater efficacy through CB1 and CB2. 2-AG is a full agonist of CB1 as determined by activation of G_i and G_o as well as by inhibition of cAMP accumulation (71, 198, 200,). Likewise, 2-AG also produces maximal inhibition of cAMP accumulation through CB2 activation (200).

While modulation of ion channels by AEA has been the focus of a number of published studies, fewer studies have explored the effects of 2-AG upon ion channel activation. A recently published study found, however, that 2-AG inhibits L-type calcium channels and activated inwardly rectifying potassium channels (Girk 1 and 4) through CB1 in rat sympathetic neurons (249). Additionally, 2-AG also mediates a transient calcium release in neuroblastoma cells, which is inhibited with a CB1 antagonist (24, 25, 250, 251). Likewise, 2-AG induces a transient calcium current in HL-60 cells, which is blocked with a CB2 antagonist and pertussis toxin (27).

Like AEA, 2-AG modulates MAP kinase pathways in a variety of different models. Activation of ERK1 and ERK2 by 2-AG in HL-60 cells appears to be mediated by CB2 as it can be blocked with a CB2 antagonist as well as by pertussis toxin (252). Similarly, 2-AG also activates ERK 1 in microglial cells in a CB2-dependent manner, which results in increased proliferation (232). In murine hippocampal slices, 2-AG activates p38 MAP kinase in wild-type mice, but not in CB1 null mice (208). Interestingly, activation of ERKs by 2-AG, like AEA, results in downregulation of prolactin receptors and subsequently reduced proliferation of breast cancer cells (211, 253, 254).

III. Therapeutic potential of endogenous cannabinoids

There has been considerable interest in the therapeutic potential of AEA and 2-AG. In particular, there has been considerable energy devoted to the inhibitors of FAAH and the putative AMT, which could be used clinically to raise endogenous AEA levels. There is diverse array of clinical conditions which might benefit from increased AEA concentrations, such that FAAH/AMT inhibitors are currently being investigated for their use as analgesics, anxiolytics, antispasmodics, cancer chemotherapeutics, anti-emetics, and for the treatment of neuropsychiatric disorders, such as schizophrenia (173, 254-268). Arguably, the analgesic effects of the FAAH/AMT inhibitors have received the majority of interest thus far. FAAH null mice have been found to possess a higher baseline for pain perception, which may be because AEA levels have been found to be 15 times higher in FAAH null mice than wild-type controls (255). Additionally, treatment of wild-type mice with a CB1 antagonist or antisense oligonucleotides against CB1 mRNA results in hyperalgesia, which is thought to be due to constitutive endocannabinoid tone (269). Moreover, several AMT inhibitors have been shown to produce analgesia in mice treated with a subeffective dose of AEA (173, 256, 257, 270). While the majority of focus has been upon the increase of endogenous AEA through FAAH/AMT inhibitors, a similar strategy could be employed for 2-AG. With an increasing number of studies reporting physiological effects of 2-AG that are of therapeutic interest, the clinical benefits of increased levels of 2-AG for a number of different conditions are currently being investigated.

IV. Other putative endogenous cannabinoids

In addition to AEA and 2-AG, there are several other putative endogenous cannabinoids which have been identified. Early binding studies revealed the existence of two other lipids in brain tissue, homo-gamma-linolenylethanolamide and docosatetranylethanolamide, which also bind to CB1 (271). Additionally, palmitoylethanolmine (PEA) was shown to have analgesic effects, similar to AEA but of longer duration, which were blocked with a CB2 antagonist (170, 272). Notably, it was later determined that PEA does not bind to CB2 and therefore cannot activate the receptor directly. It is still unclear whether the ability of the CB2 antagonist, SR144528, to block PEA-mediated analgesia is due to non-specific effects, blockage of a CB2-like receptor, or some other mechanism. There have also been a number of metabolically stable analogs of both AEA and 2-AG that have been developed to avert rapid hydrolysis in tissue culture as well as in whole animal studies. Methanandamide and fluoromethanandamide are more resistant to hydrolysis by FAAH than AEA (273-275). In contrast, 2-fluoroanandamide is equally susceptible to hydrolysis by FAAH as AEA, but has increased binding affinity for CB1 (192). Likewise, 2-AG ether was developed as a metabolically stable analog of 2-AG (25). In contrast to the synthetic AEA analogs, 2-AG ether has been detected in murine brain tissue and consequently has been named as another endogenous cannabinoid (276). This may be premature, however, as other investigators report that they are unable to detect 2-AG ether in brain tissue from a variety of different mammals, including rat, mouse, and pig (277).

V. Immunomodulatory activity of cannabinoids

The earliest observations of the immunomodulatory effects of cannabinoids were in human patients. Juel-Jensen noted more frequent recurrences of herpes simplex virus infections in his patients who smoked marijuana (278). This small study observing effects in only a few patients was followed by a number of larger controlled studies. While the immune effects of marijuana in humans remains a contentious issue, published studies indicate that high doses of marijuana cause a significant drop in immunoglobulin production by B cells (279). The effect of low to moderate doses of cannabis upon immune cells in humans remains a debatable issue.

In vivo studies performed in animals indicate that Δ^9-THC exposure is correlated with a decreased resistance to bacterial, viral, and protozoan infections. Δ^9-THC has been shown to increase susceptibility of animals to herpes simplex virus as well as to enhance the progression of the infection (280, 281). Δ^9-THC has also been shown to cause an increased susceptibility to bacterial infections, such as *Listeria monocytogenes* and *Treponema pallidum* (280, 282). More recently it has been demonstrated that Δ^9-THC exacerbates brain infection by opportunistic amebae of the genus *Acanthamoeba*, which is associated with decreased production of the cytokines, IL-1 and TNFα (283). Additionally, Δ^9-THC causes immunosuppressive effects in immune cells from severely immunocompromised animals. Immune cells from mice infected with Friend leukemia virus, which causes profound immunodeficiency, were suppressed further upon Δ^9-THC exposure, such that T cells and NK cells were 100% inhibited (284).

The increased susceptibility of animals treated with Δ^9-THC to bacterial and viral infections may be due to some of the effects of Δ^9-THC upon macrophages, B cells, and

T cells. In macrophages, Δ^9-THC causes inhibition of phagocytosis as well as inhibition of NO production (285-287). In addition, Δ^9-THC inhibits antigen processing, but not antigen presentation by macrophages (288). Inhibition of antigen processing in macrophages by Δ^9-THC appears to be mediated through CB2 as the effect was completely blocked by the CB2 antagonist, SR144528 (115). B and T lymphocytes have also been shown to be sensitive to cannabinoids. Proliferation of B and T cells are both suppressed by cannabinoids in the μM range, however low concentrations of synthetic cannabinoids enhance proliferation of B cells (289-291). While Δ^9-THC does not appear to affect T cell-independent production of immunoglobulins, it markedly inhibits antibody formation that is dependent upon T cells (290). Δ^9-THC as well as other plant-derived cannabinoids also cause a decrease in the production of cytokines, including IL-2 (292, 293).

Because plant-derived cannabinoids modulate a variety of different immune effects and AEA and 2-AG have been shown to mimic the effects of plant-derived cannabinoids, a number of studies have investigated the effects of AEA and 2-AG upon the immune system. AEA has been shown to modulate a number of immunological responses, including inhibition of NO and IL-6 production in macrophages as well as inhibition of TNFα and neutrophil recruitment in LPS-induced pulmonary inflammation in mice (294, 295). Additionally, AEA suppresses the release of IFNγ, TNFα, as well as the soluble TNFα receptor in human peripheral blood mononuclear cells and inhibits the migration of activated CD8+ T cells (296, 297).

Whether these effects are in fact mediated through the cannabinoid receptors has yet to be rigorously examined, however, there are reports of CB2-mediated immune

effects by AEA and methanandamide. Induction of the immunosuppressive cytokine, TGFβ by methanandamide is blocked with the CB2 antagonist, SR144528 (123). Recently published studies suggest that AEA induces apoptosis of immature dendritic cells, which can be blocked with CB1 and CB2 antagonists (298). Likewise, a number of studies have also investigated the effects of 2-AG upon immune cells. 2-AG has been reported to induce calcium influx in HL-60 cells, increase NO production in human monocytes, enhance antibody formation in murine splenocytes, and to induce the migration of human peripheral blood monocytes and HL-60 cells (27, 120, 299, 300). 2-AG has also been shown to inhibit cytokine production, including TNFα release from both LPS-treated rat microglial cells as well as murine macrophages, IL-6 production in J774 macrophages, and IL-2 secretion in activated murine splenocytes (295, 301-303). While CB2 has been implicated in many 2-AG-mediated immune effects, the role of CB2 and/or CB1 in the immunosuppressive effects of 2-AG upon cytokine release has yet to be conclusively determined.

VI. Regulation of IL-2

A. IL-2 function, receptors, and clinical importance

IL-2 is an autocrine/paracrine factor secreted by activated T cells and is important for T cell survival, proliferation, and in some cases, differentiation (304). As such, IL-2 is a central cytokine for the development of an adaptive immune response. IL-2 is highly regulated both transcriptionally through a number of different transcription factors and post-transcriptionally through the stabilization of IL-2 mRNA. There are three IL-2 receptors: the high-affinity receptor, containing the IL-2Rα, IL-2Rβ, and IL-2Rγ chains,

the intermediate receptor, containing the IL-2Rβ and IL-2Rγ chains, and the low-affinity receptor, which consists of IL-2Rα alone. The IL-2Rγ chain is a shared component of several other cytokine receptors, including IL-4, IL-7, IL-9, and IL-15. Consequently, γ chain deficiency in humans results in severe combined immunodeficiency. Conversely, IL-2Rα-deficient mice exhibit automimmunity, inflammatory bowel disease, and premature death. Similarly, IL-2Rβ-null mice exhibit elevated IgG, IgE, and autoantibodies as a result of overdifferentiation of B cells into plasma cells.

B. NFAT

The IL-2 minimal essential promoter comprises a number of different response elements, including the NFAT, NFκB, AP-1, Oct, and CD28RE sites (Figure 5). NFAT binds to the IL-2 promoter in two well-characterized sites, referred to as the proximal and distal NFAT binding sites, but at least three other putative NFAT binding sites have also been identified within the IL-2 promoter (305, 306). The binding of NFAT to the IL-2 promoter is essential for IL-2 transcription (307). The distal NFAT site requires cooperative binding of both nuclear and cytosolic NFAT components (308). The nuclear NFAT component is a heterodimer of jun and fos family members. The cytosolic NFAT component is related to the rel family of proteins and requires dephosphorylation by the Ca^{+2}/calmodulin-dependent phosphatase, calcineurin, for activation (305, 309). Upon dephosphorylation of cytosolic NFAT by calcineurin, the nuclear localization sequence is exposed, causing translocation of cytosolic NFAT to the nucleus, where it can then bind with the nuclear component to the IL-2 promoter (310). Additionally, there is evidence

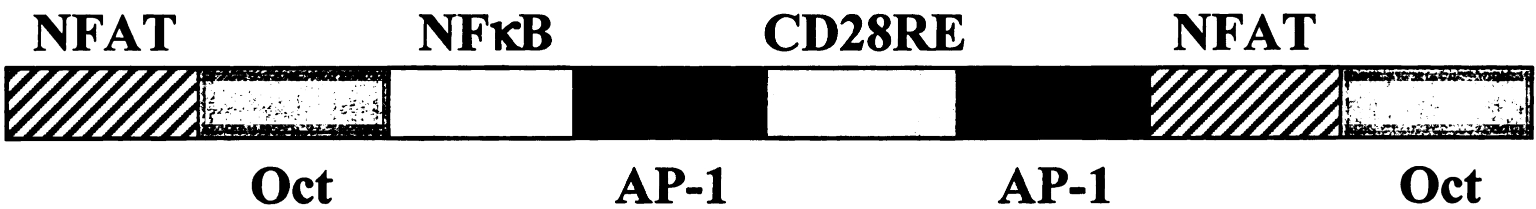


Figure 5. Schematic representation of the IL-2 minimal essential promoter region.

to suggest that NFAT may also play a cooperative role with other transcription factors in binding to the Oct-1 and CD28RE binding sites (306, 311, 312).

C. NFκB, AP-1, Oct, and CD28

Nuclear factor for κ chain in B cells (NFκB) has one binding site in the IL-2 promoter (307). In resting T cells, NFκB exists as a dimer with inhibitor of NFκB (IκB) in the cytoplasm. Upon T cell activation, IκB is phosphorylated by a number of different kinases. The primary kinase responsible for phosphorylation of IκB is IκB kinase (IKK), but IκB can also be phosphorylated by PKA and PKC (313-317). Phosphorylation of IκB causes it to dissociate from NFκB, such that NFκB can then translocate to the nucleus and bind to the IL-2 promoter.

AP-1 binds to the IL-2 promoter at two different sites, the proximal and distal AP-1 binding sites (318). While AP-1 binding at the distal site enhances IL-2 transcription, AP-1 binding at the proximal site seems to be essential for IL-2 transcription (318, 319). The AP-1 protein is best known as a heterodimer of jun and fos family members (320, 321). Additionally, the AP-1 complex may also consist of other members of the b-ZIP superfamily, such as CREB and CREB family members (321).

Oct has two binding sites in the IL-2 promoter, which are bound by the constitutively expressed Oct-1 and the lymphoid-specific Oct-2 (307, 322-324). The Oct-1 binding site is actually a composite NFAT:AP-1:Oct site, as cooperative binding occurs with both AP-1 and NFAT (306).

Maximal IL-2 production requires a costimulatory signal in addition to the activation of the T cell receptor (325). CD28 is a membrane protein that provides a

costimulatory signal during T cell activation, which results in the binding of transcription factors to the CD28RE. The stimulation of the T cell receptor and CD28 need to be temporally coincident and spatially proximal to produce full T cell activation (326). While the CD28 costimulatory pathway has not yet been fully elucidated, there is evidence to support that CD28 ligation results in activation of NFAT and NFκB (326-330). Furthermore, it has been determined that both NFAT and NFκB can bind to the CD28RE, in addition to other members of the rel family of proteins (311, 312).

D. Modulation of IL-2 production by cannabinoids

A number of different cannabinoids have been shown to inhibit IL-2 production, including Δ^9-THC, cannabinol, cannabidiol, WIN55,212-2, WIN55,212-3 and 2-AG (28, 292, 293, 331). While much insight has been gained into the mechanism of action of some of these compounds, much has yet to be learned about how cannabinoids inhibit IL-2 production. Cannabinol inhibits IL-2 secretion through inhibition of ERK MAP kinase activation, which causes a decrease in nuclear c-fos and ultimately leads to a decrease of AP-1 binding in the IL-2 promoter (42). In contrast, 2-AG inhibits NFAT binding and to a lesser extent NFκB binding to the IL-2 promoter in activated splenocytes (303). Conversely, cannabinol, cannabidiol and CP55,940 have also been shown to increase IL-2 production in suboptimally activated cells (332). Cannabinol-mediated enhancement of IL-2 transcription is likely to occur due to activation of NFAT by calcium calmodulin kinase II and PKC (332, 333).

VII. Cyclooxygenase

Cyclooxygenases (COX) are enzymes that catalyze the two-step formation of PGH_2 from arachidonic acid and oxygen, which is the committed step in prostanoid biosynthesis. The cyclooxygenase activity of COX initially converts arachidonic acid to PGG_2, which subsequently is reduced to PGH_2 by a peroxidase reaction. PGH_2 is the common substrate for an array of different synthase enzymes that produce prostaglandins and thromboxanes, although some prostanoids can be produced from PGH_2 nonenzymatically (334). Two isoforms of COX have been identified. COX-1 is constitutively produced in most cell types, although the levels can vary during different developmental stages and can be modulated in certain cell types (335, 336). COX-1 produces an array of different PGs and TXs, which function to maintain homeostasis in a variety of different tissues, including the gastrointestinal tract, the kidneys, and in platelets. COX-2 is undetectable in most tissues, but is rapidly upregulated in certain cell types in response to a variety of agents, including growth factors, cytokines, LPS, tumor promoters, and hormones (335, 336). Because COX-2 is believed to be the predominant isoform involved in prostanoid production during inflammation, a number of drugs have been developed to specifically inhibit COX-2 without affecting COX-1 activity. Both COX enzymes have been found in most cell types within the immune system, including T cells (334). In purified human T cells and Jurkat T cells, COX-1 is constitutively expressed at relatively low levels, whereas COX-2 is induced with PMA/ionomycin treatment (337).

There is evidence to support that a number of different prostanoids can cause suppression of IL-2 secretion, including PGE_2, 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2), and PGI_2

(338-340). Of the aforementioned eicosanoids, 15d-PGJ_2, is the most recent to be recognized to suppress IL-2 secretion. There has been growing interest in 15d-PGJ_2 in a number of different research areas due to its identification as one of the most potent endogenous ligands of peroxisome proliferator activated receptor γ (341).

VIII. Peroxisome Proliferator Activated Receptors (PPARs)

A. PPAR subtypes

PPARs are members of the nuclear receptor superfamily. There are three subtypes, PPARα, PPARδ (also called PPARβ and NUC1), and PPARγ. PPARs form heterodimers with retinoid X receptors (RXR), which are also nuclear receptors, and bind to peroxisome proliferator response elements (PPREs) found in the promoter regions of PPAR target genes (342). PPARα is highly expressed in metabolically active tissues, such as liver, heart, kidney, and muscle, where it plays a key role in regulating lipid metabolism (343). PPARα has also been recently discovered in B and T lymphocytes (342). Clofibrate, and other members of the fibrate class of drugs, as well as a number of fatty acids are agonists of PPARα. Interestingly, a 15-LOX metabolite of 2-AG, 15-HETE-G, is also an agonist of PPARα (247). PPARδ is ubiquitously expressed and although its physiological role is not well characterized, PPARδ may also play a role in lipid metabolism (343). PGD_2 and other fatty acids are ligands of PPARδ.

B. PPARγ isoforms

PPARγ has three different mRNA isoforms, γ_1, γ_2, and γ_3. PPARγ_1 is expressed in immune cells, such as macrophages, B cells, and T cells, and seems to play a role in the

regulation of a number of immune effects (342). Interestingly, activated PPARγ_1 sequesters NFAT such that NFAT cannot bind to the IL-2 promoter causing an inhibition of IL-2 transcription, a process called transrepression (340). PPARγ_1 has also been reported to transrepress NFκB and AP-1 in T cells and macrophages (340, 344, 345). PPARγ_2 is predominantly expressed in adipose tissue and plays a role in lipid homeostasis. The protein expressed by PPARγ_3 is identical to that expressed by PPARγ_1. The ligand binding domains of the PPARγ subtypes are identical (342). Troglitazone and its analogs, as well as 15d-PGJ$_2$, and other eicosanoids are agonists of PPARγ. In addition to specific agonists, there are two commercially-available PPARγ antagonists, GW9662 and T0070907. While PPARγ knockout mice are embryonic lethal, PPARγ null macrophages have been developed from the creation of PPARγ null embryonic stem cells via homologous recombination and the subsequent in vitro differentiation of the stem cells into macrophages (346).

C. PPARγ immune effects

A number of immune effects are attributed to PPARγ, which have generally been suppressive in nature. In macrophages, PPARγ ligands suppress inflammatory cytokines, such as IL-6, TNFα, IL-1β, IL-10, and IL-12 (344, 347, 348). Conversely, PPARγ activation has also been demonstrated to result in the upregulation of pro-inflammatory surface receptors, including CD14, CD11b/CD18, and CD36 (349, 350). Decreased expression of inducible NO synthase and induction of apoptosis have also been attributed to PPARγ in macrophages (351). The interpretation of some of these studies may need to be reviewed, however, as a recent report demonstrated that troglitazone, ciglitazone,

and 15d-PGJ_2 suppress TNFα and IL-6 production in both wild-type and PPARγ null macrophages (346).

While relatively few studies have investigated the role of PPARγ in B cells, a number of effects have been attributed to PPARγ in T cells. PPARγ activation has been associated with decreased cytokine production, inhibition of proliferation, as well as both the induction or prevention of apoptosis (340, 344, 352-354). The effects of PPARγ upon apoptosis in T cells may be dependent upon a variety of factors, including activation state and the particular model (transformed cells appear to be more sensitive to PPARγ –mediated apoptosis) (353-359). While apoptosis may be responsible for some of the immunosuppressive effects of PPARγ in T cells, a number of studies have reported that PPARγ activation suppresses cytokines, such as IL-2, IL-4, and IFNγ (340, 345, 352, 360-362).

Many investigators have suggested that the suppression of cytokine production by PPARγ has pathophysiological and clinical implications. Consequently, there has been considerable interest in the therapeutic potential of PPARγ ligands for autoimmune diseases. Indeed, a number of different animal models of autoimmune disease have been ameliorated with PPARγ agonist treatment, including allergic asthma and experimental crescentic glomerulonephritis (363-365). The therapeutic effects of PPARγ agonist treatment appear to involve suppression of T cell function in certain autoimmune models, such as autoimmune myocarditis, autoimmune diabetes, and experimental allergic encephalomyelitis (EAE), which is an animal model of multiple sclerosis (MS) (366-369). EAE, in particular, has been the focus of a number of studies, which have demonstrated that PPARγ agonists suppress T cell proliferation and cytokines, including

IFNγ, IL-4, and IL-10 in this model (367, 368, 370). Furthermore, PPARγ deficient heterozygous mice develop an exacerberated form of EAE, which suggests that constitutively expressed PPARγ may be protective against EAE (367). Moreover, PPARγ agonists have been shown to inhibit T cell proliferation and suppress cytokine production in T cells derived from human MS patients as well (371). Interestingly, 2-AG and AEA also ameliorate the symptoms of EAE (258). Suppression of T cell function by PPARγ may also involve secondary indirect mechanisms, as PPARγ activation has been shown to decrease major histocompatibility complex (MHC) II receptor expression on atheroma-associated cells (372). MHC II receptors are responsible for antigen presentation to T cell receptors and as such are critical for T cell activation.

IX. Rationale

It is widely established that 2-AG and AEA modulate a variety of immune responses, including suppression of cytokine production, inhibition of immunoglobulin secretion, and suppression of $CD8^+$ T cell migration. It has also been demonstrated that 2-AG and AEA are produced by a number of different immune cell types upon activation, including macrophages, dendritic cells, basophils, and microglial cells (108, 160, 172, 232, 234-236). Consequently, 2-AG and AEA may play an important role in immune regulation and the maintenance of immune homeostasis. Additionally, the growing interest in the therapeutic uses of AEA and 2-AG, particularly as anti-emetics, anti-spasmodics, and appetite stimulants, in immunocompromised patients, necessitates the characterization of their effects in the immune system. While previous studies from this laboratory have determined that suppression of IL-2 production by 2-AG occurs

through inhibition of NFAT, the upstream events leading to the impaired response have not yet been elucidated. The mechanism of the suppression of IL-2 by AEA is less well characterized than that of 2-AG. Because the role of the cannabinoid receptors had not yet been determined in the suppression of IL-2 by AEA and 2-AG, the initial studies focused upon CB1 and CB2. Subsequent studies examined the involvement of other targets, including COX-2 and PPARγ. The overall goal of the project was to determine the mechanism of IL-2 suppression by 2-AG and AEA through the investigation of the following hypothesis: 2-AG and AEA suppress IL-2 secretion in activated T cells through PPARγ independent of CB1 and CB2 (Figure 6).

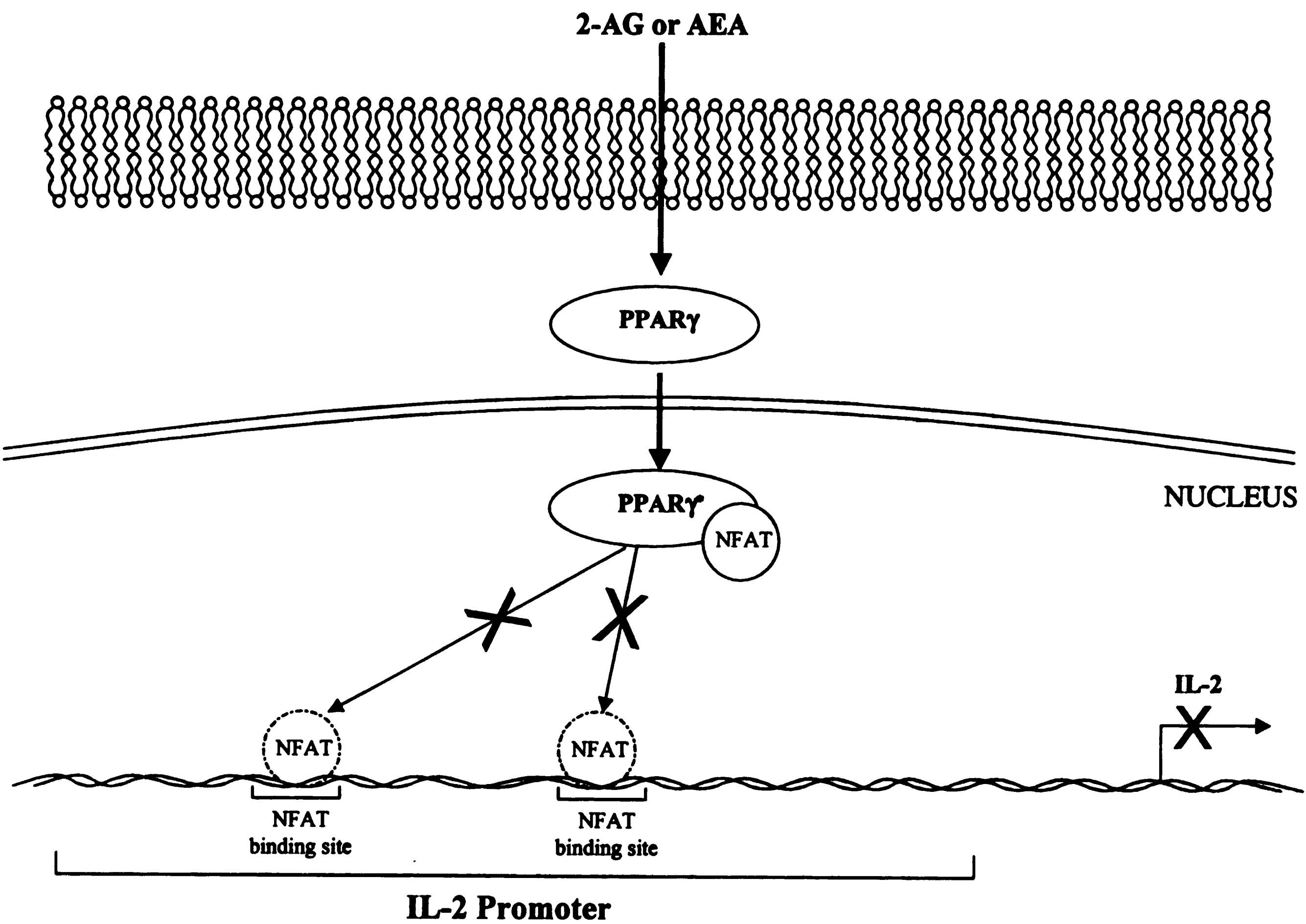


Figure 6. Schematic representation of the hypothesis.

MATERIALS AND METHODS

I. Reagents

AEA, 2-AG, CP55,940, cannabidiol, SR141716A and SR144528 were provided by the National Institute of Drug Abuse. JWH133, FR122047 and piroxicam were purchased from Tocris Cookson (Ellisville, MO). Arachidonic acid, 2-AG ether, PGE_2-ethanolamine, 15d-PGJ_2, PGJ_2, ciglitazone, GW9662, AM404, UCM707, MAFP, NS398, SC560, and T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Due to the lability of arachidonic acid and its derivatives, the following compounds were prepared as 50 mM stock in ethanol, aliquoted, and stored under nitrogen at -80°C for no longer than 30 days prior to use: arachidonic acid, AEA, 2-AG, ETYA, and 2-AG ether. Similarly, PGE_2-ethanolamine, 15d-PGJ_2, and PGJ_2 were dissolved in ethanol immediately prior to use and were stored no longer than 24 h due to their lability.

II. Animals

CB1 knockout mice ($CB1^{-/-}$) were developed in C57BL/6J mice by replacing most of the CB1 receptor coding sequence with the neomycin resistance gene through homologous recombination in MPI2 embryonic stem cells (84). CB2 knockout mice ($CB2^{-/-}$) were developed by replacing 341 bp of the CB2 receptor sequence with the neomycin resistance gene through homologous recombination (116). Chimeric mice were generated by morula aggregation or blastocyst injection with the targeted embryonic

cell line 129. For both $CB^{-/-}$ and $CB2^{-/-}$, chimeric mice were backcrossed with C57BL/6J mice. CB1/CB2 receptor double knockout ($CB1^{-/-}$/$CB2^{-/-}$) mice were obtained with the expected Mendelian frequency by mating mice heterozygous for both receptors (84). The mice heterozygous for both receptors, in turn, were obtained by mating $CB1^{-/-}$ mice with $CB2^{-/-}$ mice. The continued breeding of the $CB1^{-/-}$/$CB2^{-/-}$ mice was performed by University Laboratory Animal Resources at Michigan State University.

Female B6C3F1 mice, 6 weeks of age, were purchased from Charles River (Dortage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage), and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum*. Mice were not used for experimentation until their body weight was 17-20 g. Animal holding rooms were kept at 21-24°C and 40-60% relative humidity with a 12 h light/dark cycle. Spleens were isolated aseptically and made into single-cell suspensions (1 x 10^6 c/ml). Cells were cultured in RPMI-1640 supplemented with 100 units penicillin/ml, 100 units streptomycin/ml, 50 µM 2-mercapoethanol, and 2% bovine calf serum.

III. Cell Lines

Jurkat E6-1 and 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). Jurkat T cells were cultured in RPMI supplemented with 100 units streptomycin/ml, 100 units penicillin/ml, 10mM nonessential amino acids, 100mM sodium pyruvate, and 10% bovine calf serum. 3T3-L1 murine fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units

streptomycin/ml, 100 units penicillin/ml, 10mM nonessential amino acids, 100mM sodium pyruvate, and 10% bovine calf serum.

IV. IL-2 protein quantification

Splenocytes were cultured in triplicate (1 x 10^6 c/ml) in 48-well culture plates (800 μL/well). The cells were treated as described in the figure legends. The supernatants were collected 24 h after stimulation and IL-2 protein was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). Immulon IV coated wells (Dynatech, Chantilly, VA) were coated 1 h at 37°C (or overnight at 4°C) with 1 μg/ml purified rat anti-mouse IL-2 antibody (for splenocytes) or 2 μg/ml purified mouse anti-human IL-2 antibody (for human Jurkat cells). Wells were washed between each step with PBS-tween buffer (0.02% Tween in PBS). Prior to loading, the wells were blocked with blocking buffer (3% bovine serum albumin in PBS-tween buffer). Standard curves were generated with either mouse or human recombinant IL-2 protein. Standards and samples were loaded into wells and incubated for 1.25 h at 37°C, after which they were removed and biotinylated anti-mouse or anti-human IL-2 antibody was added. Following a 1.25 h incubation, the biotinylated antibodies were removed and replaced with streptavidin peroxidase (Sigma, St. Louis, MO). Following a 1 h incubation the streptavidin was removed and each well was developed with tetramethylbenzidine substrate (6 mg/ml in dimethyl sulfoxide). The reaction was terminated with 6N H_2SO_4. The plates were read at 450 nm wavelength on a Bio-Tek EL-808 plate reader. All IL-2 recombinant proteins and antibodies were purchased from Pharmingen (San Diego, CA).

V. 3T3-L1 Differentiation Assay and Oil Red O Staining

3T3-L1 cells, grown to confluence, were cultured in growth media (DMEM supplemented with 100 units streptomycin/ml, 100 units penicillin/ml, 10mM nonessential amino acids, 100mM sodium pyruvate, and 10% BCS) alone or in the presence of either 2-AG, 2-AG ether, ciglitazone, or vehicle (0.1% ethanol) for 7 days. The cells were then washed in PBS, fixed in 10% formalin, stained with Oil Red O (0.2% in 60% isopropanol) for 10 min, rinsed in 60% isopropanol, washed in tap water for 1-2 min, counterstained with 6% hematoxylin for 30 sec, and washed in PBS. The number of differentiated cells was quantified by enumerating the total number of differentiated cells per well.

VI. Real-time PCR

A. Reverse transcription and amplification

Total RNA was isolated from 3T3-L1 cells or splenocytes using TRI reagent (Sigma, St. Louis, MO), following the manufacturer's protocol. The relative expression levels of aP2, IL-4, and IFNγ, were determined by TaqMan one-step real-time multiplex PCR (Applied Biosystems, Foster City, CA). Relative mRNA expression for aP2, IL-4, and IFNγ, was normalized to the endogenous reference, 18S ribosomal RNA. Primers and probe for aP2 were designed to exclude the detection of genomic DNA using PrimerExpress software (Applied Biosystems, Foster City, CA), were synthesized by Applied Biosystems, and are as follows: forward primer, 5' AAGTGGGAGTGGGCTTTGC, reverse primer, 5' TCCCCATTTACGCTGATGATC, and probe, 5' CAGGCATGGCCAAGCCCACC. The probe was labeled with 6FAM dye

on the 5' end and TAMRA quencher on the 3' end. TaqMan predeveloped primers and probe were used for the detection of IL-4, IFNγ, and 18S ribosomal RNA.

B. Quantification by ΔΔCt protocol

The amplification of each sample was plotted as the change in fluorescence dye versus cycle number by the ABI Prism 770 Sequence Detection System (Applied Biosystems, Foster City, CA). Following the completion of the PCR reaction, the amplification plots were examined and a threshold was set manually within the exponential phase of amplification for the genes of interest. The thresholds are set separately for the target gene and the endogenous reference (18S ribosomal RNA). The cycle number at which the amplified product of each sample reaches the set threshold is termed the Ct value. The target gene is normalized to the endogenous reference through the subtraction of the Ct value of the endogenous reference from the Ct value of the target gene for every sample, which produces a value termed ΔCt. The relative mRNA levels are determined through the subtraction of the ΔCt value of the control sample from the ΔCt values of each sample of interest, which produces a value termed ΔΔCt. The relative mRNA levels are then calculated through the formula: $2^{-\Delta\Delta Ct}$.

VII. Transient transfection assay

A. 3T3-L1 cells (PPARγ-LBD/Gal4-DBD, Gal4-luc)

3T3-L1 cells (1.38 x 10^6) were cultured in growth medium for 16-20 h. The cells were then incubated with 37 μg plasmid and 115 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA), for 5 h in serum-free media. The transfected cells were then trypsinized,

washed, resuspended in growth medium, and plated (3 x 10^4 cells per well in a 24-well plate). Following another 5 h incubation, the cells were cultured in medium alone or in the presence of either 2-AG, 2-AG ether, ciglitazone, or vehicle (0.1% ethanol). Treatments were performed in triplicate. After a 16 h incubation, luciferase activity was assayed.

B. Jurkat T cells (NFAT-luc)

Jurkat T cells (5 x 10^5 c/ml) were incubated with transfection reagents (1.5 μg plasmid and 3 μl Lipofectamine 2000 for every 5 x 10^5 cells) for 12 h in RPMI 1640 medium with 2% BCS. The transfected cells were washed, resuspended in RPMI with 2% BCS, and treated with 2-AG or VH in the presence or absence of T0070907. After a 12 h incubation, luciferase activity was assayed.

C. Plasmids

The trans-acting PPARγ reporter gene plasmid was constructed by fusing the ligand-binding domain of mouse PPARγ to the DNA-binding domain of the yeast transcription factor, Gal4, under the control of the SV40 promoter. The plasmid also encoded the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element.

NFAT-luc, NFκB-luc, and AP1-luc reporter gene plasmids are commercially available from Clontech (Palo Alto, CA).

D. Luciferase Assay (Chemiluminescence method)

The cells were rinsed with PBS prior to addition of 50 µL 1X lysis buffer (Promega, Madison, WI). The lysates were then transferred to microcentrifuge tubes, placed on ice, vortexed for 10-15 s, and centrifuged for 15 s at 12,000 g. 20 µL of the cell lysate was then added to a luminometer tube containing 100 µL Luciferase Assay Reagent (Promega, Madison, WI). The chemiluminescence was then read by a Turner Designs TD-20e luminometer (Sunnyvale, CA).

VIII. 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 (ANOVA) using SigmaStat software (Jandel Scientific, San Rafael, CA). For those experiments with two factors, a 2-way ANOVA was employed. Dunnett's two-tailed t test was used to compare treatment groups to the vehicle control when significant differences were observed (373). IC_{50} values were calculated from the average of four different concentration responses using Prism Graphpad software.

EXPERIMENTAL RESULTS

I. Effect of AEA, 2-AG, and 2-G ether upon IL-2 secretion in primary splenocytes activated with PMA/ionomycin

It well established that marijuana and plant-derived cannabinoids have a variety of effects upon the immune system, which may ultimately result in increased susceptibility to bacterial and viral infections. A number of published studies from this laboratory have shown that Δ^9-THC and other plant-derived cannabinoids modulate T cell function, including inhibition of proliferation, induction of calcium influx, and suppression of cytokines, such as IL-2 (28, 29, 292, 374). Because AEA has been shown to mimic the activity of plant-derived cannabinoids in a variety of different model systems, the effects of AEA upon IL-2 secretion were investigated. In activated splenocytes, AEA causes a concentration-dependent suppression of IL-2, which is similar to that observed with Δ^9-THC and cannabinol (Figure 7). 2-AG also causes a concentration-dependent suppression of IL-2 secretion, which is modestly more potent than that of AEA (Figure 8a). Because it has been widely reported that 2-AG is highly unstable in tissue culture due to rapid hydrolysis, the effect of 2-AG ether, a non-hydrolyzable form of 2-AG, was assessed. 2-AG ether also produces a concentration-dependent suppression of IL-2, which is similar to that observed with 2-AG, suggesting that hydrolysis does not significantly diminish the effects of 2-AG in splenocytes (Figure 8b).

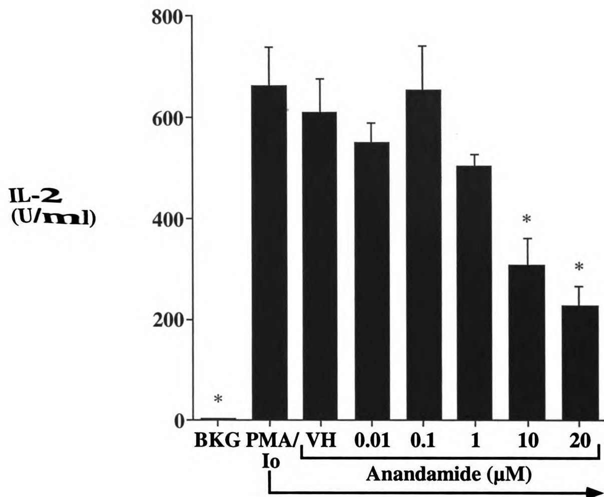
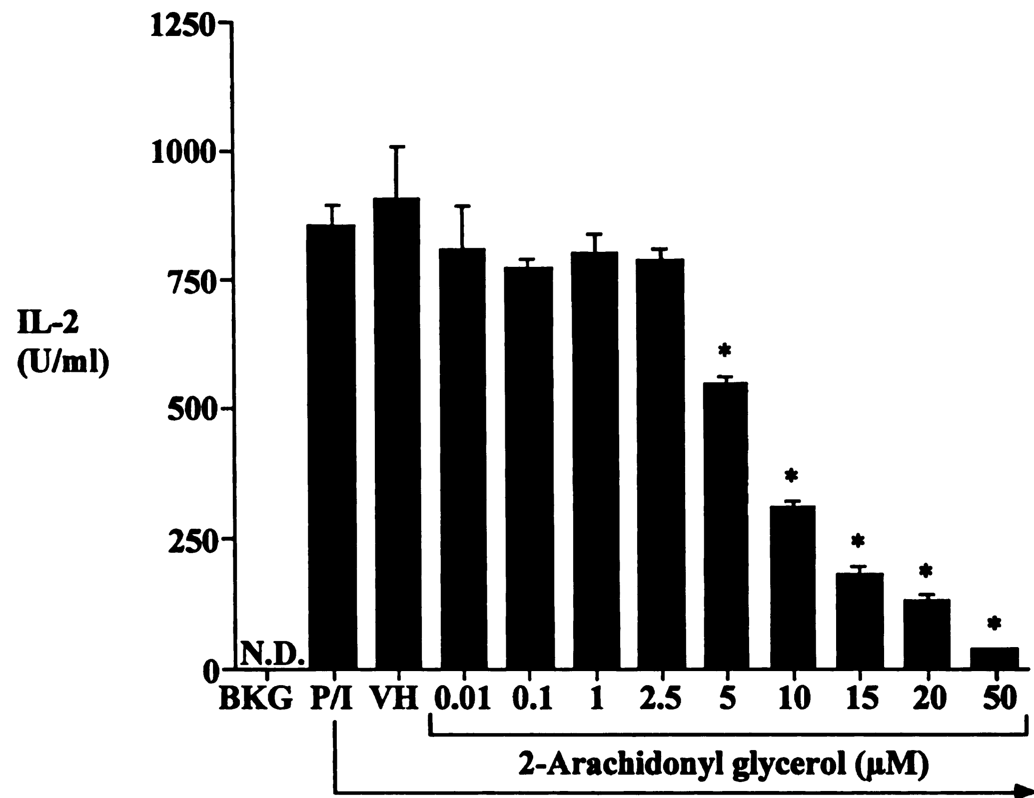


Figure 7. Effect of AEA upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with 0.01-20 μM of AEA or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH group. These data are representative of at least four separate experiments.

Figure 8. Effect of 2-AG and 2-AG ether upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with either A.) 2-AG (0.01-50 μM) or B.) 2-AG ether (0.1-50 μM) for 30 min followed by activation of the cells with PMA/ionomycin (40nM/0.5μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least three separate experiments.

A.



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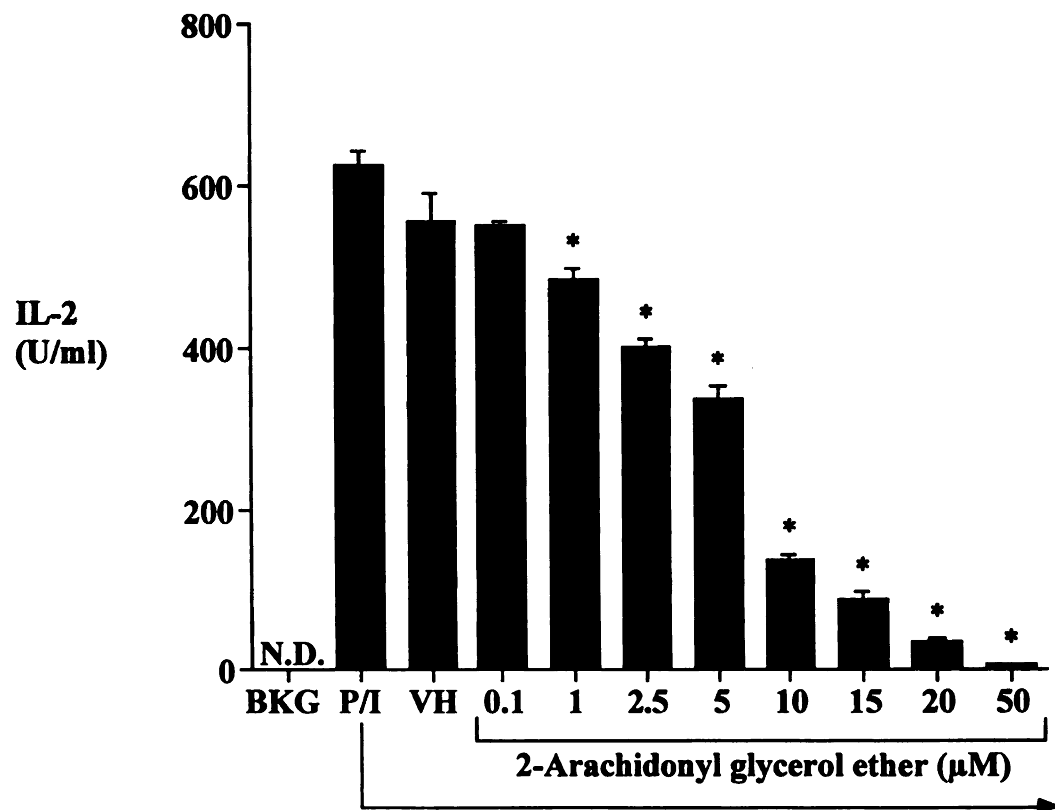


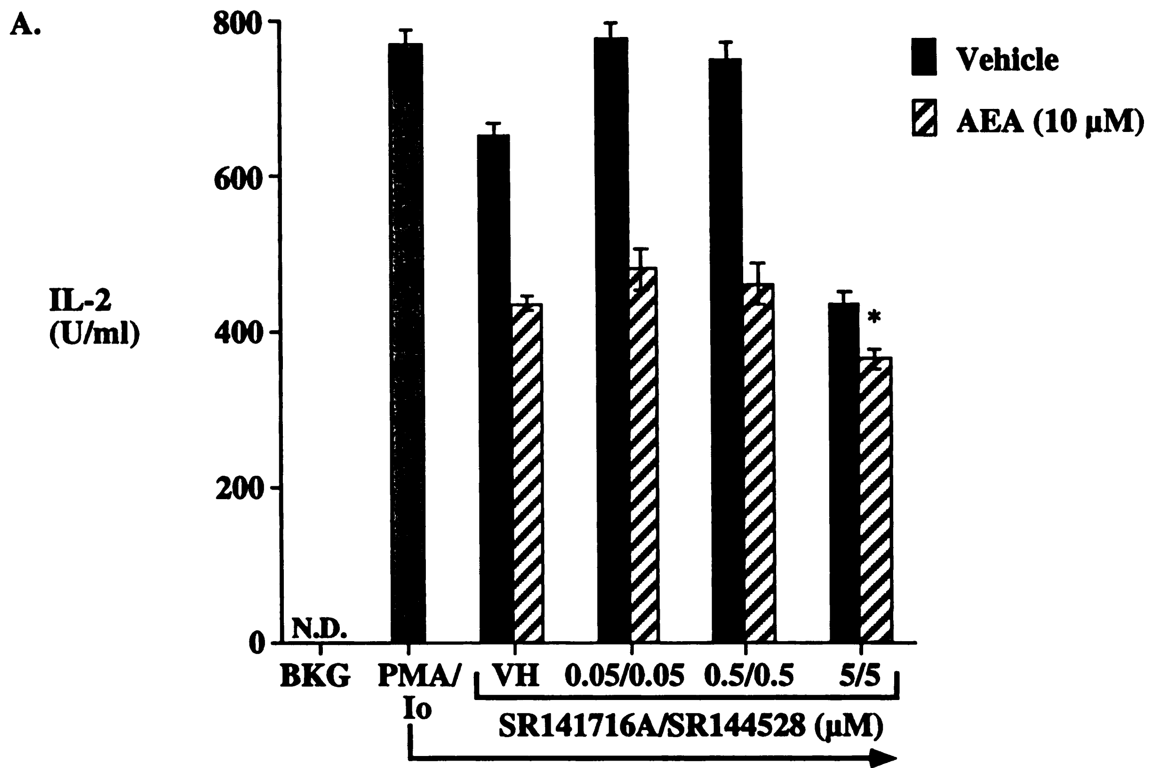
Figure 8.

II. Role of the cannabinoid receptors, CB1 and CB2, in the suppression of IL-2 secretion by AEA, 2-AG, and 2-AG ether

With the discovery that the putative endogenous cannabinoids suppress IL-2, the subsequent experiments were designed to determine the mechanism for this effect with the initial studies focusing upon the role of the cannabinoid receptors. Although CB2 is the predominant cannabinoid receptor expressed in immune cells, low levels of CB1 transcripts have also been detected in many immune cells, including T cells (104). Consequently, the role of both CB1 and CB2 in the suppression of IL-2 secretion by AEA, 2-AG, and 2-AG ether was evaluated. Pretreatment of primary splenocytes with the CB1 and CB2 antagonists, SR141716A and SR144528, used in combination (0.05/0.05, 0.5/0.5, and 5/5 μM), did not attenuate AEA-mediated suppression of IL-2 secretion (Fig. 9a). Additionally, pretreatment with SR144528 alone did not attenuate AEA-mediated suppression of IL-2 secretion (Figure 9b). Similar to AEA, pretreatment with SR141716A and SR144528, in combination, did not block the suppression of IL-2 secretion by 2-AG and 2-AG ether, suggesting that CB1 and CB2 are not involved (Figure 10). At the highest concentration used (5/5 μM), treatment of SR141716A and SR144528 alone decreased IL-2 production, which diminishes the utility of the antagonists at this level.

The role of the cannabinoid receptors in the modulation of IL-2 production was further examined through the use of the CB2-specific agonist, JWH133. Treatment of primary splenocytes with JWH133 did not suppress IL-2 except at the highest concentration used (50 μM), which further suggests that the decrease in IL-2 secretion is independent of CB2 (Figure 11). Additionally, AEA, 2-AG, and 2-AG ether all suppress

Figure 9. Effect of cannabinoid receptor antagonists on AEA-mediated suppression of PMA/ionomycin-stimulated IL-2 production. Splenocytes (1 x 10^6 cells/ml) were pretreated with either A.) both SR141716A and SR144528 or B.) SR144528 alone for 30 min followed by AEA (10 μM) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. A.) * $p<0.05$ compared to VH + AEA group. B.) None of the groups were significantly different from the 0 + AEA group. These data are representative of at least three separate experiments.



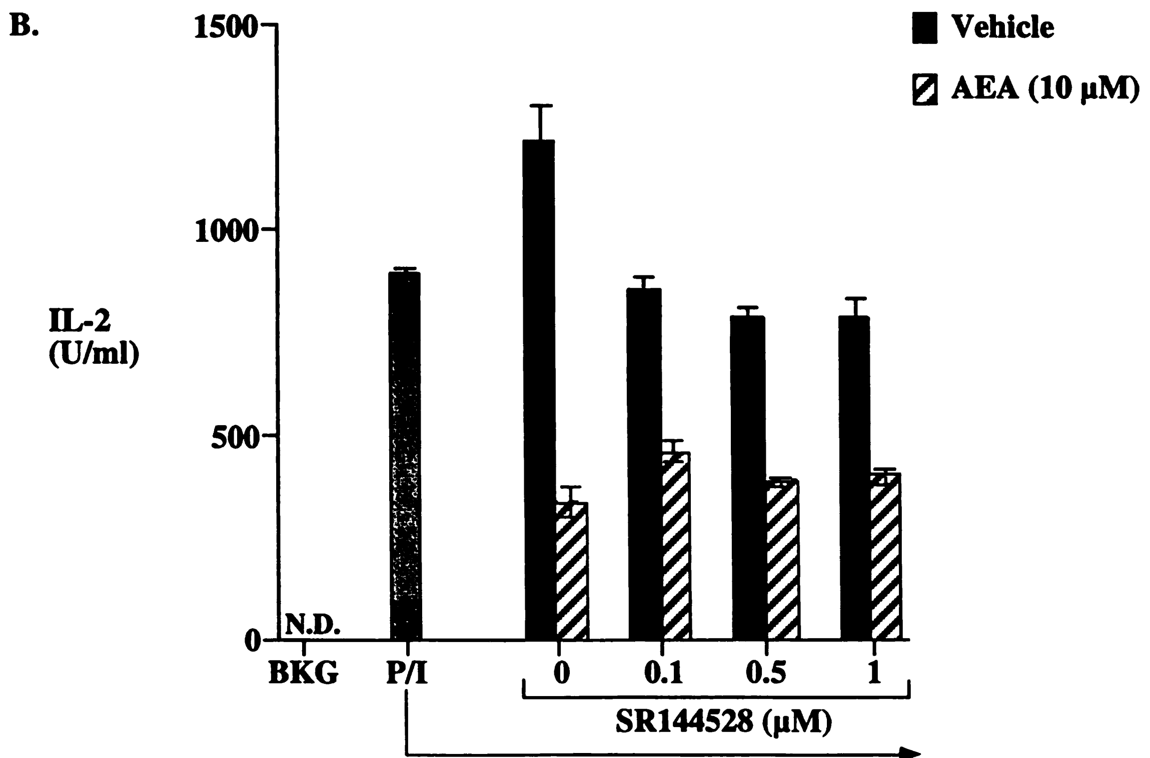


Figure 9.

Figure 10. Effect of cannabinoid receptor antagonists on suppression of IL-2 production by 2-AG and 2-AG ether. Splenocytes (1×10^6 cells/ml) were pretreated with both SR141716A and SR144528 or VH (0.1% DMSO) for 30 min followed by treatment with either A.) 2-AG (20 μM) or B.) 2-AG ether (20 μM) for 30 min. Cells were then stimulated with 40nM PMA/0.5 μM ionomycin for 24 h. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH + 2-AG ether group. None of the groups were significantly different from the VH + 2-AG group. These data are representative of at least three separate experiments.

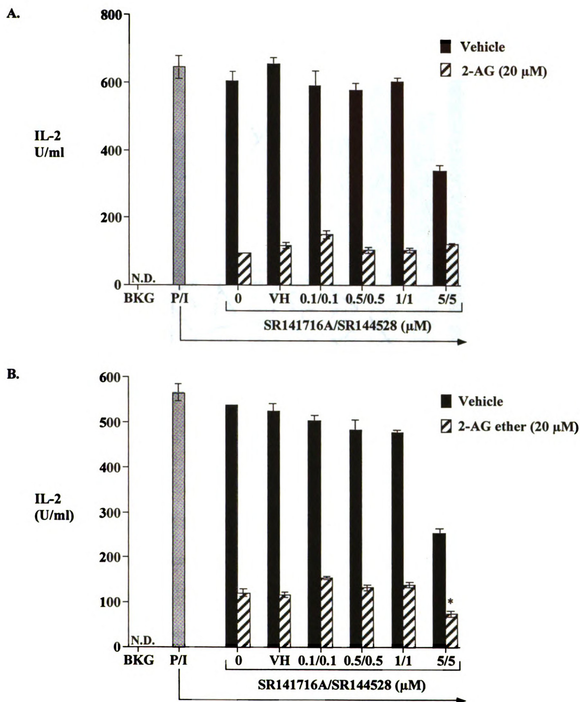


Figure 10.

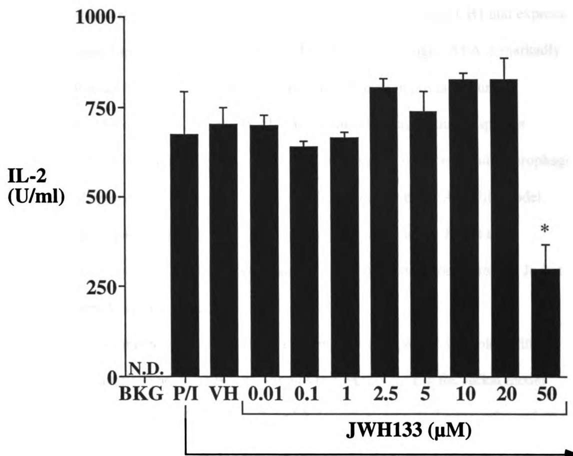


Figure 11. Effect of the CB2-specific agonist, JWH133, upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with JWH133 (0.01-50 μM) or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least two separate experiments.

PMA/Io-induced IL-2 production in the Jurkat T cell line, which lacks CB1 and expresses a nonfunctional form of CB2 (Figures 12 and 13) (53). Interestingly, AEA is markedly more potent and efficacious in the human Jurkat T cells than in primary murine splenocytes. The increased potency of AEA in Jurkat cells may be due to species differences in sensitivity to AEA. Alternatively, the presence of B cells and macrophages in the mixed splenocyte preparation may diminish the activity of AEA in this model. Although there is a pronounced difference in potency of AEA in the Jurkat and splenocyte models, both 2-AG and 2-AG ether are only modestly more potent in Jurkat cells compared to splenocytes.

While the studies with the CB1/CB2 antagonists and JWH133 coupled with the ability of the putative endogenous cannabinoids to suppress IL-2 in the Jurkat model suggested that the effects of AEA, 2-AG, and 2-AG ether upon IL-2 were independent of CB1 and CB2, the evidence was not entirely conclusive. Due to the relatively few number of laboratories investigating the immune effects of cannabinoids, there was a dearth of published studies with SR144528 to confirm its utility as a CB2 antagonist. Furthermore, the suppression of IL-2 by SR141716A and SR144528 treatment alone, confounded their ability to act as antagonists. Additionally, the presence of the aberrant CB2 transcripts in the Jurkat model was problematic because it was initially unclear whether the aberrant CB2 receptor was functional. Moreover, the lack of specific CB2 antibodies made it difficult to determine whether CB2 protein is expressed in Jurkat cells. Although previous studies from this laboratory demonstrated that, unlike normal CB2, the aberrant CB2 receptor is not capable of inhibiting cAMP production, it was unknown whether the aberrant receptor could couple to other second messengers and

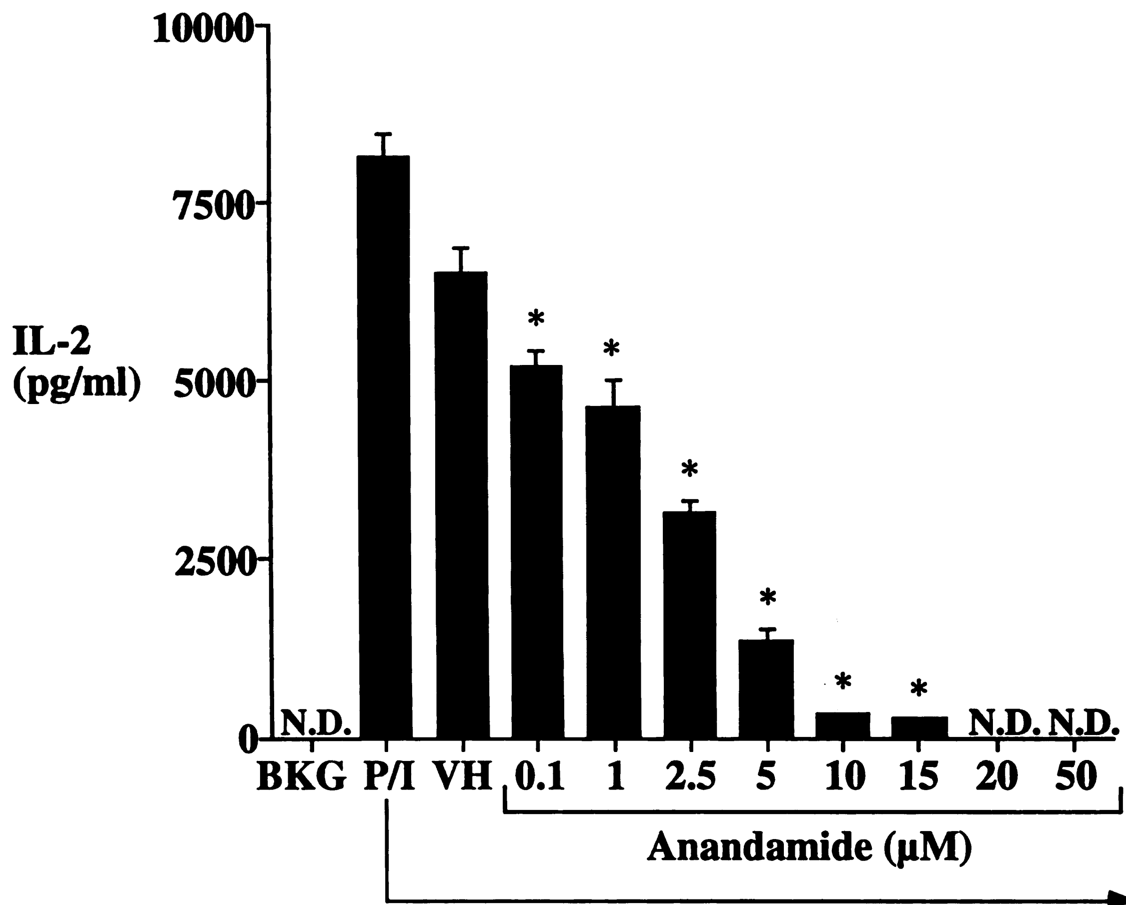
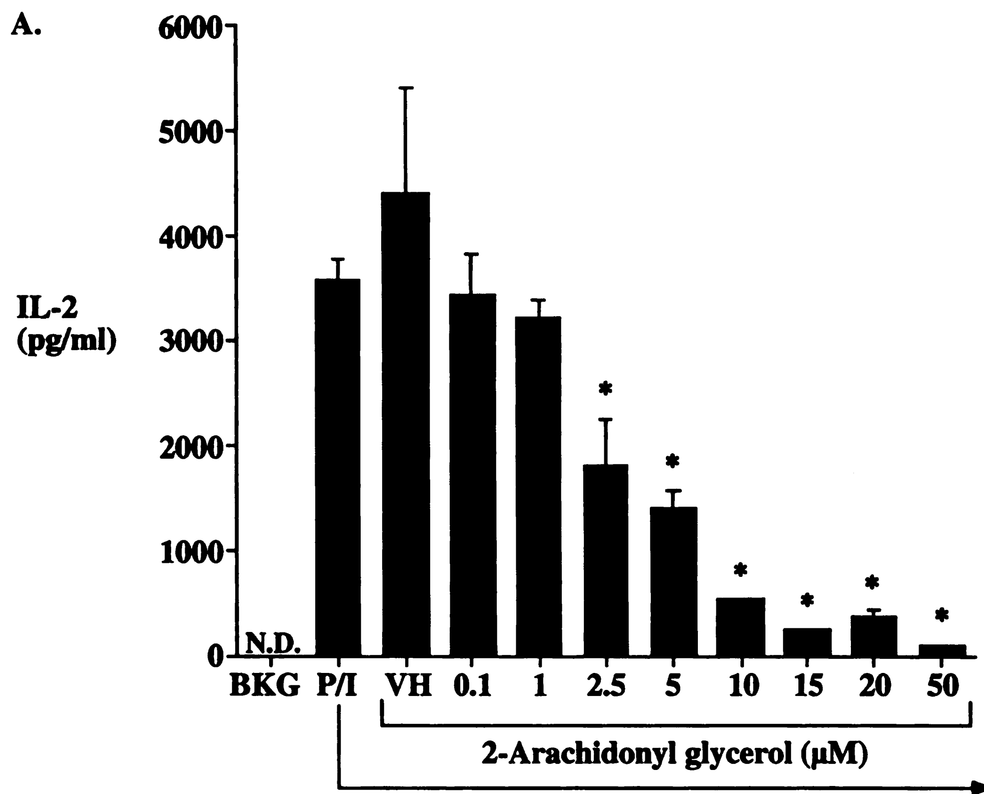


Figure 12. Effect of AEA upon PMA/ionomycin-stimulated IL-2 production in human Jurkat T cells. Jurkat cells (5 x 10^5 cell/ml) were treated with 0.1-50 μM of AEA or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, p<0.05 compared to VH group. These data are representative of at least two separate experiments.

Figure 13. Effect of 2-AG and 2-AG ether upon PMA/ionomycin-stimulated IL-2 production in Jurkat T cells. Jurkat T cells (5×10^5 cell/ml) were treated with 0.1-50 μM of either A.) 2-AG or B.) 2-AG ether for 30 min followed by activation of the cells with PMA/ionomycin (40nM/0.5μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH group. These data are representative of at least two separate experiments.



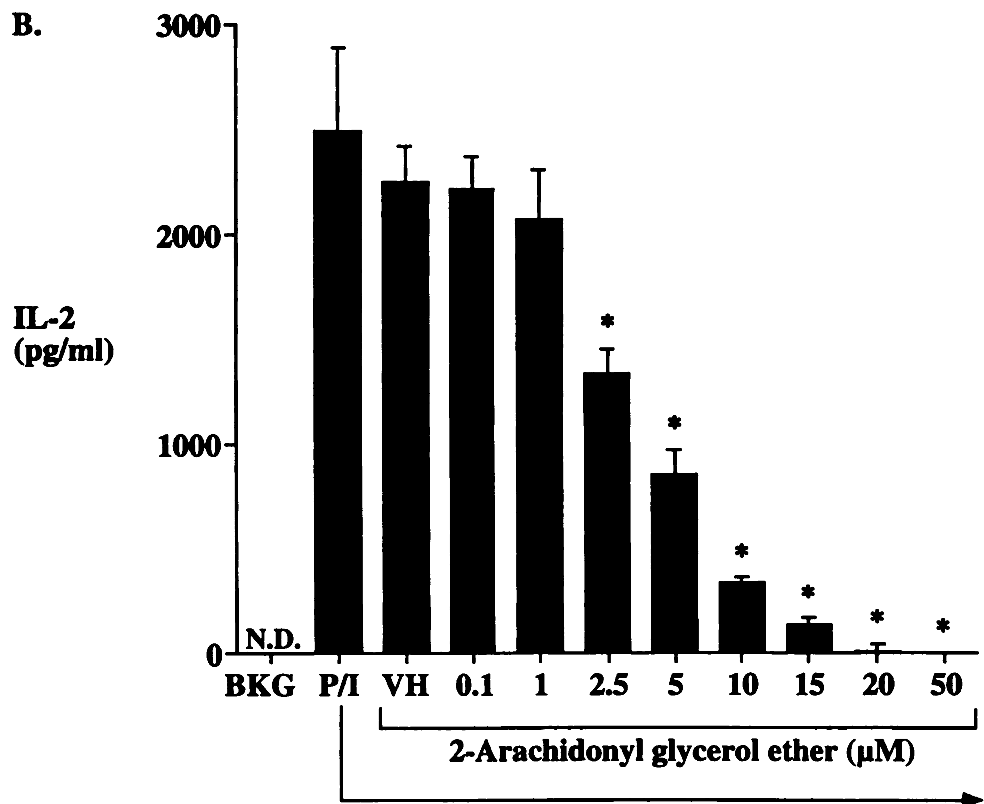


Figure 13.

thereby function in other capacities. Consequently, the involvement of CB1 and CB2 in IL-2 suppression by the putative endocannabinoids was examined further through the use of CB1/CB2 double-knockout mice. AEA causes a suppression of IL-2 in splenocytes derived from CB1/CB2 null mice which is similar to that observed in splenocytes derived from wild-type controls, confirming that CB1 and CB2 are not involved (Figure 14). Likewise, both 2-AG and 2-AG ether also produce a comparable decrease in IL-2 in CB1/CB2 null splenocytes to that observed in wild-type controls (Figures 15 and 16).

III. The role of the vanilloid receptor, VR1, in AEA-mediated IL-2 suppression

Because AEA has also been shown to activate the vanilloid receptor, VR1, the role of VR1 in AEA-mediated IL-2 suppression was investigated (214). The effect of VR1 activation upon PMA/Io-induced IL-2 secretion was initially assessed with the VR1 agonist, capsaicin. In contrast to the positive control, CP55940, increasing concentrations of capsaicin had little effect upon IL-2 secretion (Figure 17). Furthermore, pretreatment with the VR1 antagonist, capsazepine, did not block AEA-mediated suppression of IL-2 secretion. Collectively, the aforementioned results suggest that VR1 is not involved (Figure 18).

IV. Effect of 2-AG and AEA upon calcium influx in resting splenocytes and activated splenocytes and thymocytes

The activation of T cells requires both the induction of the MAP kinase pathway as well as a rise in intracellular calcium, which in turn activates the Ca^{+2}-dependent phosphatase, calcineurin, and ultimately results in the dephosphorylation and

Figure 14. Effect of AEA upon PMA/ionomycin-stimulated IL-2 production in splenocytes derived from CB1/CB2 null and wild-type mice. Splenocytes (1 x 10^6 cells/ml) were treated with 0.1-50 μM of AEA for 30 min followed by activation of the cells with PMA/ionomycin (40nM/0.5μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least two separate experiments.

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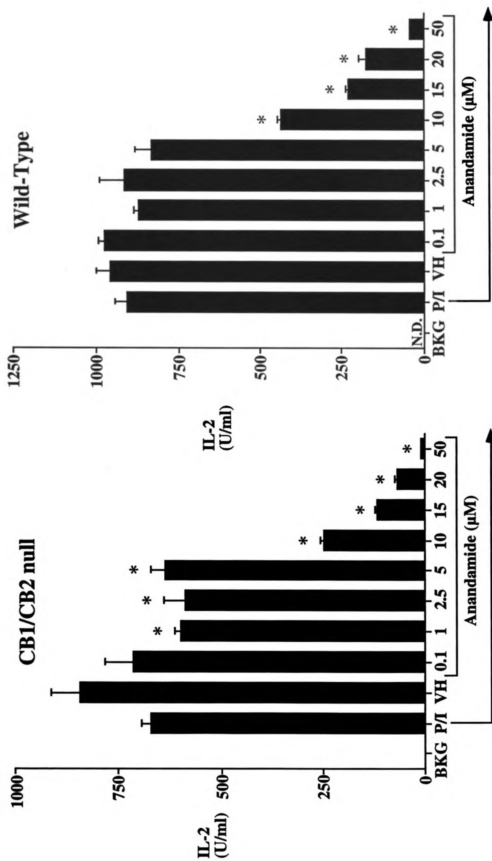


Figure 14.

Figure 15. Effect of 2-AG upon PMA/ionomycin-stimulated IL-2 production in splenocytes derived from CB1/CB2 null and wild-type mice. Splenocytes (1 x 10^6 cells/ml) were treated with 2-AG (0.1-50 μM) for 30 min followed by activation of the cells with PMA/ionomycin (40nM/0.5μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least two separate experiments.

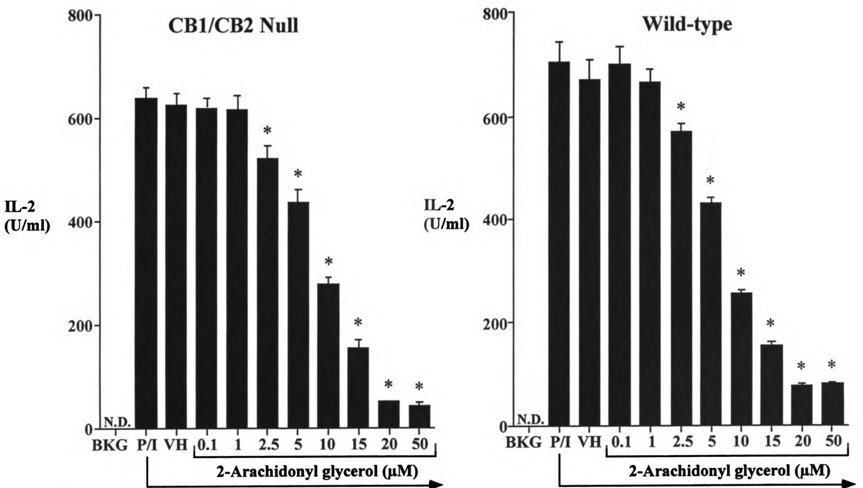


Figure 15.

Figure 16. Effect of 2-AG ether upon PMA/ionomycin-stimulated IL-2 production in splenocytes derived from CB1/CB2 null and wild-type mice. Splenocytes (1 x 10^6 cells/ml) were treated with 2-AG ether (0.1-50 μM) for 30 min followed by activation of the cells with PMA/ionomycin (40nM/0.5μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH group. These data are representative of at least two separate experiments.

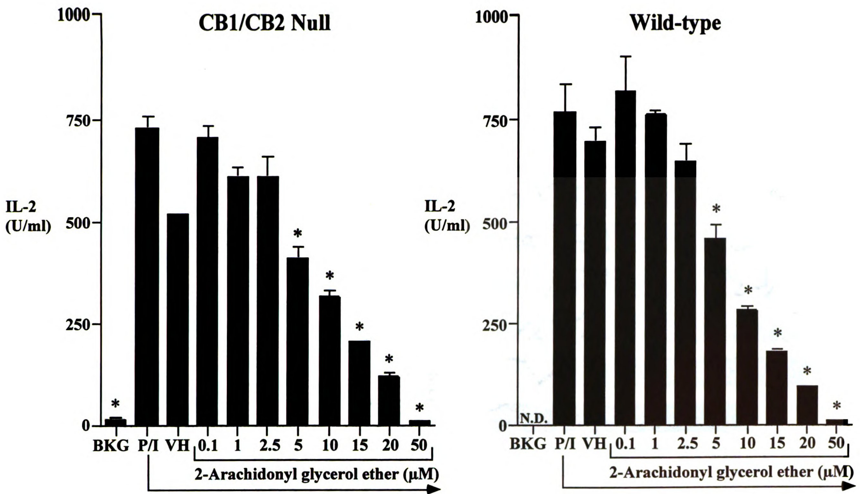


Figure 16.

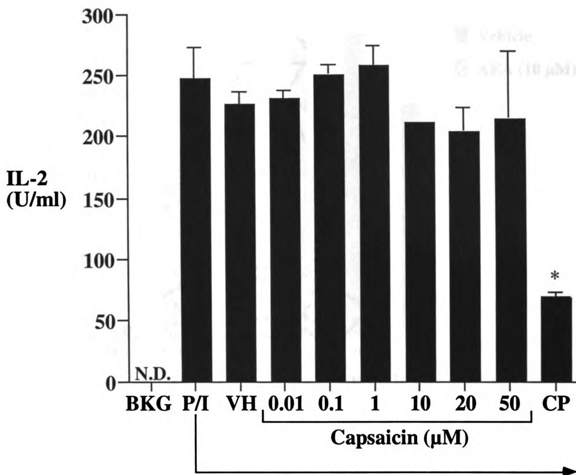


Figure 17. Effect of capsaicin upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with capsaicin (0.01-50 μM), CP55940 (10 μM), which served as a positive control, or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH group. These data are representative of two separate experiments.

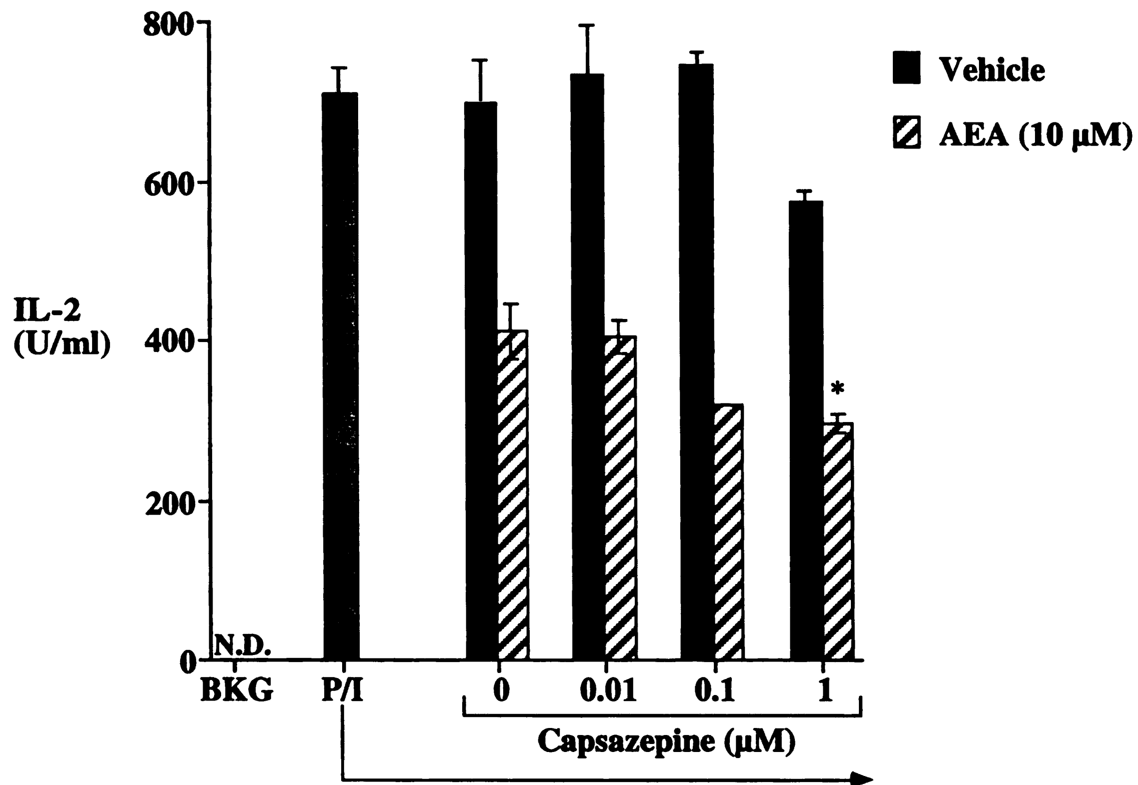


Figure 18. Effect of the vanilloid receptor antagonist, capsazepine, on AEA-mediated suppression of PMA/ionomycin-stimulated IL-2 production. Splenocytes (1×10^6 cells/ml) were pretreated with capsazepine (0.01-1 μM) for 15 min followed by AEA treatment for 15 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to 0 + AEA group. These data are representative of two separate experiments.

translocation of NFAT to the nucleus. As a result, disruption of the normal Ca^{+2} influx associated with T cell activation would inhibit the binding of NFAT to the IL-2 promoter and suppress transcription. Conversely, the initiation of a calcium signal in the absence of simultaneous MAP kinase activation generally results in T cell anergy (375). Furthermore, published studies suggest that both AEA and 2-AG modulate calcium channels, including inhibition of voltage-gated calcium channels in a variety of cell types and induction of transient calcium influx in HL-60 cells (17, 20, 27). Collectively, these results suggest that 2-AG and AEA could mediate their effects upon IL-2 through modulation of the calcium signal, which thereby could either induce T cell anergy or inhibit activation of NFAT. Unlike the plant-derived cannabinoids, however, neither 2-AG nor AEA induce calcium influx in primary murine splenocytes (Figure 19). In addition, calcium influx by ionomycin or concanavalin A is not inhibited by 2-AG pretreatment, suggesting that modulation of intracellular calcium is not the major mechanism of 2-AG-mediated IL-2 suppression (Figure 20).

V. The effect of arachidonic acid upon IL-2 secretion and the roles of FAAH, MAG lipase, and the AMT in the suppression of IL-2 by AEA and 2-AG

Because it was unclear whether the inhibitory effect of AEA upon IL-2 secretion was due to the parent molecule or a hydrolysis product of AEA, the effect of arachidonic acid upon IL-2 secretion was also evaluated. Treatment of primary splenocytes with various concentrations of arachidonic acid (0.01 – 20 μM) caused a concentration-dependent decrease in IL-2 secretion (Figure 21). The magnitude of the decrease in IL-2 secretion produced by arachidonic acid was very similar to that observed with AEA as

Figure 19. Effect of AEA and 2-AG upon intracellular calcium. Splenocytes (5 x 10^6 c/ml) were loaded with fura-2 AM dye for 30 minutes at room temperature in the dark. Cells were harvested and washed four times in Ca^{+2} KREB buffer to remove excess fura-2 AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration determined. At 300 sec, either A.) AEA (20 µM) or B.) 2-AG (20 µM) was added. In a second run, cannabidiol (10 µM) was added at 300 sec, which served as a positive control. Maximum and minimum values were obtained with 0.1% Triton-X and 0.5 mM EGTA, respectively. Calcium concentration was calculated from the change in ratio of bound to free calcium. These data are representative of at least four separate experiments.

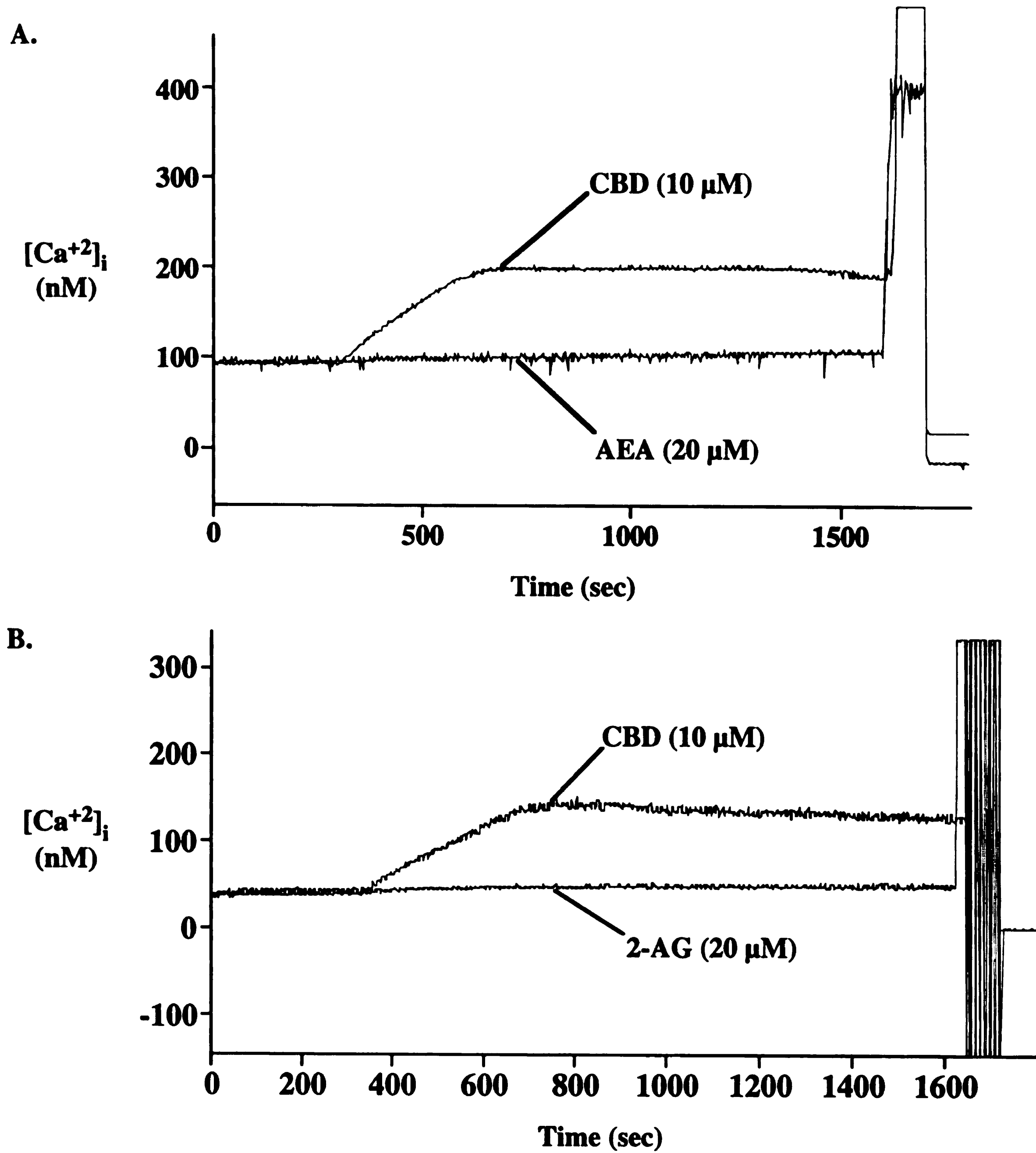
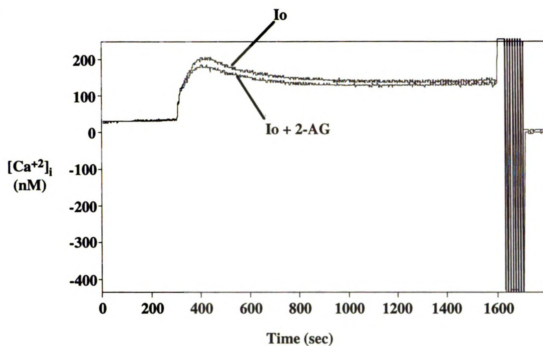


Figure 19.

Figure 20. Effect of 2-AG upon calcium influx by ionomycin and concanavalin A. Splenocytes (5 x 10^6 c/ml) were loaded with fura-2 AM dye for 30 minutes at room temperature in the dark. Cells were harvested and washed four times in Ca^{+2} KREB buffer to remove excess fura-2 AM dye from the buffer. Three ml of cells were added to a quartz cuvette and were either pretreated with 2-AG (20 µM) or left untreated. At 300 sec, either A.) ionomycin (0.5 µM) or B.) concanavalin A (10 µg/ml) was added. Maximum and minimum values were obtained with 0.1% Triton-X and 0.5 mM EGTA, respectively. Calcium concentration was calculated from the change in ratio of bound to free calcium. These data are representative of at least two separate experiments.

A.



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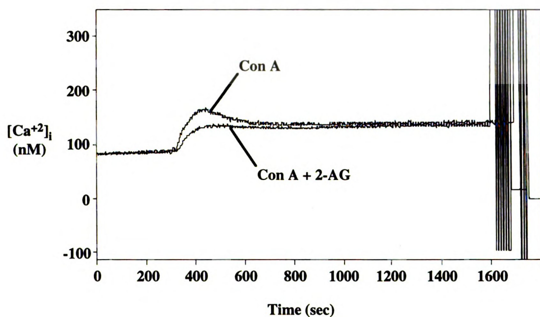


Figure 20.

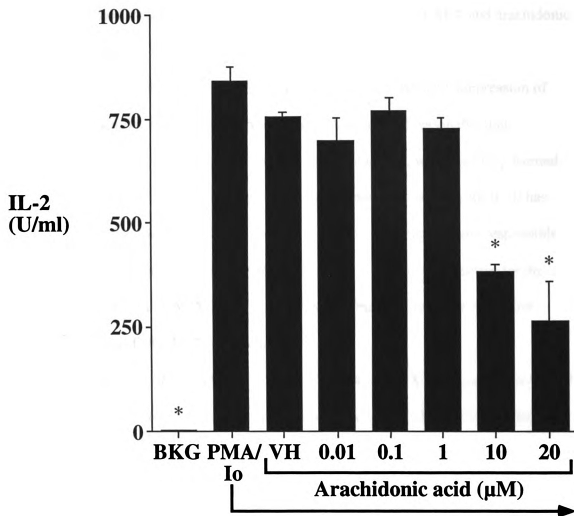


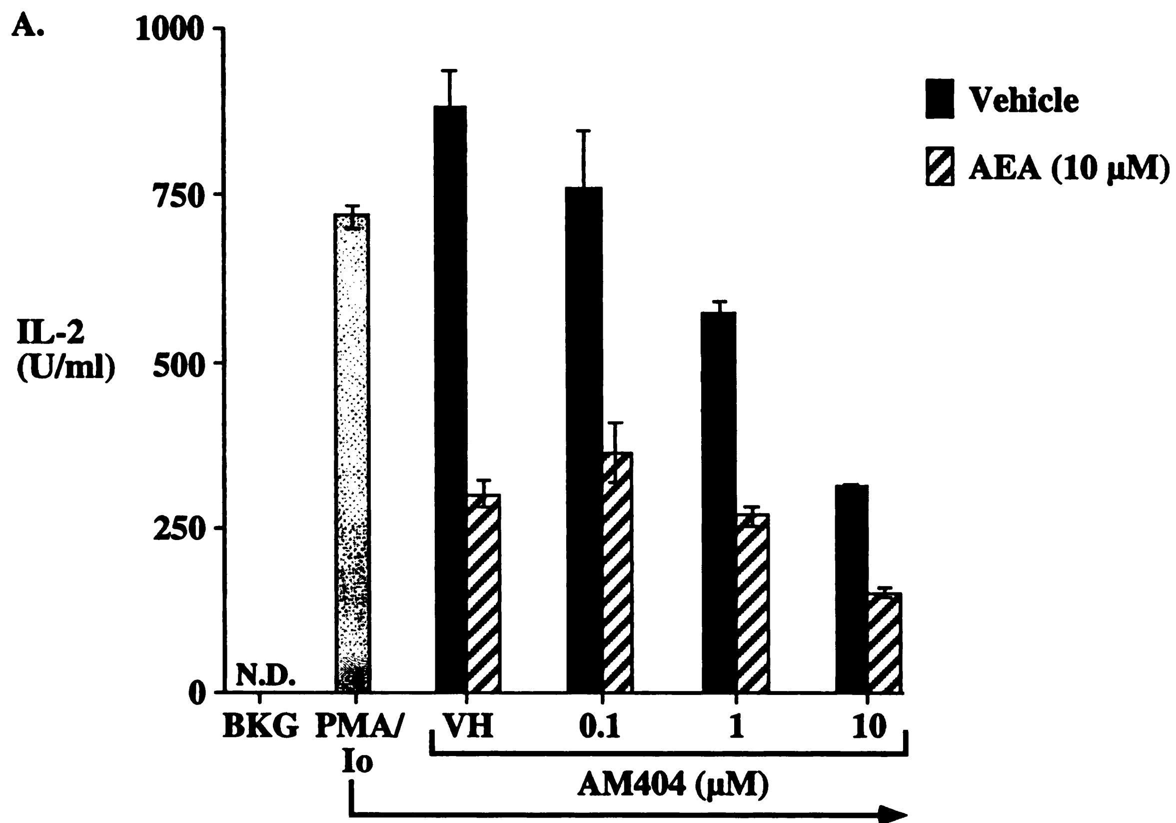
Figure 21. Effect of arachidonic acid upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with 0.01-20 μM of arachidonic acid or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least four separate experiments.

evidenced by the calculated IC_{50} values: 11.4 μM and 10.3 μM for AEA and arachidonic acid, respectively.

Because arachidonic acid produced a concentration-dependent suppression of IL-2 secretion similar to the one observed with AEA, it seemed reasonable that suppression of IL-2 by AEA was mediated by arachidonic acid, which is likely formed from the hydrolysis of AEA, rather than by the parent molecule of AEA itself. It has been postulated that the AMT may be coupled to FAAH, the chief enzyme responsible for AEA hydrolysis and that these two proteins represent a major mechanism for the uptake and catabolism of AEA (376). As such, the potential roles of the AMT and FAAH in AEA-mediated IL-2 suppression were studied.

Pretreatment with the AMT inhibitors, AM404 and UCM707 (IC_{50} values = 1μM and 0.8 μM, respectively), at various concentrations, did not attenuate AEA-mediated suppression of IL-2 secretion, suggesting that the AMT is not involved (Figure 22). At the highest concentration used (10 μM), AM404 alone caused substantial suppression of IL-2. In the presence of AEA, 10 μM AM404 modestly potentiated the decrease in IL-2 secretion by AEA, but this was not statistically significant. Conversely, UCM707 had little effect upon IL-2 secretion in the absence of AEA. With the elimination of the AMT as a potential target of AEA, the role of FAAH was subsequently examined using the potent, irreversible FAAH inhibitor, MAFP (IC_{50} = 2.5 nM). Pretreatment with a broad range of concentrations of MAFP (0.001 – 1 μM) did not attenuate the suppression of IL-2 secretion by AEA (Figure 23a). It has been demonstrated that in addition to the hydrolysis of AEA, FAAH also catalyzes the reverse reaction, which results in the synthesis of AEA from arachidonic acid and ethanolamine. Consequently, it is possible

Figure 22. Effect of AMT inhibitors on suppression of IL-2 secretion by AEA. Splenocytes (1 x 10^6 cells/ml) were pretreated with either A.) AM404 (0.1-10 μM) or B.) UCM707 (1-20 μM) for 30 min followed by treatment with AEA for 30 min. Cells were then stimulated with 40nM PMA/0.5 μM ionomycin for 24 h. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. Neither the groups that were treated with AM404 and AEA nor those that were treated with UCM707 and AEA were significantly different from the control groups, VH + AEA. The data are representative of at least three separate experiments.



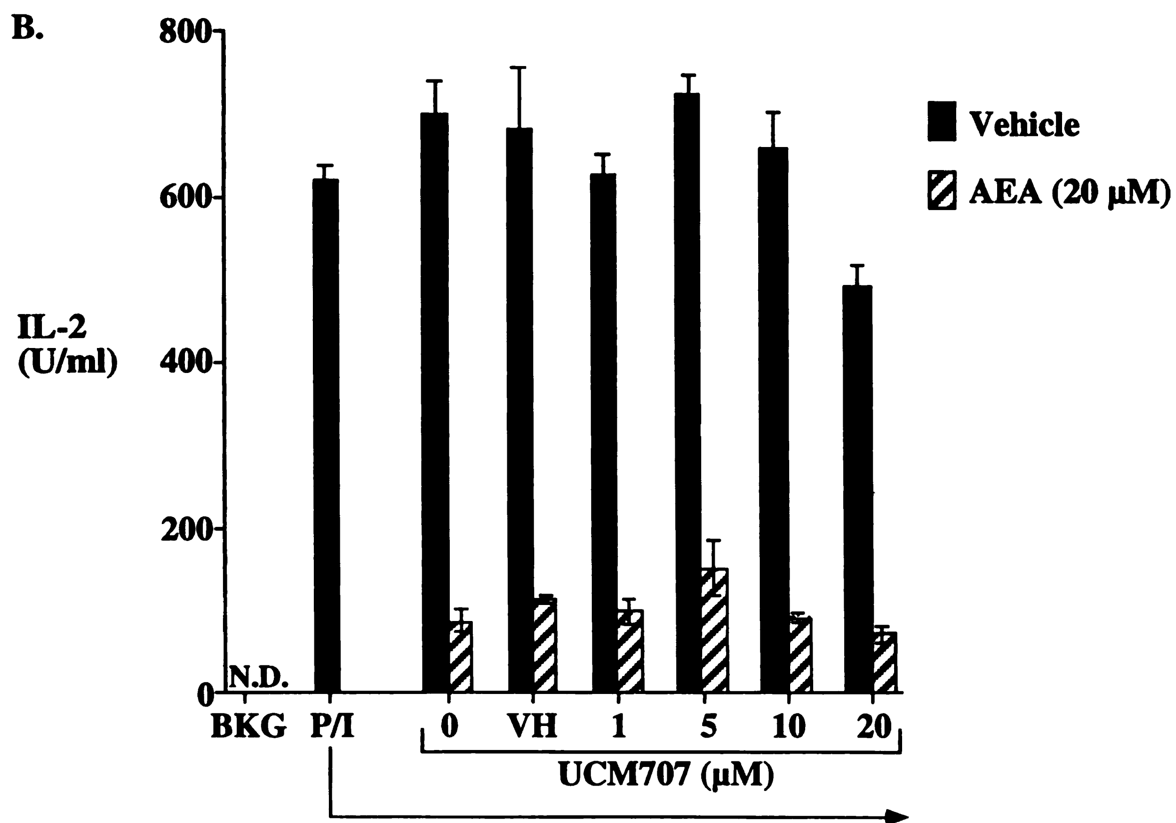
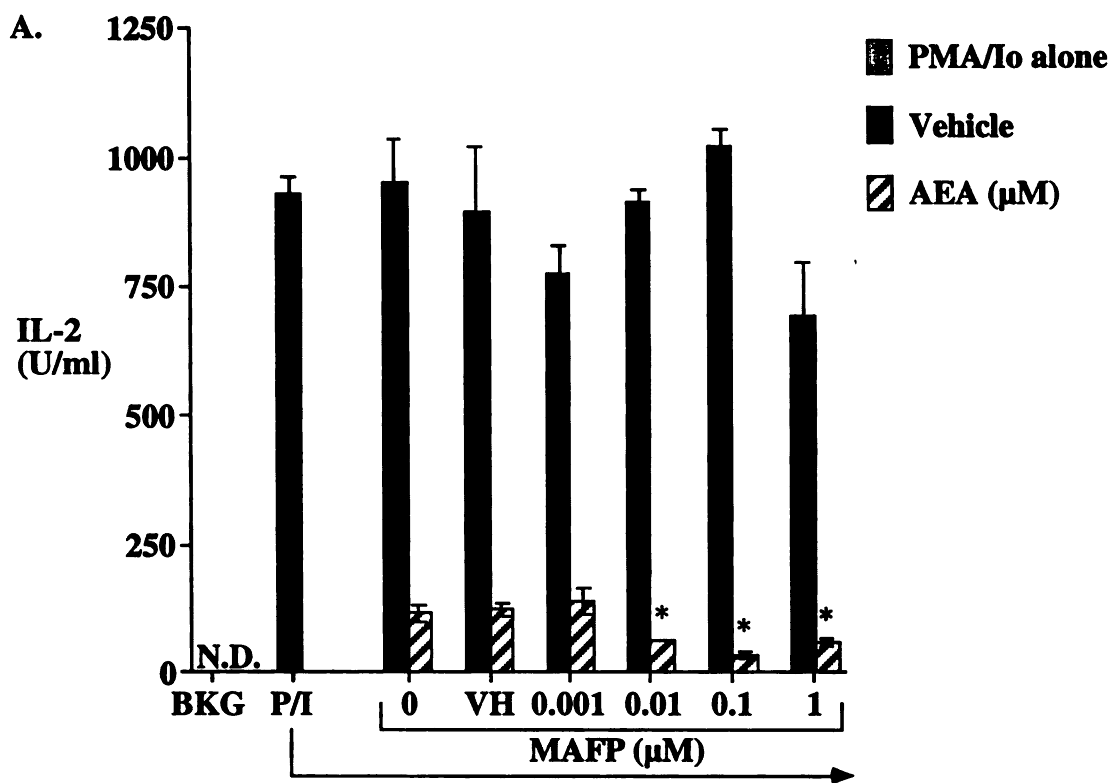


Figure 22.

Figure 23. Effect of the FAAH inhibitor, MAFP, on suppression of IL-2 secretion by AEA and arachidonic acid. Splenocytes (1 x 10^6 cells/ml) were pretreated with MAFP at various concentrations for 30 min followed by treatment with either A.) AEA or B.) arachidonic acid for 30 min. Cells were then stimulated with 40nM PMA/0.5 μM ionomycin for 24 h. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH + AEA group. None of the groups treated with arachidonic acid were significantly different from the control group, 0 + arachidonic acid. These data are representative of at least three separate experiments.



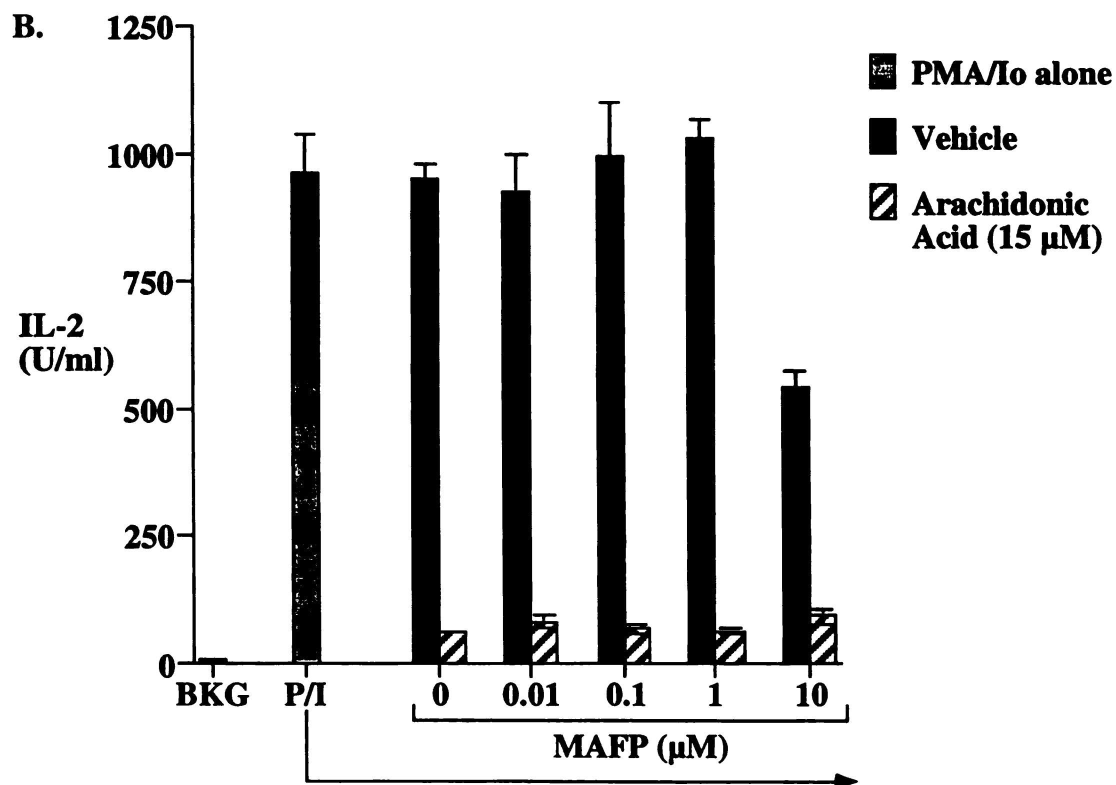


Figure 23.

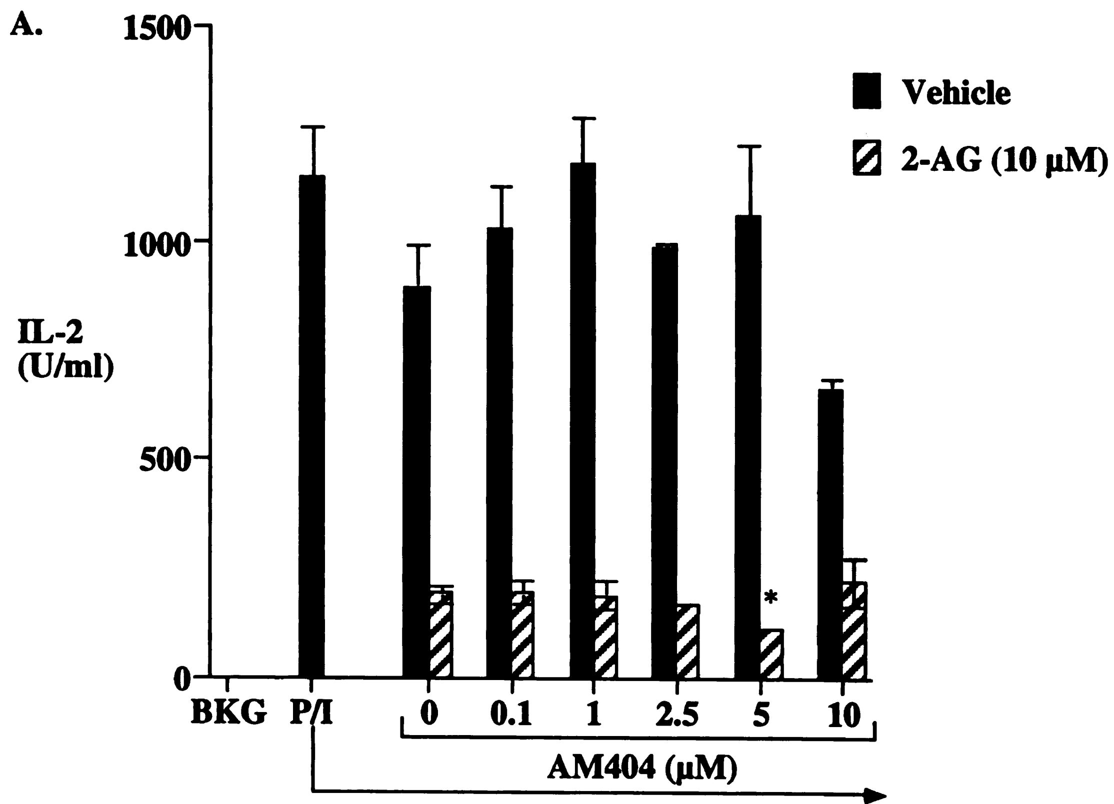
that the observed effects of arachidonic acid upon IL-2 secretion are actually mediated by newly-synthesized AEA, produced by FAAH. As such, the effect of MAFP upon the suppression of IL-2 by arachidonic acid was studied. Pretreatment with a broad range of concentrations of MAFP (0.01 – 10 μM) did not attenuate the decrease in IL-2 secretion by arachidonic acid (Figure 23b). At the highest concentration used (10 μM), MAFP alone caused significant inhibition of IL-2 secretion. Because MAFP is also a PLA_2 inhibitor and previous studies from this laboratory have shown that PLA_2 inhibitors cause a decrease in IL-2 production, the inhibitory effect of MAFP upon IL-2 secretion was not unexpected (331).

In addition to studies suggesting that the AMT catalyzes the transport of AEA across cell membranes, there is also evidence to suggest that the AMT can also participate in 2-AG transport. Likewise, FAAH has also been found to hydrolyze 2-AG in addition to AEA. In addition to FAAH, 2-AG is also hydrolyzed by MAG lipase, which is also inhibited by MAFP. As such, the roles of the putative AMT, FAAH, and MAG lipase in the inhibitory effect of 2-AG upon IL-2 were investigated. Neither pretreatment with AM404 nor MAFP affected suppression of IL-2 by 2-AG, thus ruling out the involvement of either the AMT, FAAH, or MAG lipase in IL-2 suppression by 2-AG (Figure 24).

VI. The role of COX in the suppression of IL-2 by AEA and 2-AG

Because arachidonic acid suppresses IL-2 with a potency comparable to that of AEA and 2-AG, the role of the COX enzymes was investigated. Initial evaluation of concentration responses of flurbiprofen determined 50 μM to be an optimum

Figure 24. Effect of the AMT inhibitor, AM404, and the FAAH inhibitor, MAFP, on suppression of IL-2 secretion by 2-AG. Splenocytes (1 x 10^6 cells/ml) were pretreated with either A.) AM404 (0.1-10 µM) or B.) MAFP (0.1-10 µM) for 30 min followed by treatment with 2-AG (10 µM) for 30 min. Cells were then stimulated with 40nM PMA/0.5 µM ionomycin for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH + 2-AG group. These data are representative of two separate experiments.



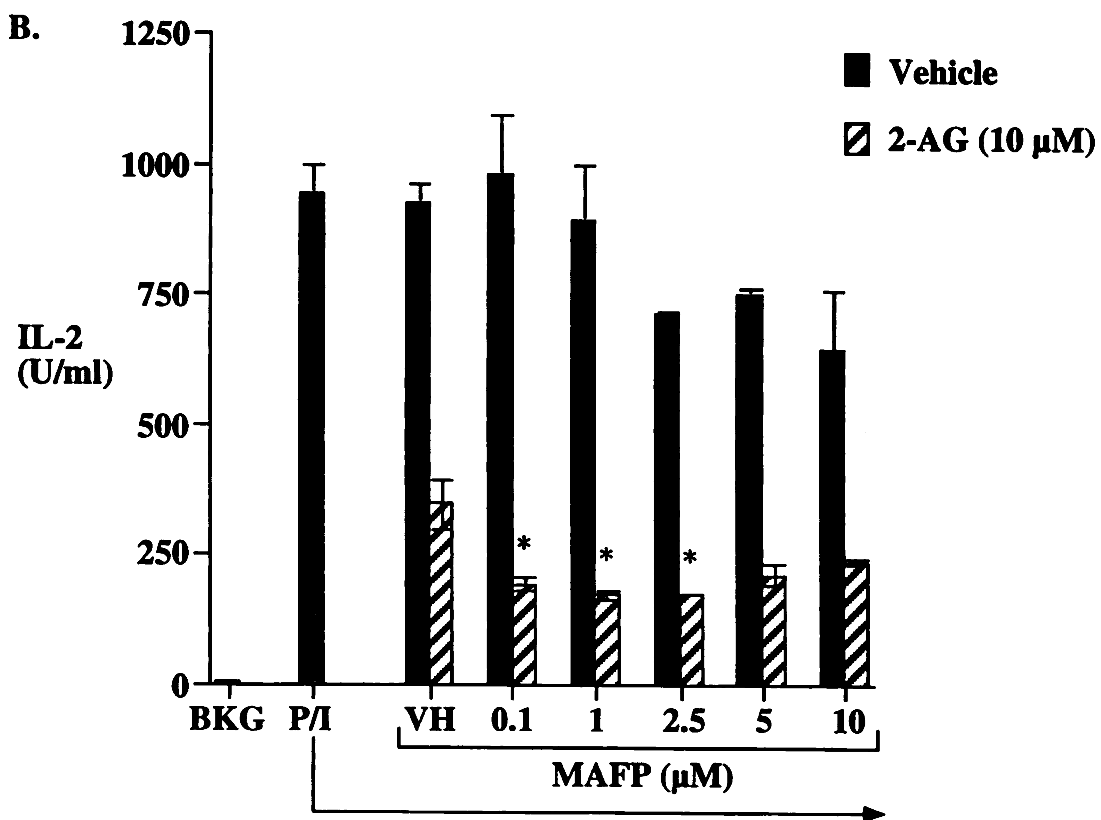
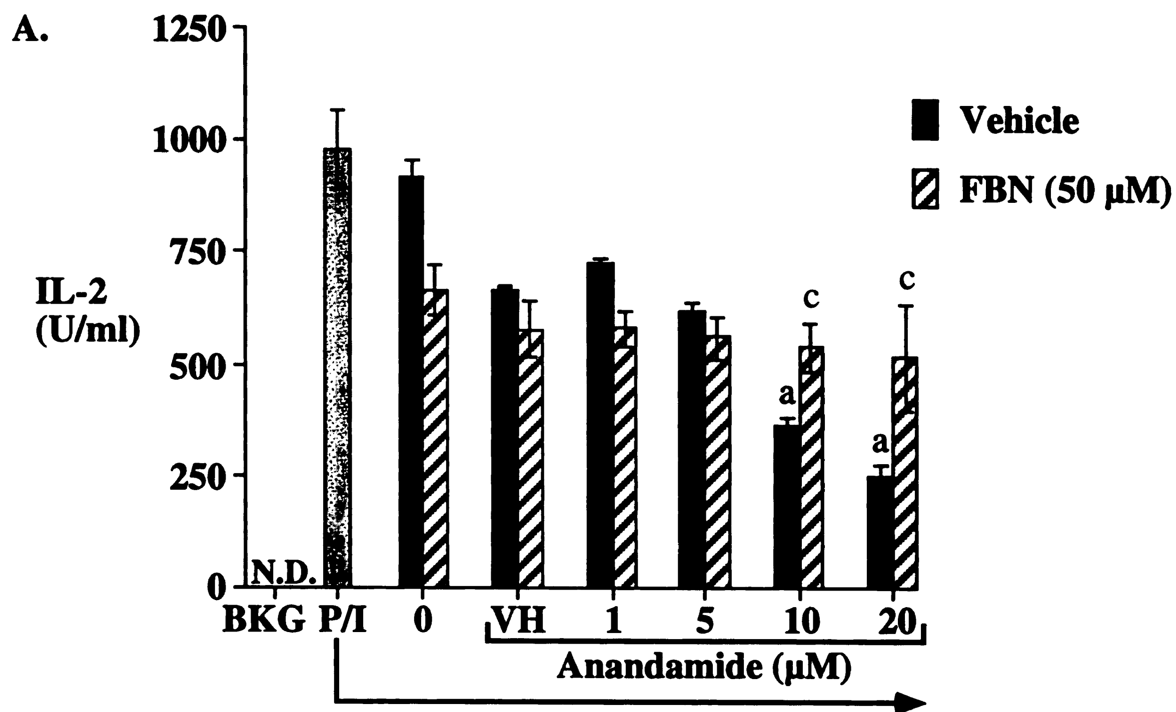


Figure 24.

concentration for inhibition of COX-1 and COX-2 in splenocytes (data not shown). The higher concentration of 100 μM flurbiprofen caused marked suppression of IL-2 by itself, which is not surprising since flurbiprofen (in the range of 100 – 1000 μM) is known to inhibit NFκB, an important transcription factor for IL-2 transcription (377). Although 50 μM flurbiprofen also caused decreased IL-2 secretion in the absence of AEA or arachidonic acid, the decrease was far less robust than that which was produced by 100 μM flurbiprofen and nonetheless still caused an almost complete reversal of AEA-mediated IL-2 inhibition (Figure 25a). Likewise, flurbiprofen pretreatment also significantly attenuated the suppression of IL-2 mediated by arachidonic acid (Figure 25b). Similar to flurbiprofen, pretreatment of primary splenocytes with piroxicam partially attenuated the inhibitory activity of both AEA as well as arachidonic acid upon IL-2 secretion (Figure 26). Moreover, the effect of piroxicam upon AEA-mediated suppression of IL-2 is concentration-dependent (Figure 28a).

In order to determine which COX subtype is involved, the ability of NS398, a COX-2 specific inhibitor, to attenuate suppression of IL-2 by AEA and arachidonic acid was evaluated. Similar to flurbiprofen and piroxicam, NS398 also attenuated the decrease in of IL-2 secretion by AEA and arachidonic acid (Figure 27). In addition, the attenuation of AEA-mediated decrease in IL-2 secretion by NS398 is also concentration-dependent (Figure 28b). Conversely, the COX-1 specific inhibitors, SC560 and FR122047 (IC_{50} values = 9nM and 28 nM, respectively), had no effect upon the suppression of IL-2 by AEA (Figure 29). Likewise, pretreatment with SC560 did not attenuate the decrease in IL-2 by arachidonic acid (Figure 30). Collectively, this suggests that COX-2 plays a role in the inhibitory effects of AEA and arachidonic acid upon IL-2.

Figure 25. Effect of the nonselective COX inhibitor, flurbiprofen, on suppression of IL-2 secretion by AEA and arachidonic acid. Splenocytes (1 x 10^6 cells/ml) were pretreated with 50 μM flurbiprofen (FBN) or VH (0.05% ethanol) for 30 min followed by treatment with either A.) AEA (1-20 μM) or B.) arachidonic acid (1-20 μM) for 30 min. Cells were then stimulated with 40nM PMA/0.5 μM ionomycin for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a denotes $p<0.05$ compared to VH + VH group. b denotes $p<0.05$ compared to FBN + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three separate experiments.



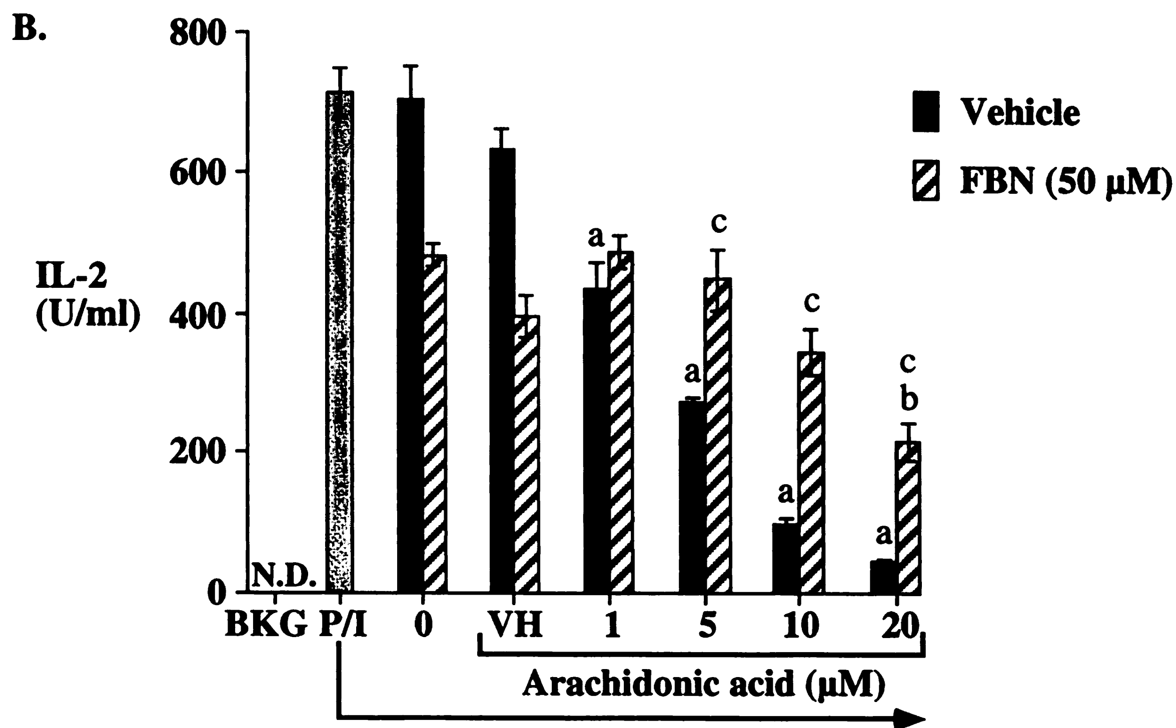
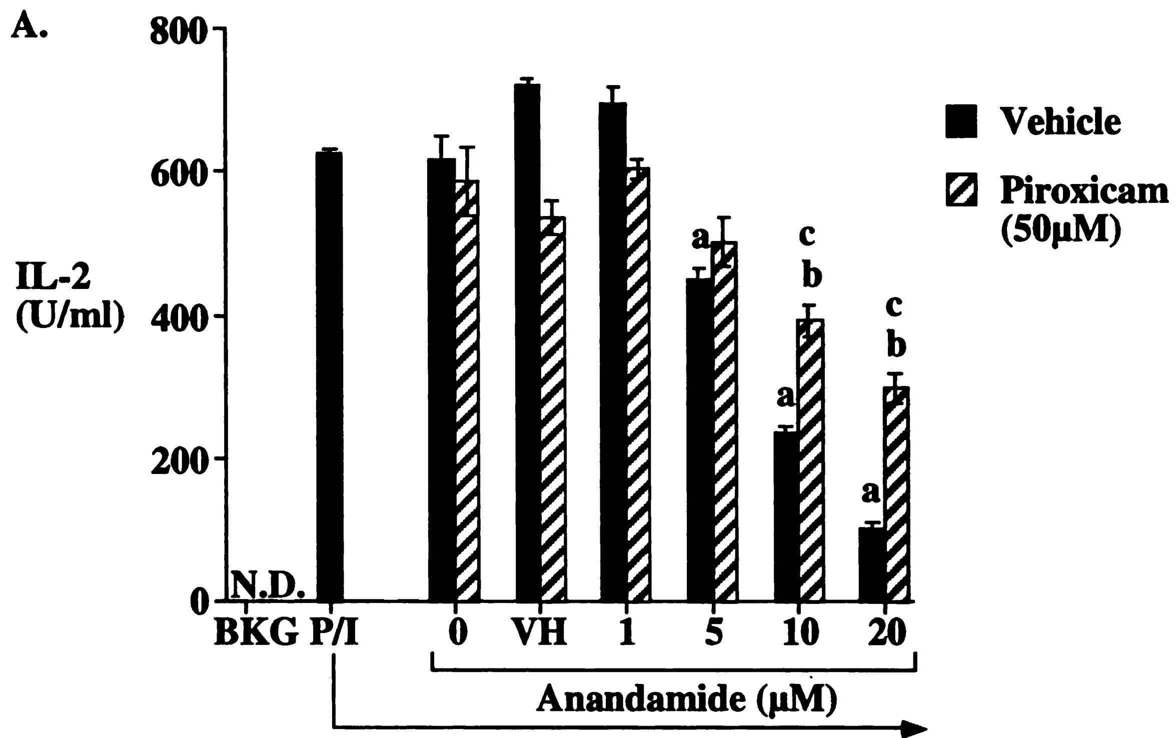


Figure 25.

Figure 26. Effect of the COX-1 selective inhibitor, piroxicam, upon suppression of IL-2 secretion by AEA and arachidonic acid. Splenocytes (1 x 10^6 cells/ml) were pretreated with piroxicam (20 or 50 µM) or VH (0.1% ethanol) for 30 min followed by treatment with either A.) anandamide (AEA) or B.) arachidonic acid (AA) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a denotes $p<0.05$ compared to VH + VH group. b denotes $p<0.05$ compared to piroxicam + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three separate experiments.



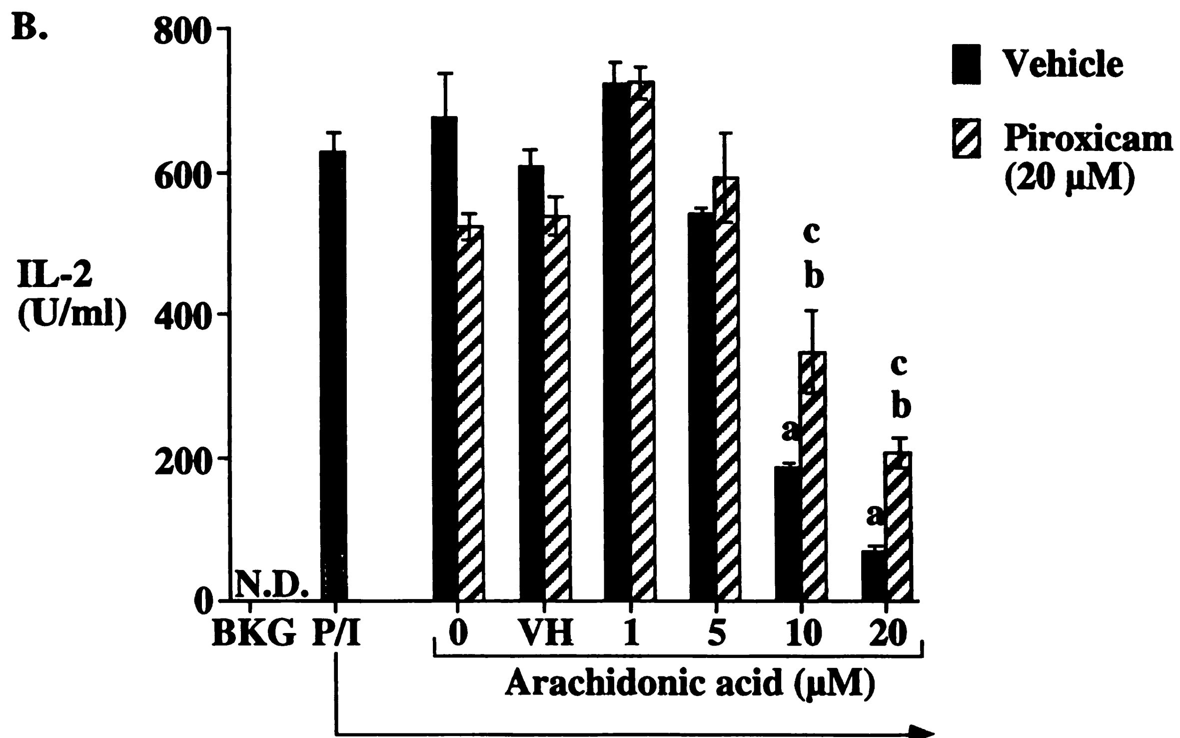
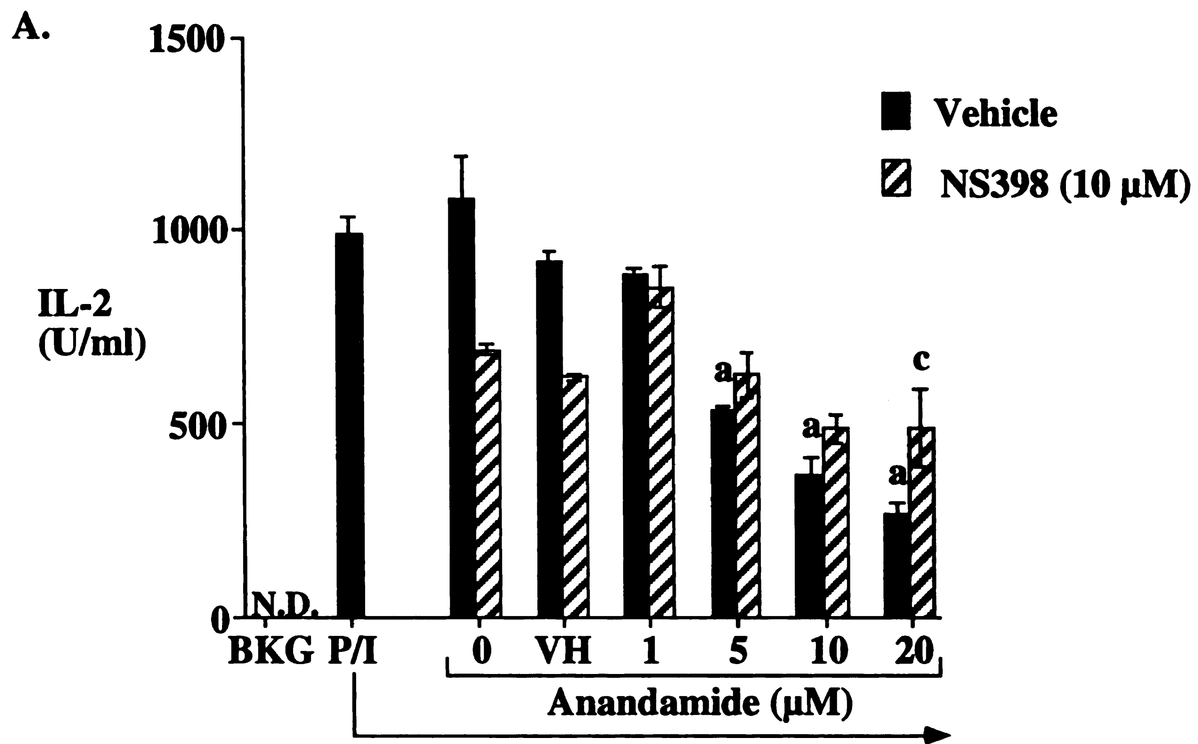


Figure 26.

Figure 27. Effect of the COX-2 specific inhibitor, NS398, on the suppression of IL-2 secretion by AEA and arachidonic acid. Splenocytes (1×10^6 cells/ml) were pretreated with NS398 (10 μM) or VH (0.02% ethanol) for 30 min followed by treatment with either A.) anandamide or B.) arachidonic acid for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a denotes $p<0.05$ compared to VH + VH group. b denotes $p<0.05$ compared to NS398 + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three separate experiments.



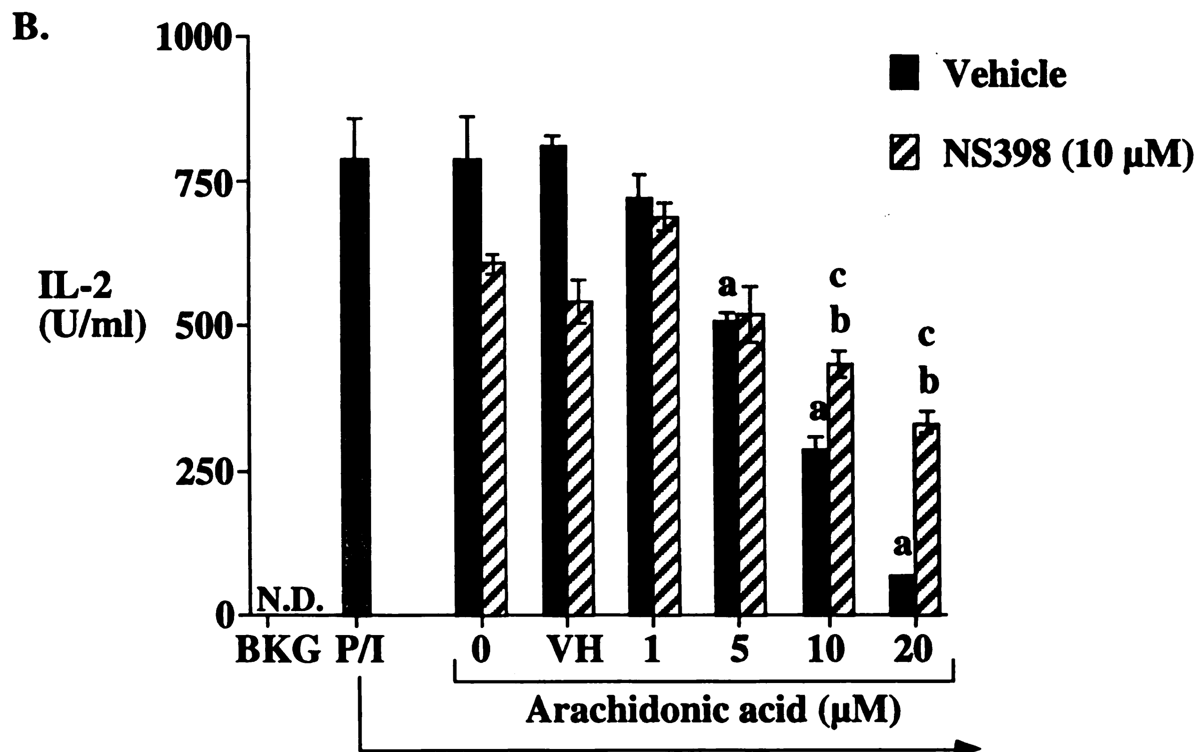
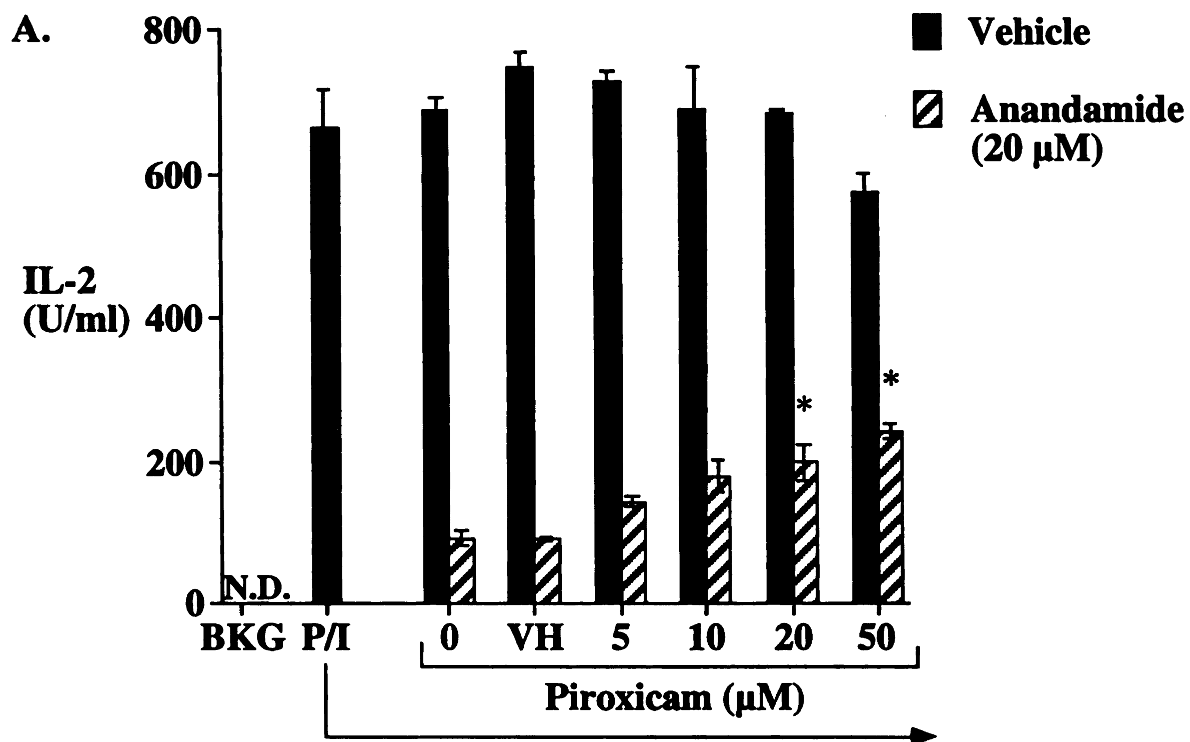


Figure 27.

Figure 28. Piroxicam and NS398 attenuate the suppression of IL-2 secretion by AEA in a concentration-dependent manner. Splenocytes (1 x 10^6 cells/ml) were pretreated with either A.) piroxicam or B.) NS398 for 30 min followed by treatment with either anandamide (AEA) or VH (0.04% ethanol) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * denotes $p<0.05$ compared to VH + anandamide group. These data are representative of least three separate experiments.



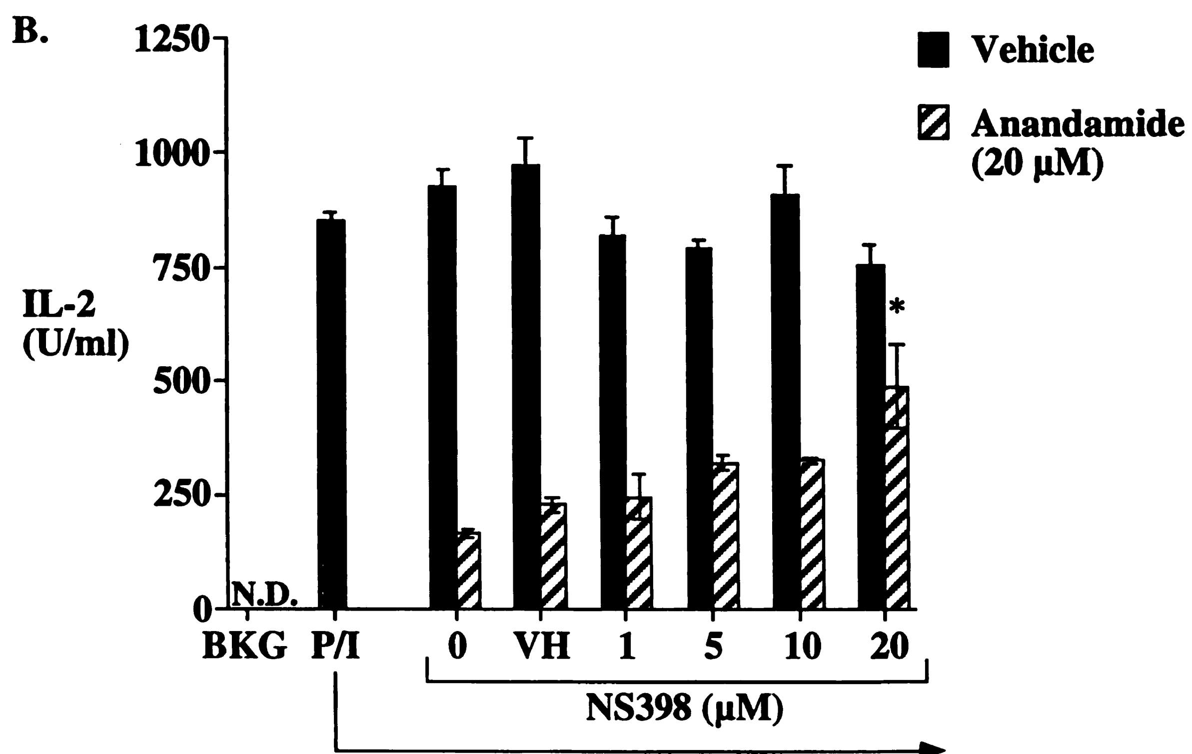
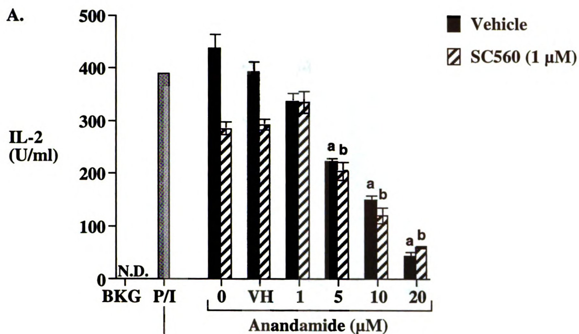


Figure 28.

Figure 29. Effect of the COX-1 specific inhibitors, SC560 and FR122047, on the suppression of IL-2 secretion by AEA. Splenocytes (1 x 10^6 cells/ml) were pretreated with either A.) SC560 (1 μM) or B.) FR122047 (0.01-5 μM) for 30 min followed by treatment with AEA or VH (0.04% ethanol) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a, $p<0.05$ compared to VH + VH group. b, $p<0.05$ compared to VH + SC560 group. *, $p<0.05$ compared to VH + AEA group. These data are representative of at least two separate experiments.



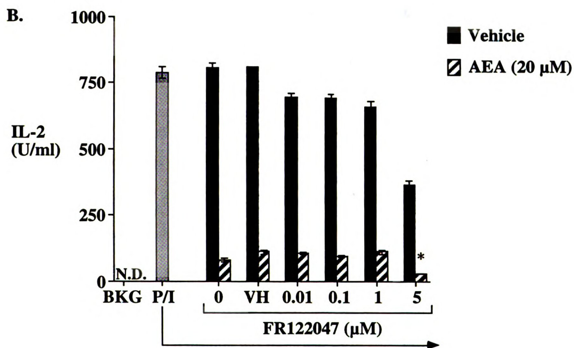


Figure 29.

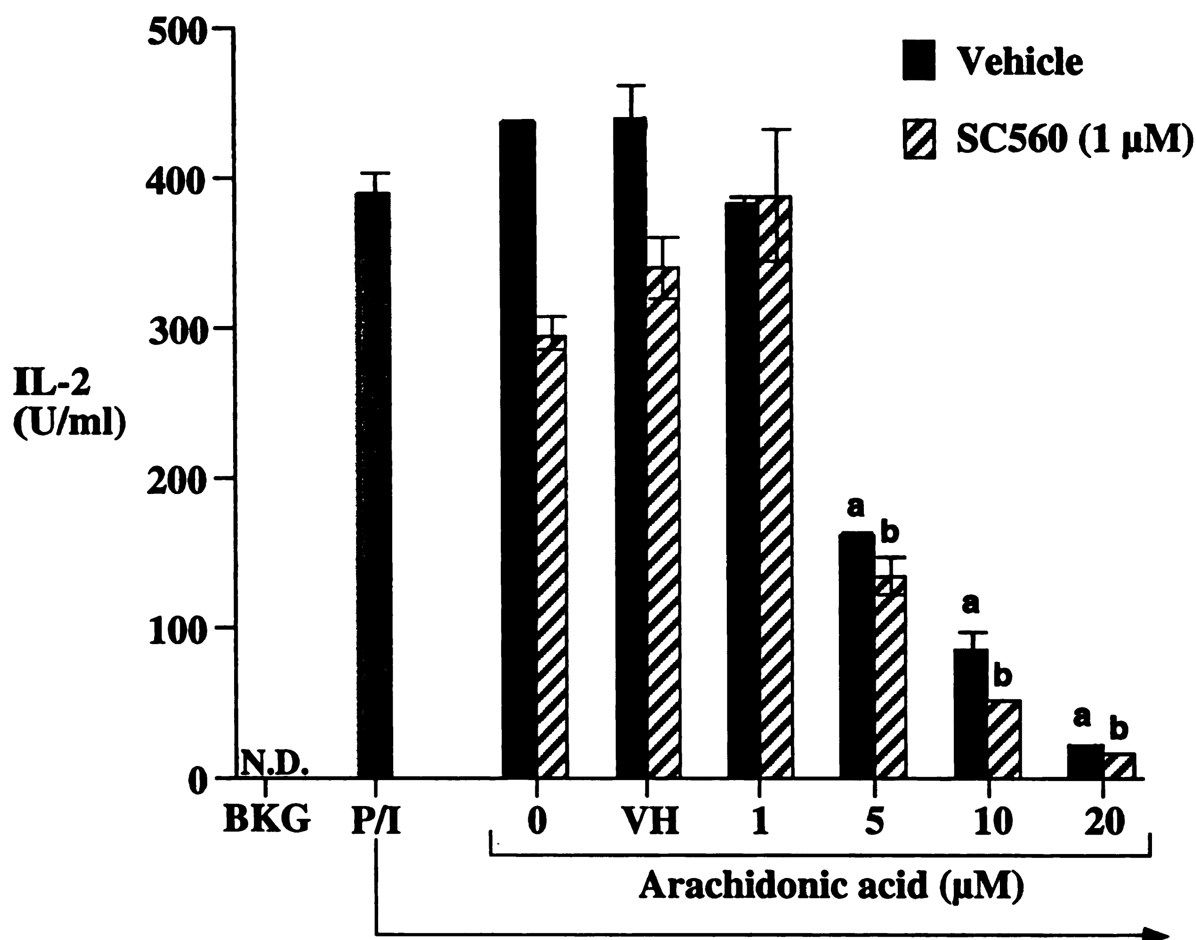


Figure 30. Effect of the COX-1 specific inhibitor, SC560, on arachidonic acid-mediated inhibition of PMA/ionomycin-stimulated IL-2 production. Splenocytes (1 x 10^6 cells/ml) were pretreated with SC560 (1 μM) for 30 min followed by arachidonic acid or VH (0.04% ethanol) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a, $p<0.05$ compared to VH + VH group. b, compared to VH + SC560 group. These data are representative of at least two separate experiments.

Similar to the attenuation of AEA-mediated IL-2 suppression in primary splenocytes, flurbiprofen pretreatment also blocked inhibition of IL-2 secretion by 2-AG in Jurkat T cells (Figure 31). In contrast to AEA and arachidonic acid, however, piroxicam only modestly attenuated 2-AG-mediated suppression of IL-2 In Jurkat cells (Figure 32). This is likely because piroxicam has been shown to be highly selective for human COX-1 over human COX-2 (378-380). Furthermore, pretreatment with the COX-1 specific inhibitors, SC560 and FR122047, did not affect inhibition of IL-2 secretion by 2-AG in Jurkat cells (Figure 33). Conversely, the COX-2 specific inhibitor, NS398, caused a concentration-dependent reversal of 2-AG-mediated suppression of IL-2, suggesting the involvement of COX-2 in these effects (Figure 34).

VII. Effect of prostanoids and PPARγ agonists upon IL-2 production

Although the experiments with NS398 strongly suggest that both 2-AG and AEA are metabolized by COX-2 and that the metabolites are responsible for suppression of IL-2, the identity of the metabolites is unknown. The subsequent studies were designed to determine which metabolites inhibit IL-2 secretion. Initially the effect of AH6809, an antagonist of the EP1, EP2, and DP prostanoid receptors, was investigated. Although AH6809 did not antagonize the effects of AEA, it caused a significant degree of IL-2 suppression by itself (Figure 35a). As such, it was not possible to rule out the EP1, EP2 or DP receptors as targets of the metabolites of AEA. The EP receptors were of particular interest because a number of published studies demonstrated that PGE_2 causes inhibition of IL-2 secretion (339). Additionally, it has been demonstrated that PGE_2-

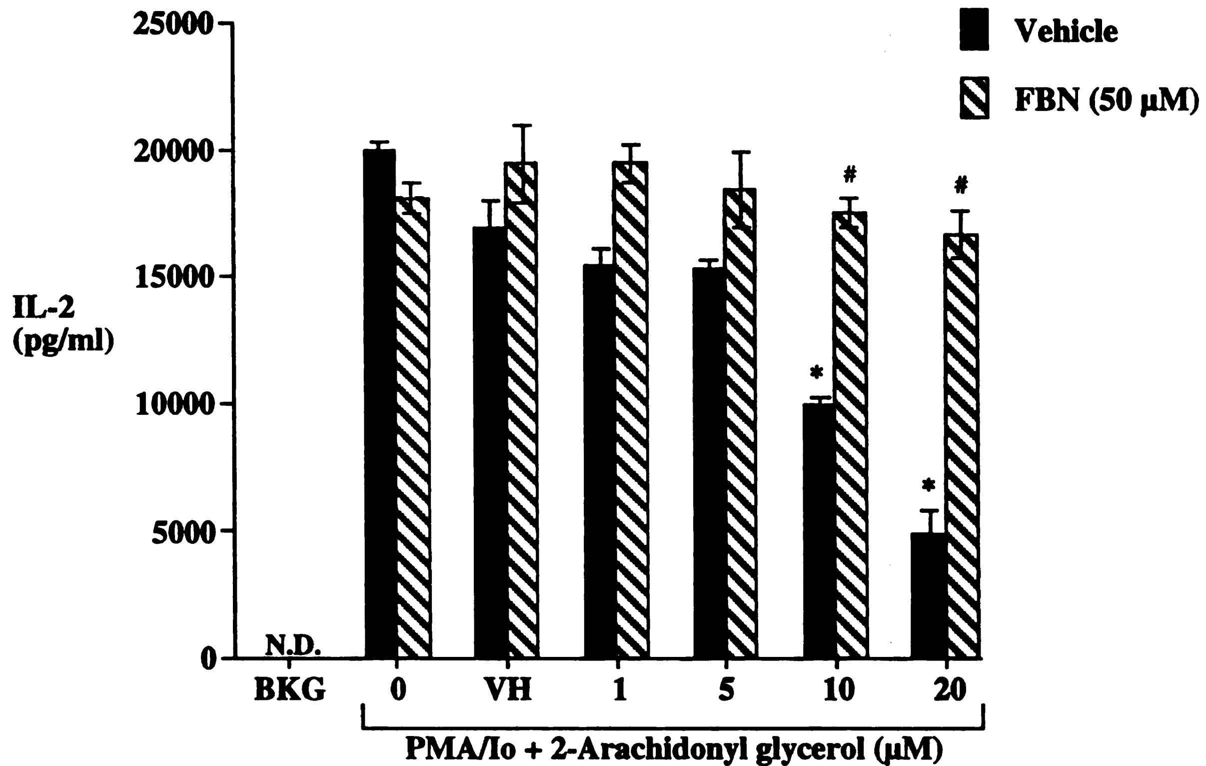


Figure 31. Effect of the nonselective COX inhibitor, flurbiprofen, on 2-AG-mediated suppression of PMA/ionomycin-stimulated IL-2 production. Jurkat T cells (5×10^5 cells/ml) were pretreated with 50 μM flurbiprofen (FBN) for 30 min followed by 2-AG (1-20 μM) or VH (0.04% ethanol) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ compared to VH + VH group. #, $p<0.05$ compared to the matched vehicle group. These data are representative of three separate experiments,.

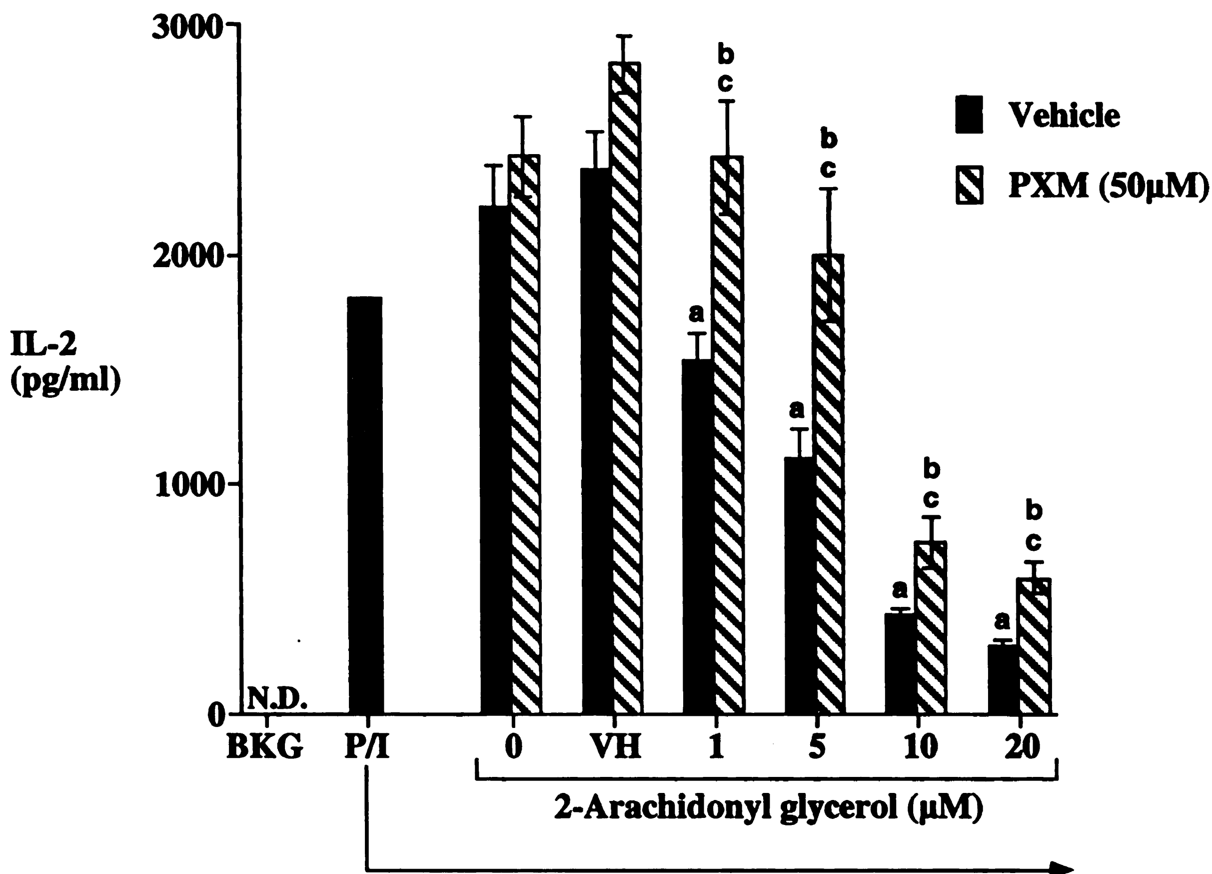


Figure 32. Effect of the COX-1 selective inhibitor, piroxicam, on 2-AG-mediated suppression of PMA/ionomycin-stimulated IL-2 production. Jurkat T cells (5 x 10^5 cells/ml) were pretreated with 50 µM piroxicam (PXM) for 30 min followed by 2-AG (1-20 µM) or VH (0.04% ethanol) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. a, $p<0.05$ compared to VH + VH group. b, $p<0.05$ compared to VH + piroxicam group. c, $p<0.05$ compared to the matched vehicle group. These data are representative of four separate experiments

Figure 33. Effect of the COX-1 specific inhibitors, SC560 and FR122047, on 2-AG-mediated suppression of PMA/ionomycin-stimulated IL-2 production. Jurkat T cells (5×10^5 cells/ml) were pretreated with either A.) SC560 (0.01-0.5 μM) or B.) FR122047 (0.01-5 μM) for 30 min followed by 2-AG (20 μM) or VH (0.04% ethanol) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. A.) None of the groups treated with both 2-AG and SC560 were significantly different from the control group, VH + 2-AG. B.) *, compared to VH + 2-AG group. These data are representative of at least two separate experiments.

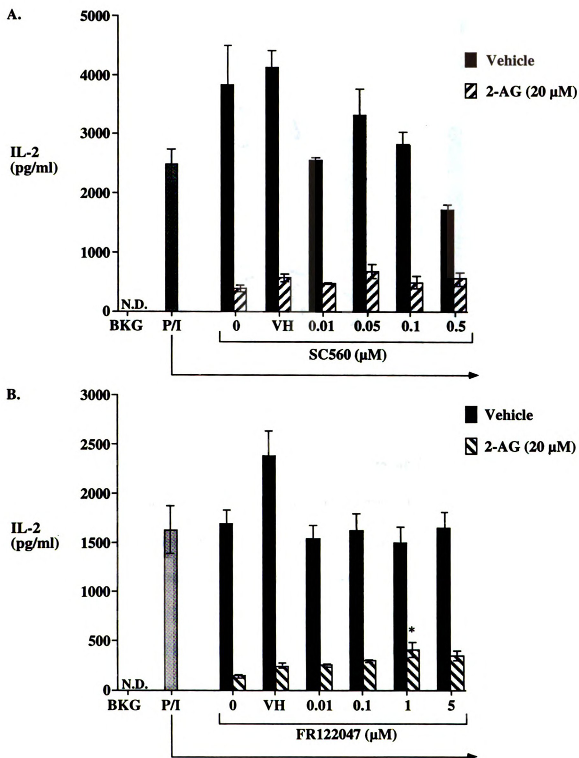


Figure 33.

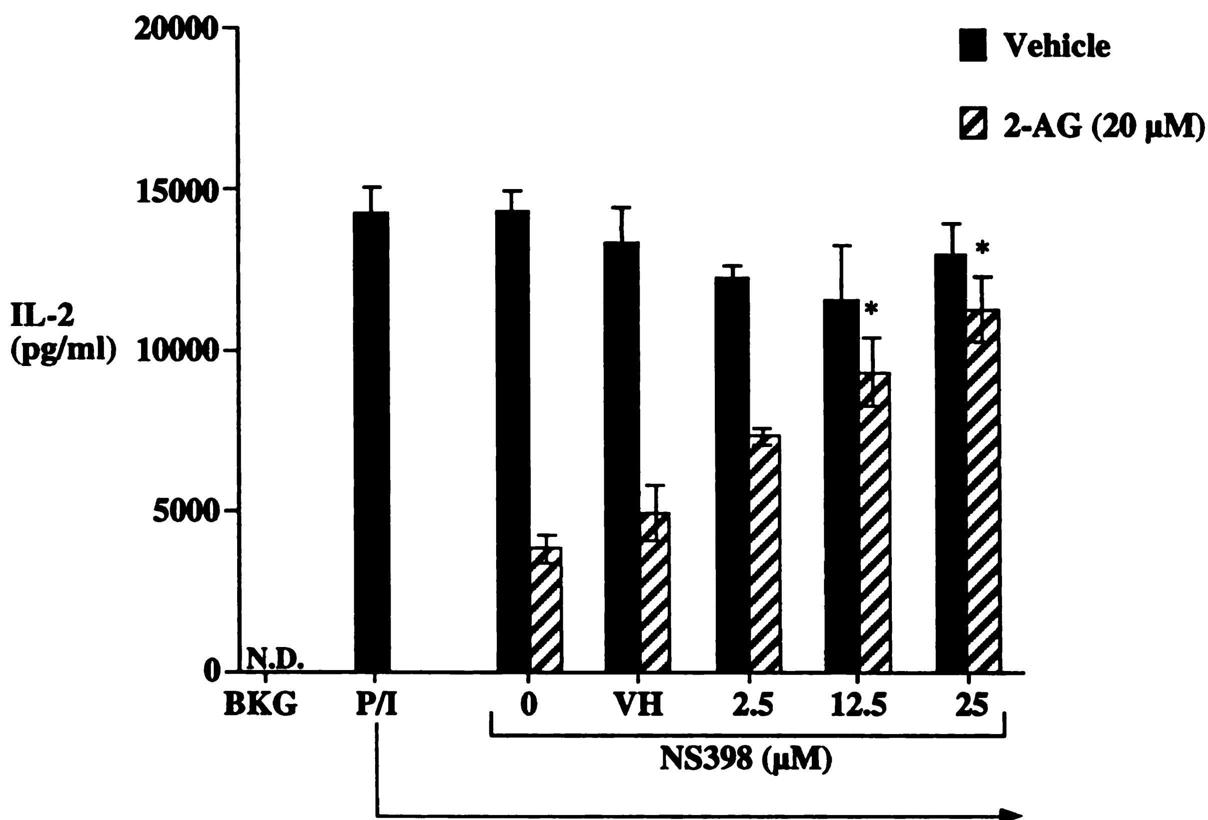
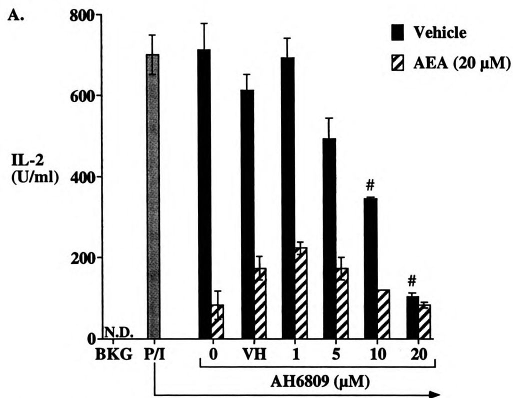


Figure 34. Effect of the COX-2 specific inhibitor, NS398, on 2-AG-mediated inhibition of PMA/ionomycin-stimulated IL-2 production. Jurkat T cells (5×10^5 cells/ml) were pretreated with NS398 (2.5-25 µM) for 30 min followed by 2-AG (20 µM) or VH (0.04% ethanol) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * , $p<0.05$ compared with VH + 2-AG group. These data are representative of at least two separate experiments.

Figure 35. Effect of the prostaglandin inhibitor, AH6809, on the suppression of IL-2 secretion by AEA and the effect of PGE_2-ethanolamine on IL-2 production.

Splenocytes (1 x 10^6 cells/ml) were either A.) pretreated with AH6809 (1-20 μM) for 30 min followed by treatment with AEA (20 μM) or VH (0.04% ethanol) for 30 min or B.) treated with prostaglandin E_2-ethanolamine (0.1-50 μM) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. A.) None of the AEA-treated groups were significantly different from the VH + AEA group. #, $p<0.05$ compared to the VH + VH group. B.) *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.



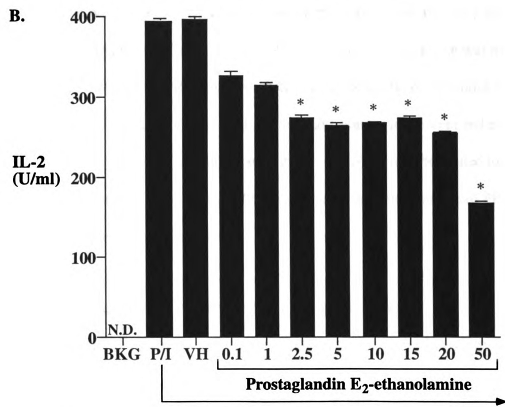


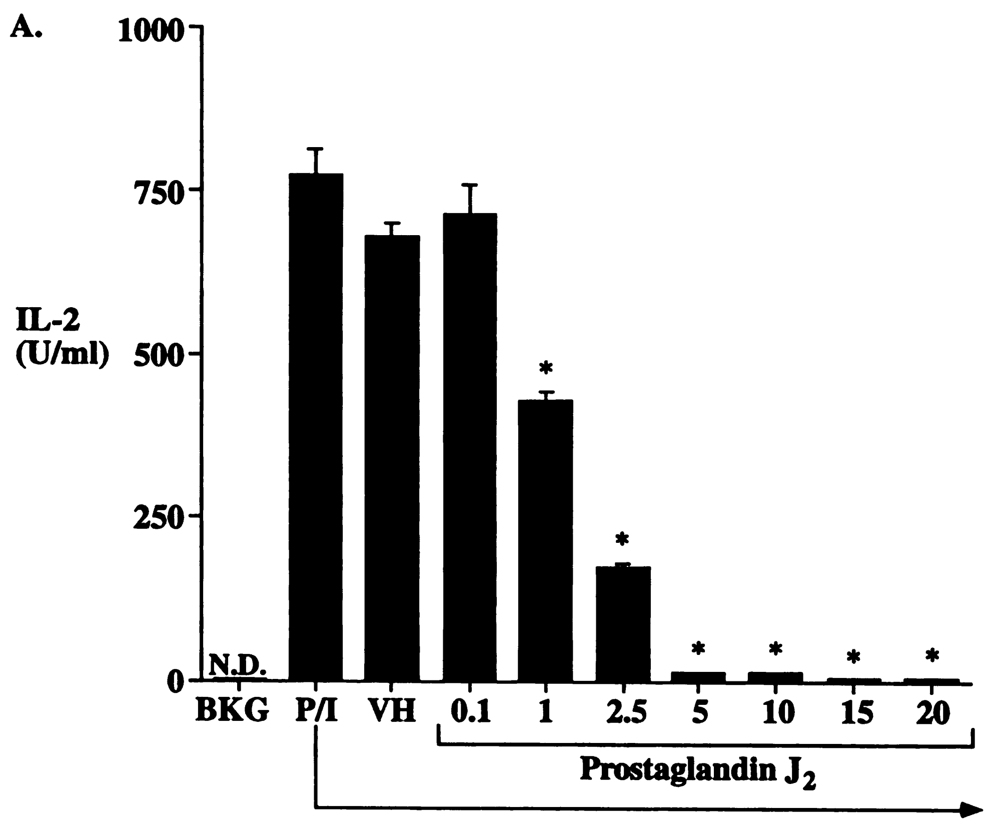
Figure 35.

ethanolamine is a COX-2 metabolite of AEA and that PGE_2-ethanolamine binds to and activates all four EP receptors (176, 178). Moreover, all four EP receptors are expressed in T cells. Consequently, the effect of PGE_2-ethanolamine upon IL-2 was assessed. IL-2 production was only modestly affected by increasing concentrations of PGE_2-ethanolamine, however, suggesting that the EP receptors are not involved (Figure 35b). In addition to PGE_2, other prostaglandins have been reported to suppress IL-2, including 15d-PGJ_2 and PGI_2 (339, 340). Treatment of primary splenocytes with PGJ_2 or 15d-PGJ_2 produced a robust concentration-dependent decrease in IL-2 secretion (Figure 36). Because both PGJ_2 and 15d-PGJ_2 have been found to activate PPARγ, the effect of other known PPARγ agonists upon IL-2 production was investigated. The PPARγ agonists, ETYA and ciglitazone, both suppressed IL-2 in a concentration-dependent manner, which supports the published findings of other laboratories that activated PPARγ inhibits IL-2 production (Figure 37). The inhibition of IL-2 secretion by ciglitazone was more modest than that of the other PPARγ agonists, which is consistent with observations of other laboratories (352). Although it is unclear why ciglitazone is less potent and efficacious than the other PPARγ agonists in the suppression of IL-2, it may be related to differences in chemical structure that result in subtle variations in the conformation of PPARγ, which are not conducive to transrepression.

VIII. The activation of PPARγ by 2-AG and 2-AG ether

The ability of PPARγ agonists to inhibit IL-2 secretion coupled with the similarity in structure of 2-AG and AEA to the putative endogenous ligands of PPARγ, suggested that PPARγ may play a role in the suppression of IL-2 by 2-AG and/or AEA. Evidence

Figure 36. Effect of PGJ_2 and 15-deoxy PGJ_2 on IL-2 secretion. Splenocytes (1 x 10^6 cells/ml) were treated with either A.) prostaglandin J_2 or B.) 15-deoxy prostaglandin J_2 for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.



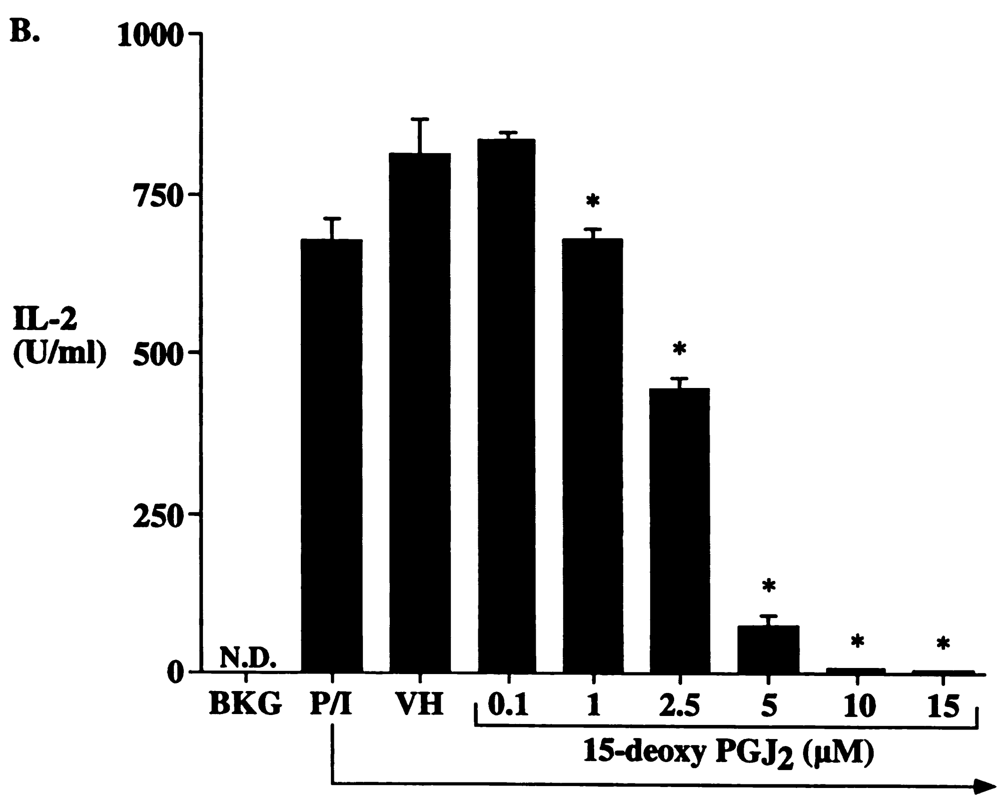


Figure 36.

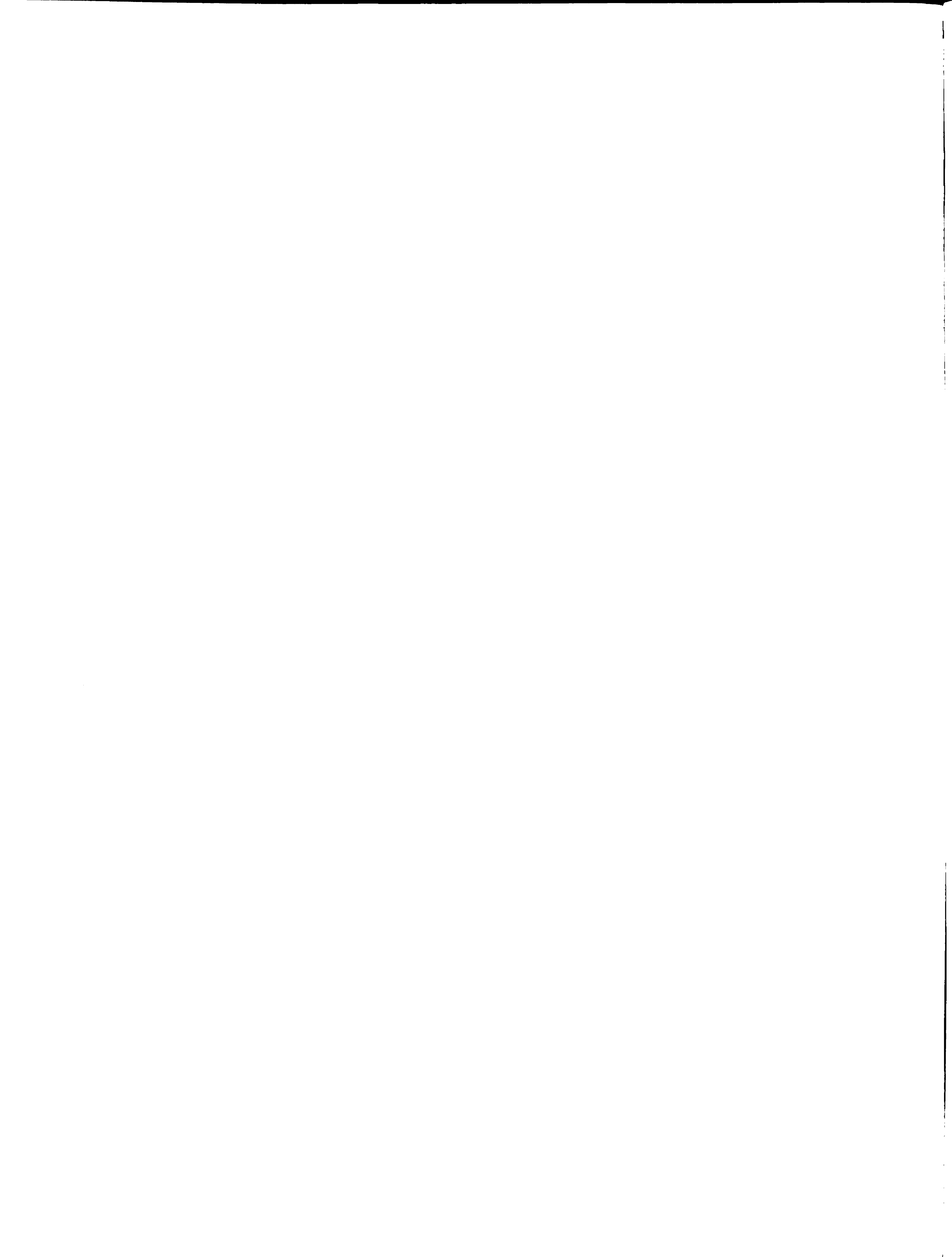


Figure 37. Effect of ciglitazone and ETYA on IL-2 secretion. Splenocytes (1×10^6 cells/ml) were treated with either A.) ciglitazone (0.1-50 μM) or B.) ETYA (0.1-50 μM) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of at least three separate experiments.

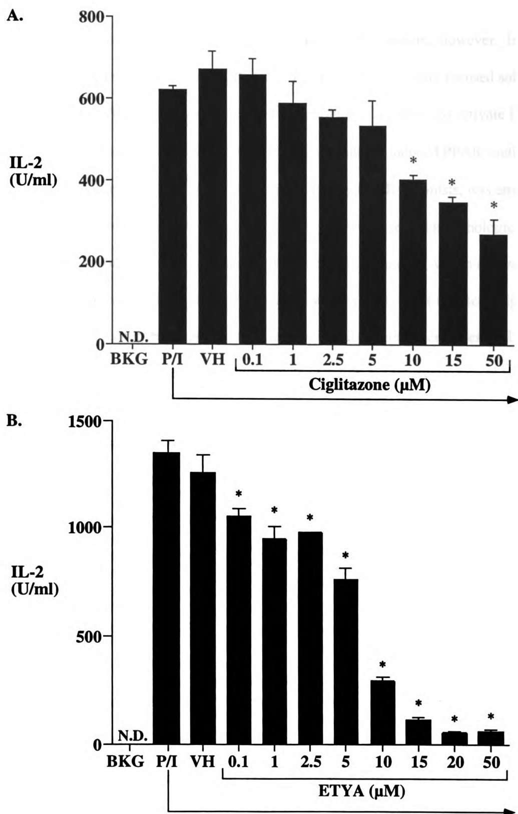


Figure 37.

that 2-AG or AEA treatment caused PPARγ activation was lacking, however. In an effort to simplify the preliminary studies, this line of investigation initially focused solely upon the ability of 2-AG and its non-hydrolyzable analogue, 2-AG ether, to activate PPARγ. To characterize whether 2-AG and/or 2-AG ether treatment induced PPARγ activation, 3T3-L1 adipogenesis, an assay widely used to identify PPARγ agonists, was employed. Differentiation of 3T3-L1 fibroblasts into adipocytes was assessed morphologically by their round shape and by the presence of prominent lipid vacuoles, which are readily stained by Oil Red O. Treatment with 2-AG as well as 2-AG ether induced adipogenesis as quantified by enumeration of the total number of differentiated cells per well (Figure 40). 2-AG ether induced more adipogenesis than 2-AG, but morphologically the changes induced by both were indistinguishable from those induced by ciglitazone (Figures 38 & 39). It is notable that because 3T3-L1 fibroblast adipogenesis requires 7 days coupled with the fact that 3T3-L1 cells efficiently absorb fatty acids and store them in the form of triglycerides, higher concentrations of 2-AG and 2-AG ether were required than in other short term assays using other models (i.e. IL-2 secretion in primary splenocytes).

Because mRNA expression is a more precisely quantified endpoint than adipogenesis, the effect of 2-AG and 2-AG ether upon mRNA levels of aP2, a gene regulated by PPARγ, was measured. The ability of PPARγ to regulate aP2 was confirmed by the induction of aP2 by ciglitazone, a PPARγ agonist, as well as by the suppression of the induction by GW9662, a PPARγ antagonist (Figure 41). Concordant with PPARγ activation, treatment of 3T3-L1 cells with either 2-AG or 2-AG ether produced a marked increase in aP2 mRNA levels. Moreover, 2-AG ether appeared to

Figure 38. Differentiation of 3T3-L1 cells by 2-AG. 3T3-L1 cells were grown to confluence and either exposed to B.) vehicle or C.) 2-AG (50μM) or A.) not exposed. Cells were cultured 7 days after exposure with fluid renewal every 2 days. Cells were then fixed in 10% formalin and stained with Oil Red O and hematoxylin. Images in this dissertation are presented in color.

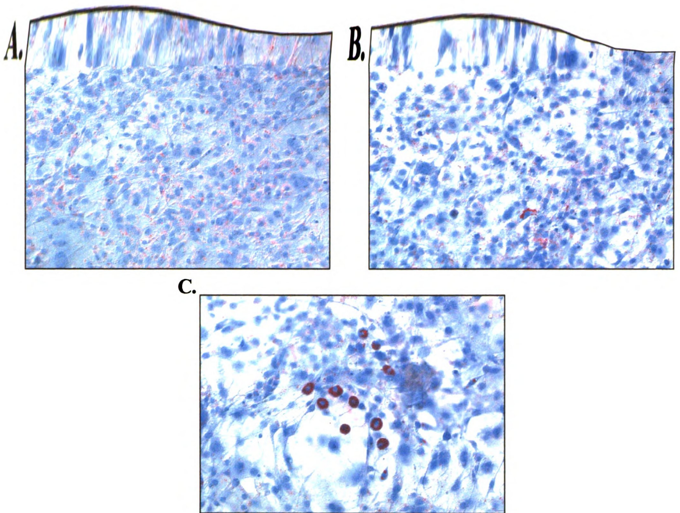
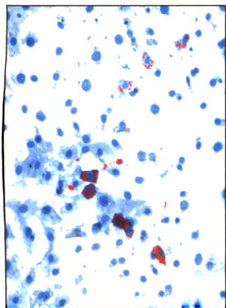


Figure 38.

Figure 39. Differentiation of 3T3-L1 cells by 2-AG ether, ciglitazone, and differentiation media. 3T3-L1 cells were grown to confluence and either exposed to A.) 2-AG ether (50 μM) or B.) ciglitazone (10μM) or C.) differentiation media (10μM dexamethasone, 10μg/ml insulin, 0.5mM IBMX). Cells were cultured 7 days after exposure with fluid renewal every 2 days. Cells were then fixed in 10% formalin and stained with Oil Red O and hematoxylin. Images in this dissertation are presented in color.



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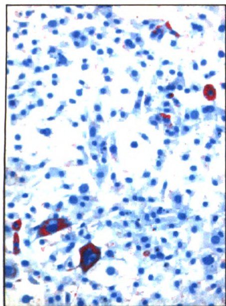

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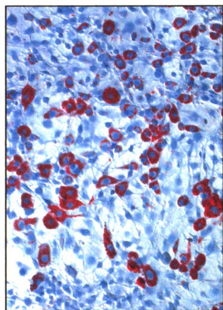

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Figure 39.

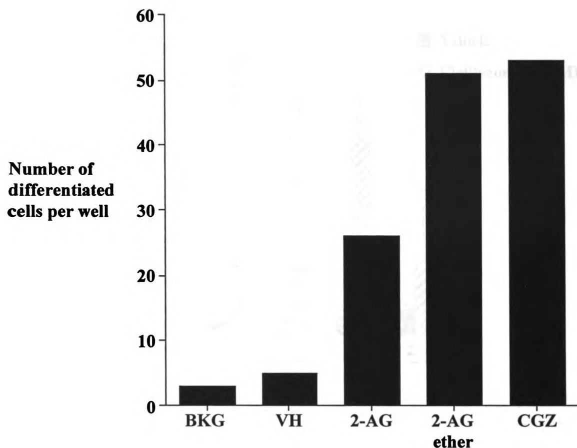


Figure 40. Quantification of 3T3-L1 differentiation. A.) 3T3-L1 preadipocytes were cultured in plastic 2-well culture slides. Upon reaching confluency, the cells were either not treated (NA) or treated with vehicle (0.1 % ethanol), 2-AG (50 μM), 2-AG ether (50 μM), or ciglitazone (10 μM). The cells were then cultured for 7 days, after which the media was removed and the cells were washed with PBS prior to staining with Oil Red O. B.) Cellular differentiation was quantified by counting the total number of differentiated cells per well.

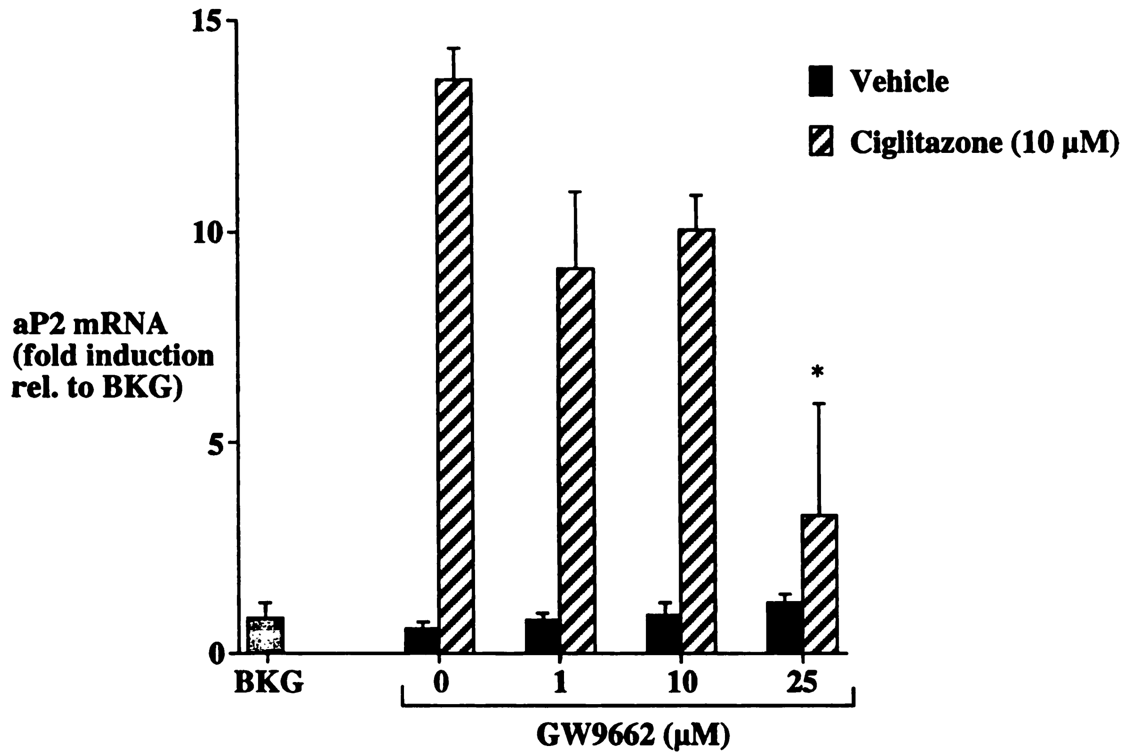


Figure 41. Induction of aP2 by the PPARγ agonist, ciglitazone, is attenuated by the PPARγ antagonist. GW9662. 3T3-L1 cells were cultured in 60 mm culture plates and allowed to grow to confluence. The cells were then either left untreated (BKG) or treated with GW9962 (0-25 μM) and either ciglitazone (10 μM) or vehicle (0.1% ethanol). Cells were cultured for 4 days following treatment at which time total RNA was isolated. aP2 mRNA was determined by real-time PCR using Taqman primers and probe. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to the 0 + ciglitazone group. The data are representative of two separate experiments.

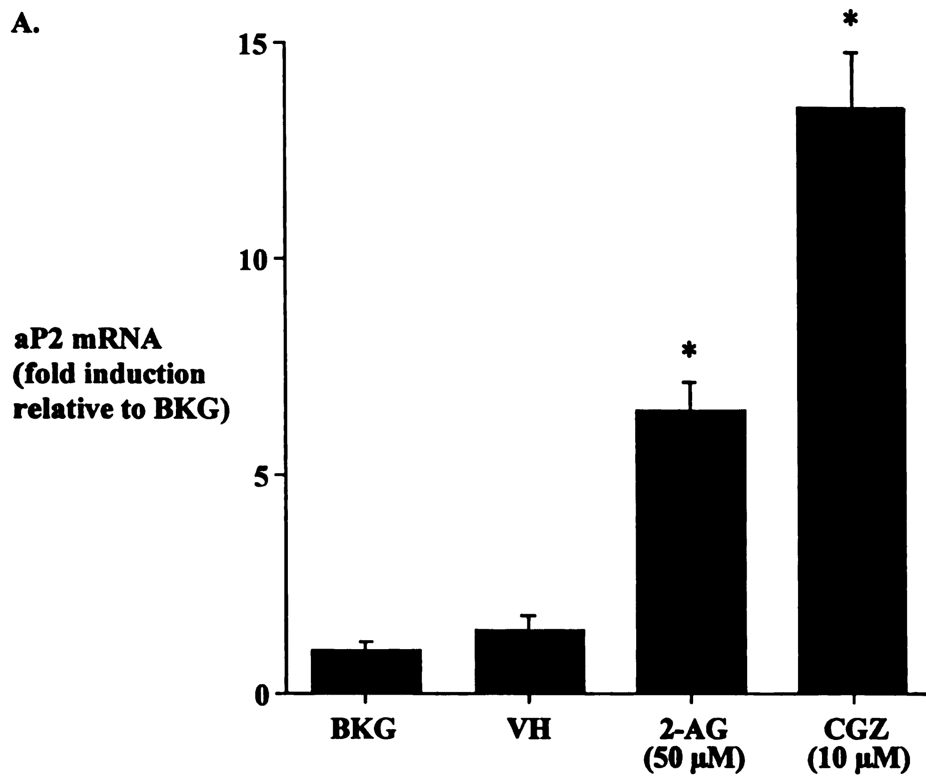
produce modestly greater induction of aP2 than 2-AG as compared to the positive control, ciglitazone, at half the concentration of 2-AG, which is likely due to the resistance of 2-AG ether to hydrolysis (Figure 42).

In order to further evaluate the specificity of 2-AG and AG ether in the activation of PPARγ, transient transfection experiments in 3T3-L1 cells were conducted using the PPARγ–LBD/Gal4-DBD Gal 4 luciferase reporter which is activated by ligands for PPARγ but not PPARα or PPARδ. Interestingly, 2-AG, 2-AG ether and the positive control, ciglitazone, all exhibited comparable potency on the activation of the PPARγ–specific luciferase plasmid. These studies confirm that treatment of 3T3-L1 cells with 2-AG and/or 2-AG ether induces PPARγ activation (Figure 43).

IX. The role of PPARγ in the suppression of IL-2 by 2-AG, 2-AG ether, and AEA

To establish a causal relationship between PPARγ activation and suppression of IL-2 by 2-AG and 2-AG ether, studies were conducted to ascertain whether the PPARγ–specific antagonist, T0070907, attenuates 2-AG and/or 2-AG ether-mediated IL-2 suppression. Pretreatment of freshly isolated splenocytes with increasing concentrations of T0070907, attenuated 2-AG-mediated IL-2 suppression in a concentration-responsive manner (Figure 44a). Notably, T0070907 treatment alone, at the highest concentration used (10 μM) produced a marked suppression of IL-2 in the absence of 2-AG or 2-AG ether, but only in splenocytes. Identical experiments were performed in the Jurkat T cell. These studies showed that Jurkat cells were more refractory to the suppressive effects of T0070907, while almost completely abrogating 2-AG and 2-AG ether-mediated inhibition of IL-2 secretion (Figures 44b and 45).

Figure 42. Effects of 2-AG and 2-AG ether upon aP2 production. 3T3-L1 cells were cultured in 60 mm culture plates and allowed to grow to confluence. The cells were then either left untreated (NA) or treated with VH (0.1% ethanol), ciglitazone (10 μM) and either A.) 2-AG (50 μM) or B.) 2-AG ether (25 μM). Cells were cultured for 4 days following treatment at which time total RNA was isolated. aP2 mRNA was determined by real-time PCR using Taqman primers and probe. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to VH group. The data are representative of at least two separate experiments.



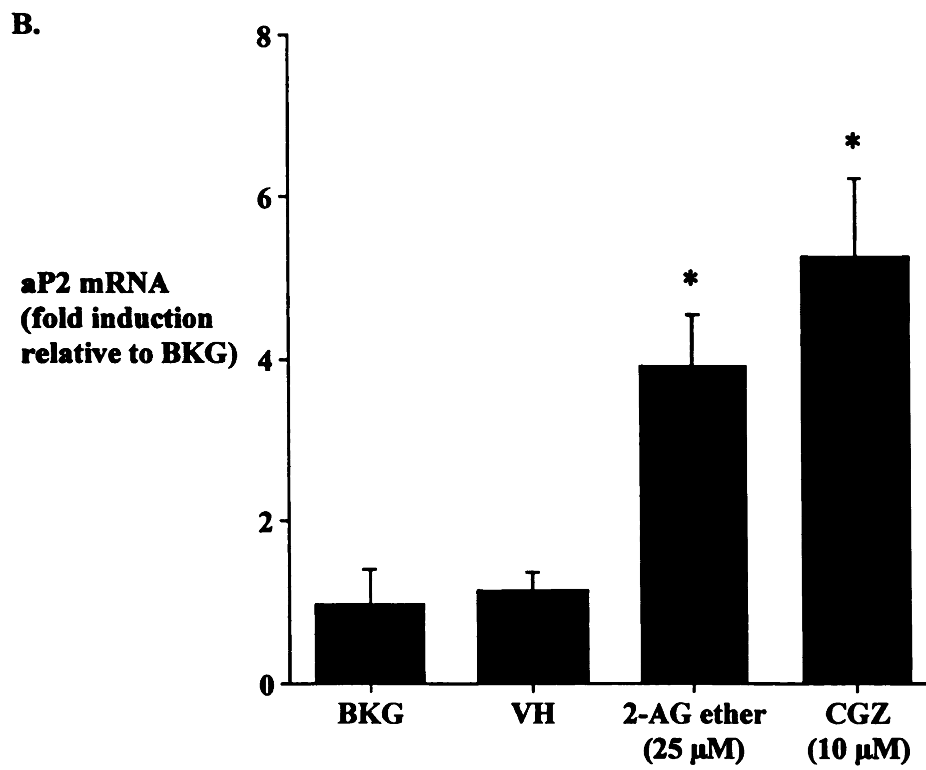


Figure 42.

Figure 43. Effects of 2-AG and 2-AG ether upon luciferase activity in 3T3-L1 cells transfected with PPARγ-LBD/Gal 4-DBD. 3T3-L1 cells were transiently transfected with the PPARγ-LBD/Gal4-DBD Gal4 luciferase reporter plasmid. Following transfection, the cells were trypsinized, washed, resuspended in DMEM with 2% BCS, and pooled together. The cells were then cultured in 24-well plates for 5 h prior to treatment. Cells were either left untreated (NA), or treated with 0.1% ethanol (VH), 10-50 μM ciglitazone (CGZ), 1-30 μM 2-AG, or 1-30 μM 2-AG ether. Cells were then incubated for 16-20 h. The luciferase activity was quantified in relative light units (RLU) by chemiluminscence assay. The results are the mean ± standard error of triplicate cultures. * denotes $p<0.05$ compared to VH group. These data are representative of two separate experiments.

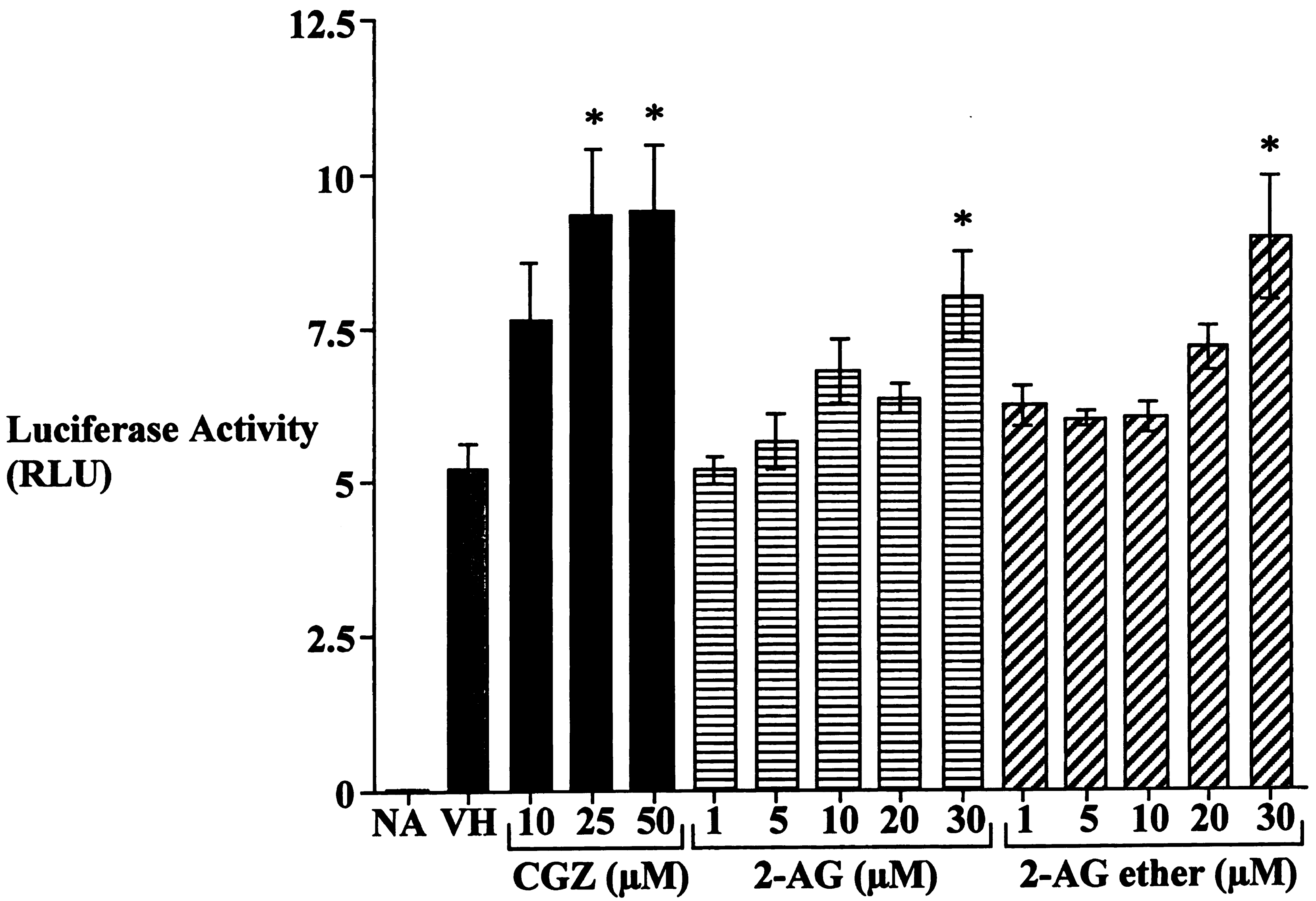
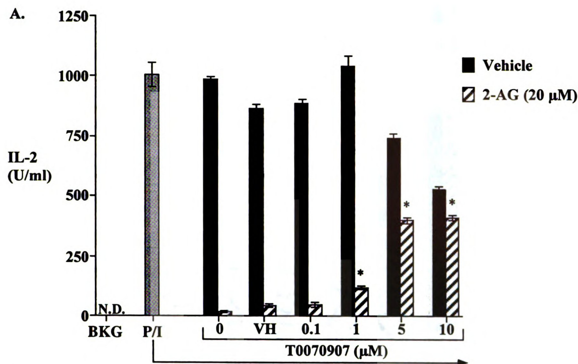


Figure 43.

Figure 44. Effect of the PPARγ antagonist, T0070907, upon suppression of IL-2 by 2-AG in primary murine splenocytes and human Jurkat T cells. A.) Splenocytes (1 x 10^6 cells/ml) or B.) Jurkat cells (5 x 10^5 cells/ml) were treated with T0070907 (0.01 – 10 μM) or vehicle (0.02% DMSO) for 30 min prior to treatment with 2-AG (20 μM). Following a 30 min incubation, the cells were then stimulated with 40 nM PMA/0.5μM ionomycin for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * denotes $p<0.05$ compared to the VH + 2-AG group. These data are representative of at least two separate experiments.



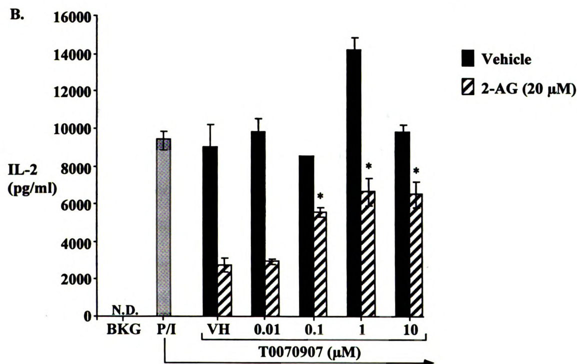


Figure. 44

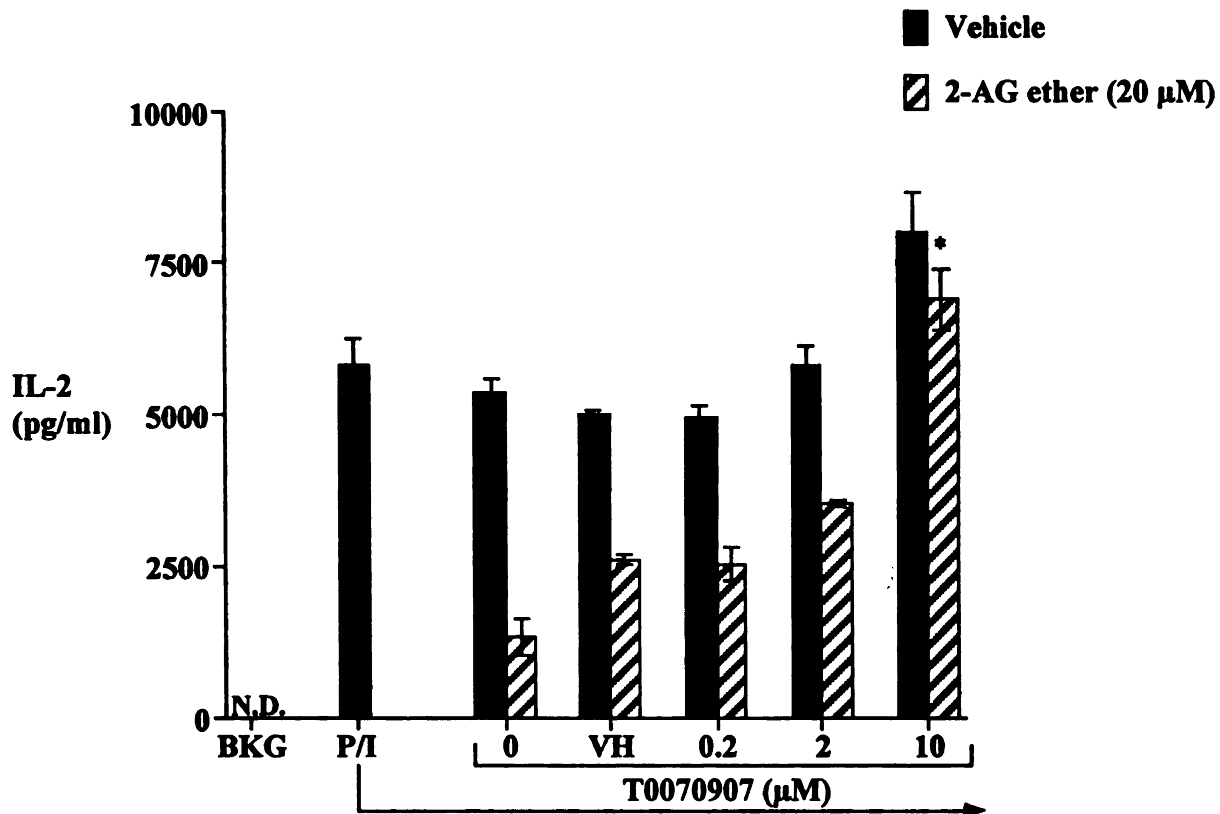


Figure 45. Effect of the PPARγ antagonist, T0070907, upon suppression of IL-2 by 2-AG ether in human Jurkat T cells. Jurkat cells (5 x 10^5 cells/ml) were treated with T0070907 (0.2 – 10 μM) or vehicle (0.02% DMSO) for 30 min prior to treatment with 2-AG ether (20 μM). Following a 30 min incubation, the cells were then stimulated with 40 nM PMA/0.5μM ionomycin for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * denotes $p<0.05$ compared to the VH + 2-AG ether group. These data are representative of two separate experiments.

Likewise, T0070907 pretreatment also blocked AEA-mediated inhibition of IL-2 production (Figure 46).

X. The role of NFAT, NFκB, and AP-1 in the suppression of IL-2 by 2-AG

Published studies from a number of laboratories have demonstrated that suppression of IL-2 by activated PPARγ likely involves the transrepression of the transcription factor, NFAT. As such, it is notable that 2-AG also inhibits the production of other cytokines which are regulated by NFAT, such as IL-4, IFNγ, and TNFα (302) (Figure 47). In addition to NFAT, there is evidence to suggest that NFκB and AP-1 can also be transrepressed by PPARγ. The subsequent studies were designed to ascertain the effect of 2-AG upon the transcriptional activity of NFAT, NFκB, and AP-1 in Jurkat T cells transfected with NFAT-luc, NFκB-luc, or AP-1-luc reporters respectively. Preliminary studies were designed to determine the kinetics of NFAT-luc activity as well as to compare different activators. The greatest level of NFAT-luc activity was induced by 80 nM PMA and 1 μM ionomycin at 12 hours and as a result was used for the subsequent experiments (Figure 48). 2-AG treatment caused a concentration-dependent decrease in NFAT transcriptional activity in Jurkat T cells transfected with NFAT-luc, which is consistent with previous studies from this laboratory in EL4 T cells transfected with NFAT-CAT (Figure 49). The magnitude of induction of NFAT-luc activity in Figure 48 is much more robust than that seen in Figure 49, which is likely due to differences in transfection efficiency between the two experiments. Consistent with the effects of 2-AG upon IL-2, the 2-AG-mediated inhibition of NFAT transcriptional activity was also blocked with the PPARγ antagonist, T0070907, suggesting that PPARγ inhibits NFAT

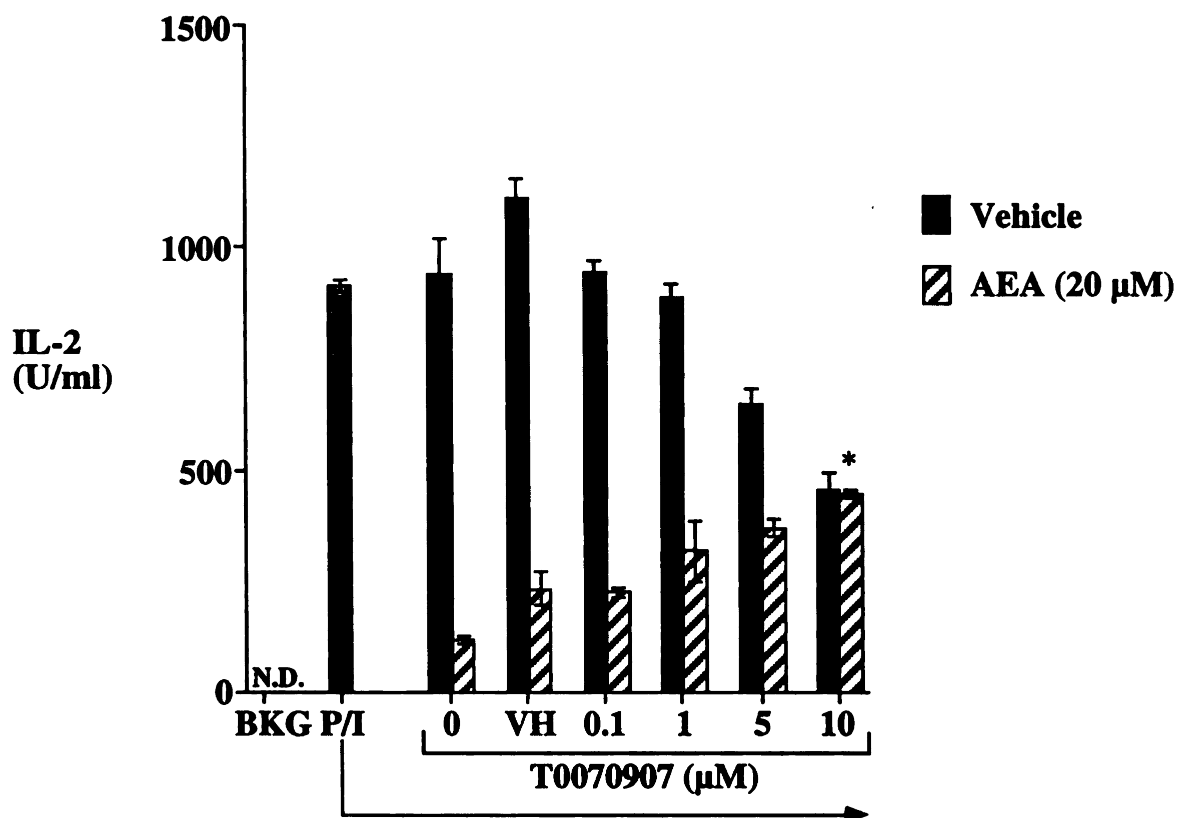
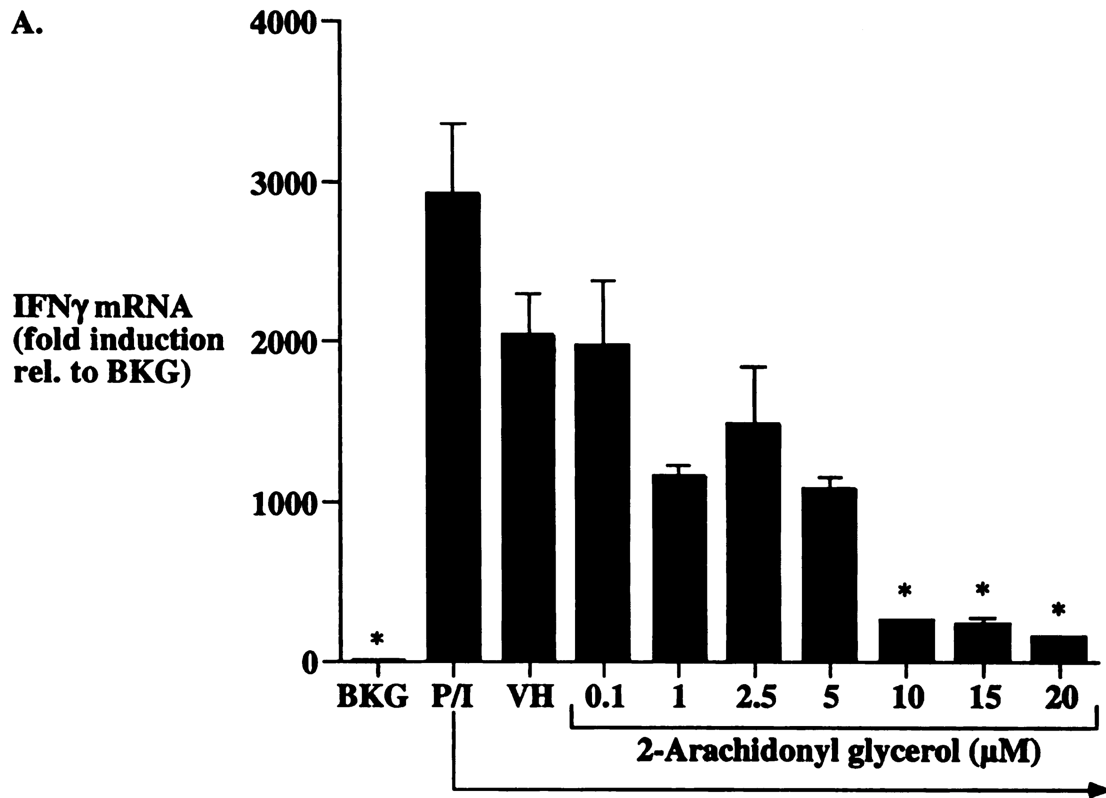


Figure 46. Effect of the PPARγ antagonist, T0070907, upon suppression of IL-2 by AEA in primary murine splenocytes. Splenocytes (1 x 10^6 cells/ml) were pretreated with T0070907 or VH (0.1% DMSO) for 30 min followed by AEA treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA analysis. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to the VH + AEA group. These data are representative of at least three separate experiments.

Figure 47. Effects of 2-AG upon IFNγ and IL-4 production in primary splenocytes. A.) Splenocytes (1 x 10^6 cells/ml) were treated with 2-AG (0.1 – 20 μM) or vehicle (0.1% ethanol) for 30 min prior to stimulation with 40 nM PMA/0.5μM. After a 6 h incubation, the cells were harvested and total RNA was isolated. IFNγ and IL-4 mRNA was determined by real-time PCR using predeveloped Taqman primers and probe from Applied Biosystems (Foster City, CA). The results are the mean ± standard error of triplicate cultures. * denotes $p<0.05$ compared to the VH group. These data are representative of two separate experiments.



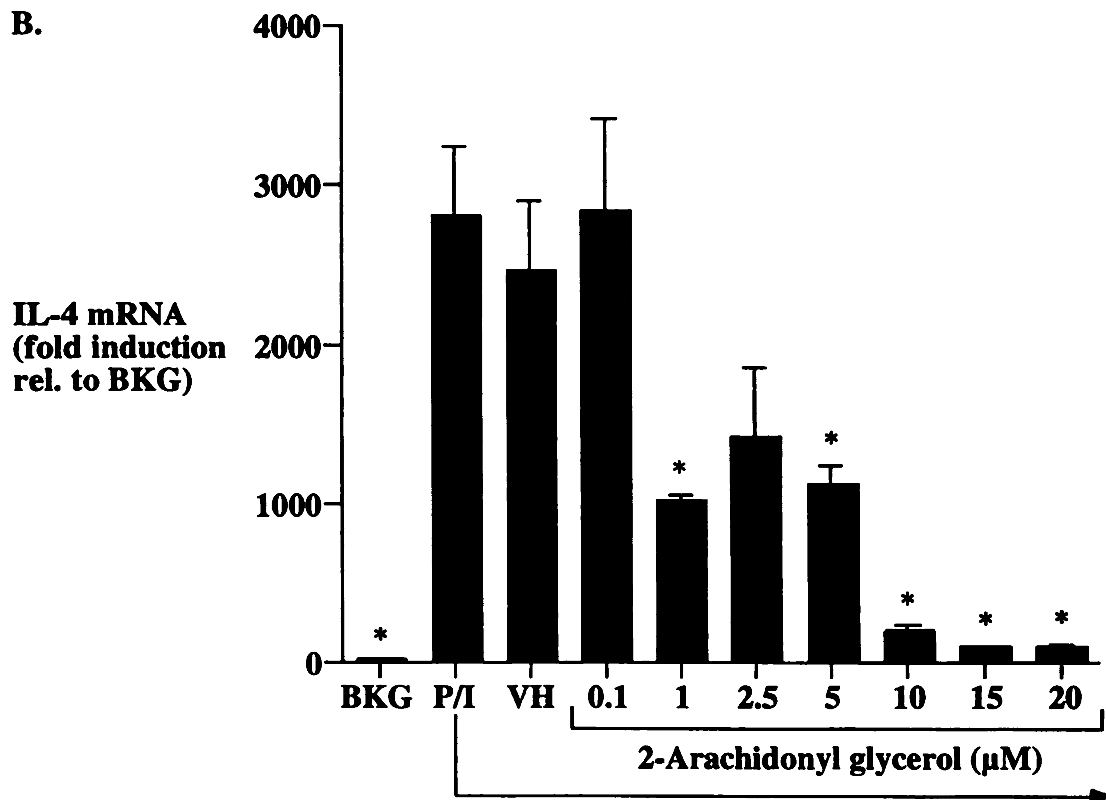


Figure 47.

Figure 48. Effect of PMA/ionomycin and PHA/PMA upon NFAT transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the NFAT-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then either left untreated (NA) or treated with 40 nM PMA/0.5 μM ionomycin, 80 nM PMA/1 μM ionomycin, or PHA/PMA and incubated for 12 hr. NFAT-luc activity was quantified by chemiluminescence assay. Jurkat T cells transfected with the pTA-luc plasmid were treated with 80 nM PMA/1 μM ionomycin and served as a negative control. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. These data are representative of two separate experiments.

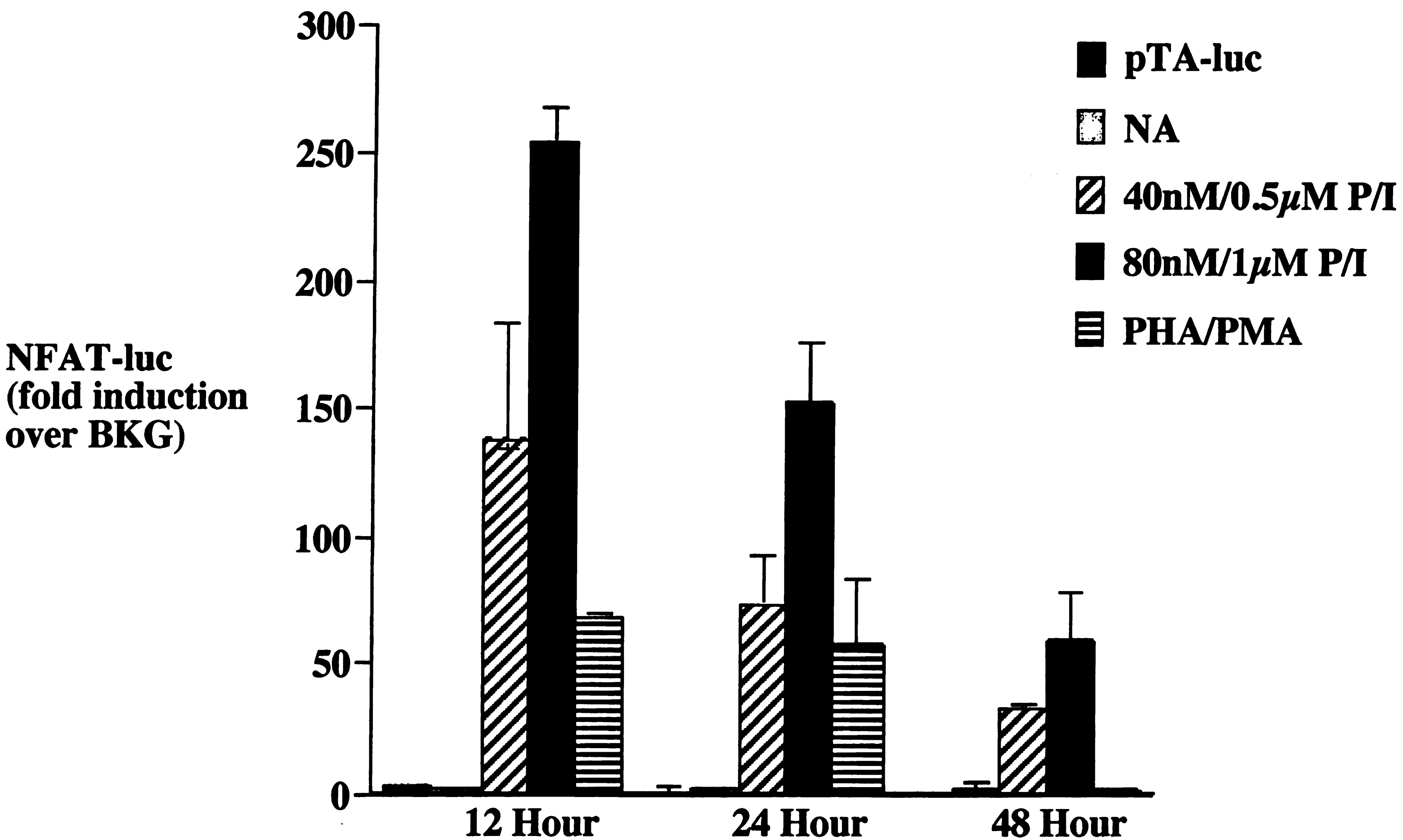


Figure 48.

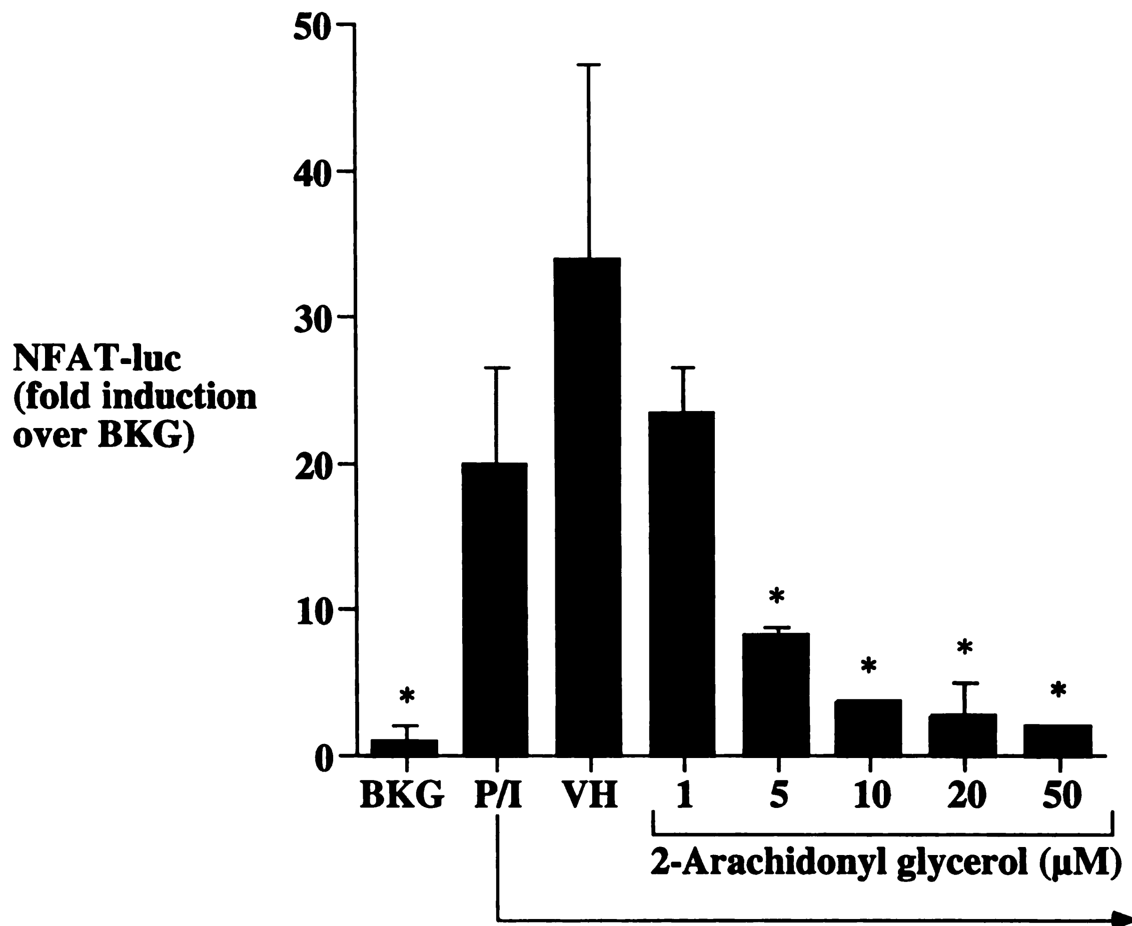


Figure 49. Effect of 2-AG upon NFAT transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the NFAT-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then treated with 2-AG (1-50 μM) for 30 min prior to treatment with 80 nM PMA/1 μM ionomycin. The cells were then incubated for 12 hr. NFAT-luc activity was quantified by chemiluminescence assay. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.

activation resulting in IL-2 suppression (Figure 50). Likewise, 2-AG also repressed the transcriptional activity of both NFκB-luc and to a lesser degree, AP1-luc (Figures 51 & 53). Similar to the effects of 2-AG upon NFAT, 2-AG-mediated inhibition of NFκB activity was also antagonized with T0070907, suggesting that transrepression of NFκB by PPARγ also plays a role in the inhibition of IL-2 by 2-AG (Figure 54). Conversely, T0070907 alone causes modest suppression of AP-1-luc activity by itself, which is comparable to that produced by 2-AG, confounding the interpretation of these results (Figure 52). Although the inhibition of AP-1 by 2-AG appears to be more robust in Figure 52 than 51, the difference in vehicle effects between the two experiments may contribute to the disparity as 2-AG-mediated inhibition appears more consistent when compared to the PMA/Io controls without vehicle. Collectively, the aforementioned observations suggest that while AP-1 is only modestly inhibited by 2-AG, NFAT and NFκB are strongly inhibited by 2-AG and that PPARγ plays a role in the inhibition of NFAT and NFκB.

Figure 50. Effect of the PPARγ antagonist, T0070907, upon 2-AG-mediated suppression of NFAT transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the NFAT-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then either treated with T0070907 (1-10 μM) or VH (0.05% DMSO) and 2-AG (20 μM). Following a 30 min incubation, the cells were then treated with PMA (80 nM) and ionomycin (1 μM) and incubated for 12 hr. NFAT-luc activity was quantified by chemiluminescence assay. Jurkat T cells transfected with the pTA-luc plasmid (pTA) were treated with 80 nM PMA/1 μM ionomycin and served as a negative control. NFAT-luc activity was quantified by chemiluminescence assay. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. These data are representative of two separate experiments.

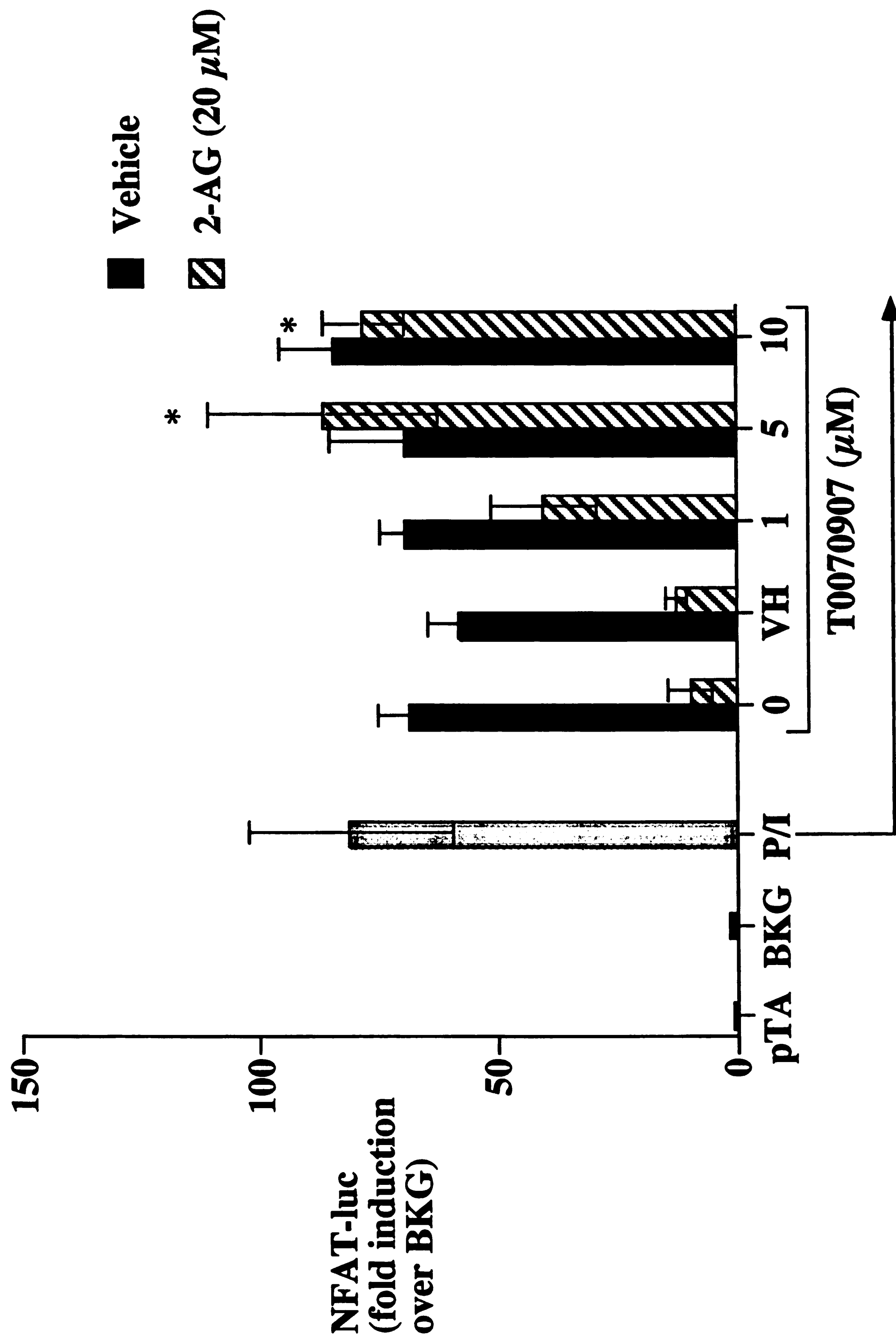


Figure 50.

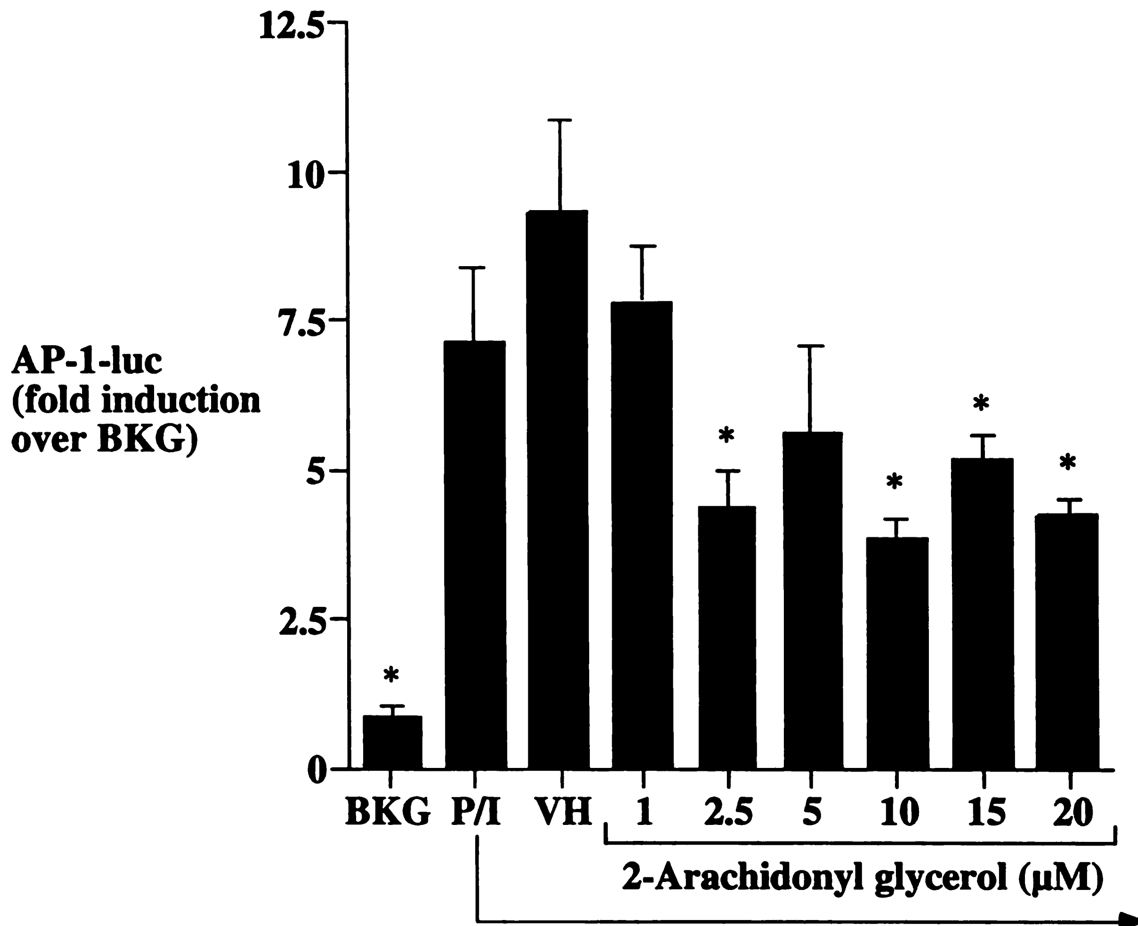


Figure 51. Effect of 2-AG upon AP-1 transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the AP-1-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then treated with 2-AG (1-20 μM) for 30 min prior to treatment with 80 nM PMA/1 μM ionomycin. The cells were then incubated for 12 hr. AP-1-luc activity was quantified by chemiluminescence assay. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.

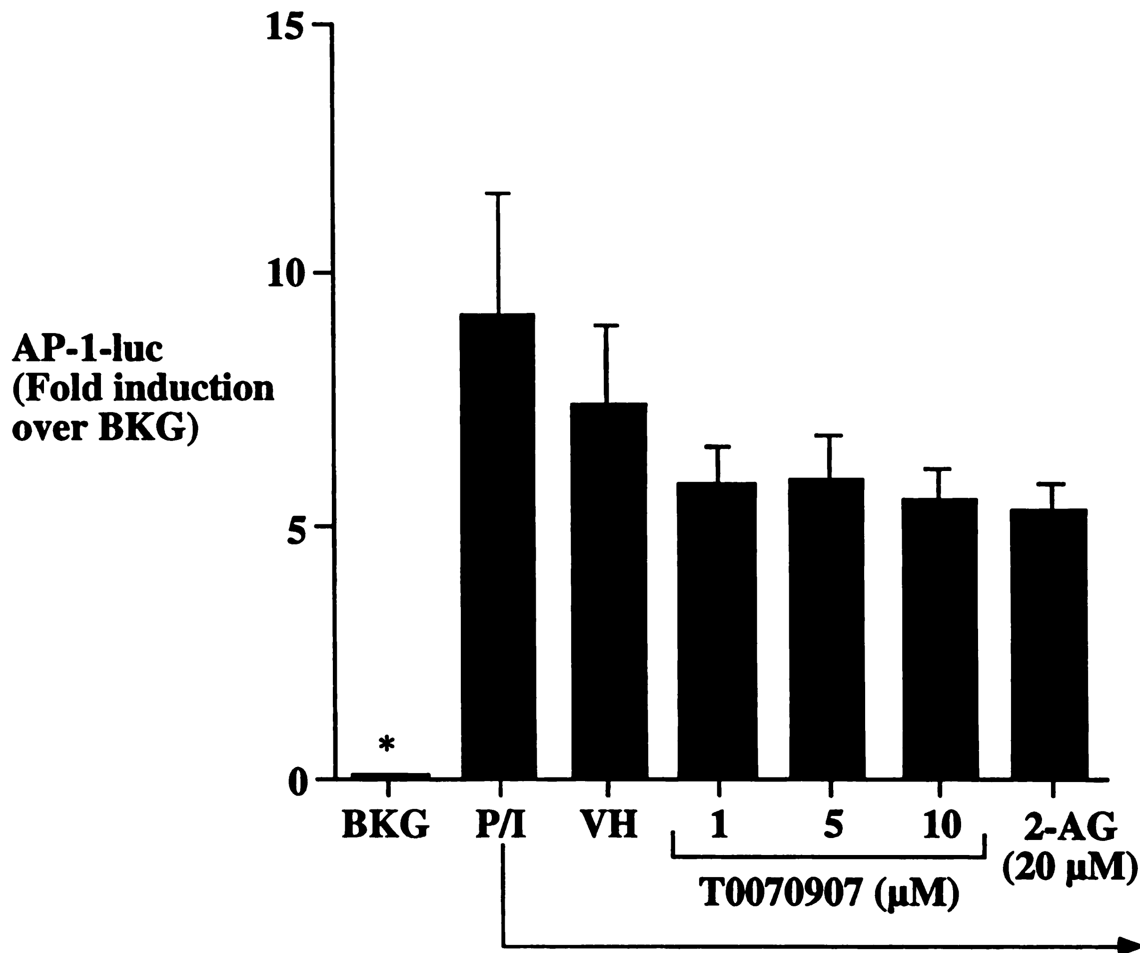


Figure 52. Effect of T0070907 and 2-AG upon AP-1 transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the AP-1-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then treated with 2-AG (1-20 μM) or T0070907 (1-10 μM) for 30 min prior to treatment with 80 nM PMA/1 μM ionomycin. The cells were then incubated for 12 hr. AP-1-luc activity was quantified by chemiluminescence assay. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.

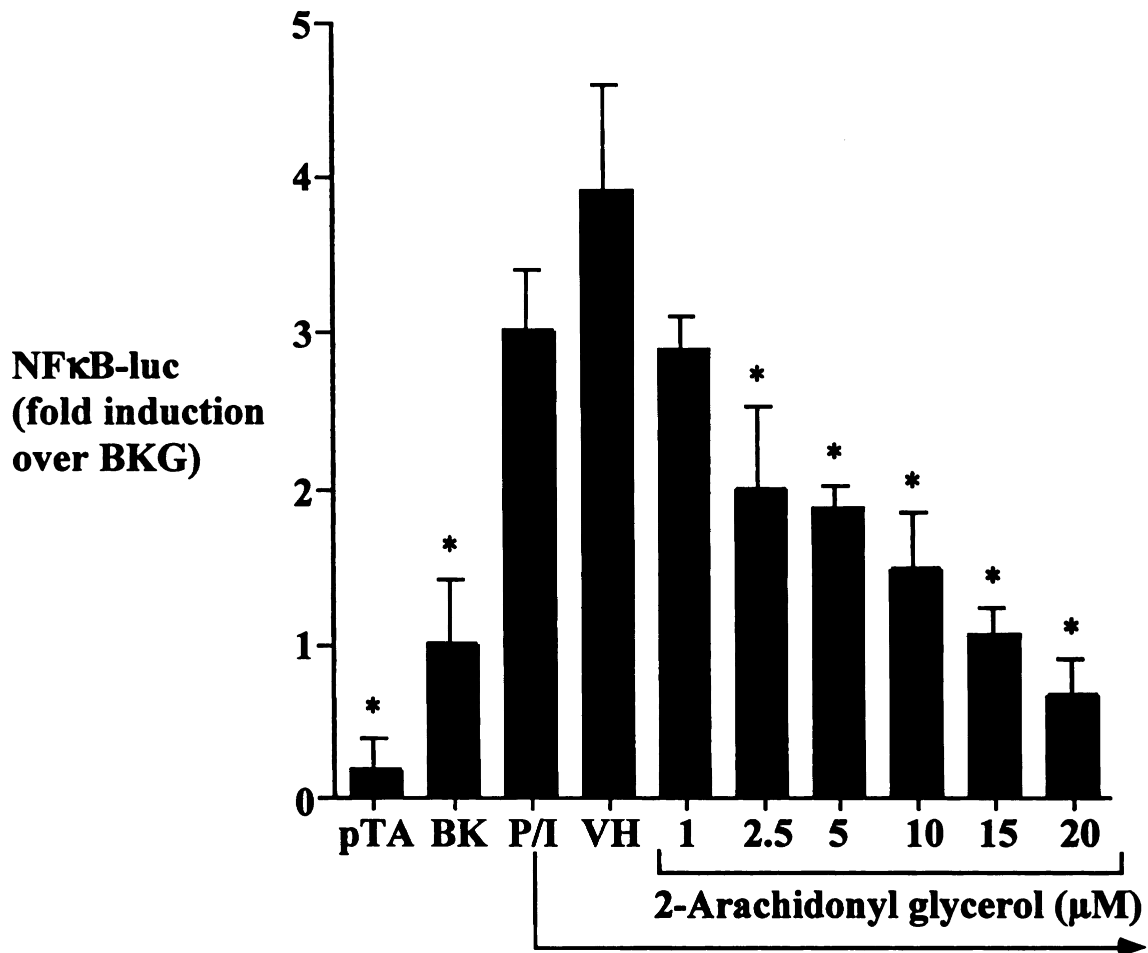


Figure 53. Effect of 2-AG upon NFκB transcriptional activity. Jurkat T cells (5 x 10^5 cells/ml) were transiently transfected with the NFκB-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then treated with 2-AG (1-20 μM) for 30 min prior to treatment with 80 nM PMA/1 μM ionomycin. The cells were then incubated for 12 hr. NFκB-luc activity was quantified by chemiluminescence assay. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. *, $p<0.05$ as compared to the VH group. These data are representative of two separate experiments.

Figure 54. Effect of the PPARγ antagonist, T0070907, upon 2-AG-mediated inhibition of NFκB transcriptional activity. Jurkat T cells (5×10^5 cells/ml) were transiently transfected with the NFκB-luc luciferase reporter plasmid. Following transfection, the cells were washed and resuspended in RPMI with 2% BCS. The cells were then either treated with T0070907 (1-10 μM) or VH (0.05% DMSO) and 2-AG (20 μM). Following a 30 min incubation, the cells were treated with PMA (80 nM) and ionomycin (1 μM) and incubated for 12 hr. NFκB-luc activity was quantified by chemiluminescence assay. Jurkat T cells transfected with the pTA-luc plasmid (pTA) were treated with 80 nM PMA/1 μM ionomycin and served as a negative control. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of quadruplicate cultures. These data are representative of two separate experiments.

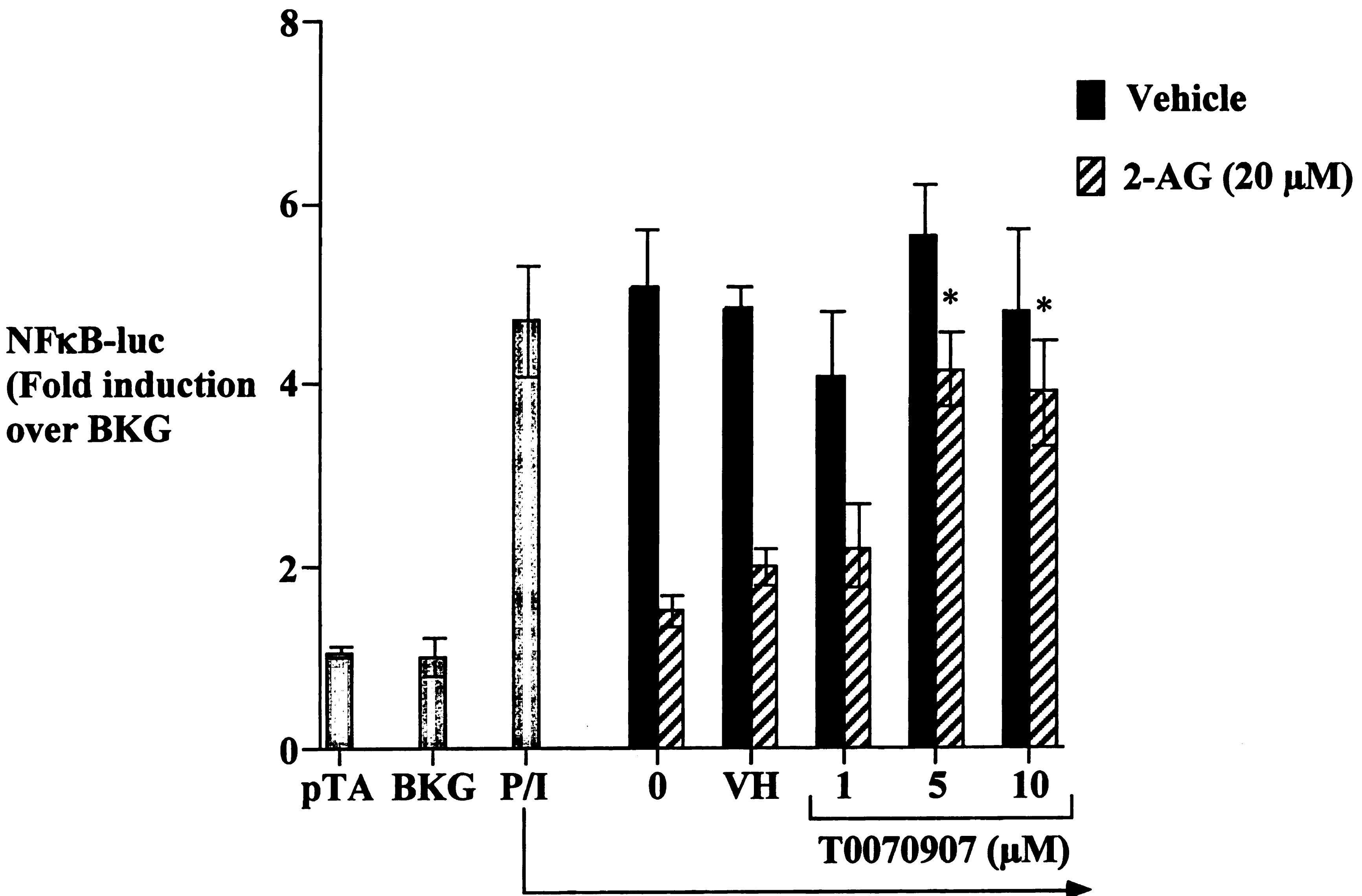


Figure 54.

DISCUSSION

I. Effects of AEA, 2-AG, and 2-AG ether upon IL-2 secretion

The putative endogenous cannabinoids, AEA, 2-AG and 2-AG ether, suppress IL-2 secretion in both murine splenocytes as well as human Jurkat T cells, activated with PMA and ionomycin. While 2-AG and 2-AG ether are somewhat more potent in Jurkat T cells than murine splenocytes, AEA is markedly more potent in Jurkat cells compared to splenocytes. Although the reason for the observed differences between the two models is unclear, species differences could result in increased sensitivity of human T cells to 2-AG and AEA. Alternatively, metabolism of AEA, and to a lesser extent 2-AG, by macrophages in the primary splenocyte population, may diminish the activity of AEA and 2-AG and thereby reduce their potency in the mixed cell preparation.

In activated Jurkat cells, suppression of IL-2 by AEA and 2-AG is observed at concentrations as low as 100 nM and 2.5 μM, respectively. While this is slightly higher than the calculated endogenous levels, concentrations of AEA and 2-AG exceeding 50 nM and 250 nM, respectively, have been detected in human plasma (381). Interestingly, it has been demonstrated that AEA can be sequestered in cells such that the intracellular concentrations of AEA can be as much as 3 orders of magnitude higher than extracellular concentrations (167). Furthermore, intracellular levels of AEA have been calculated to be as high as 2 μM in Hep2 human laryngeal cells (166). Although cellular accumulation of 2-AG is not nearly as well characterized as that of AEA, there is evidence to suggest that 2-AG is also sequestered intracellularly (244). Measurements of 2-AG and AEA are further confounded by the lability of the compounds. Due to the chemical and metabolic

instability of 2-AG and AEA, it seems likely that they are synthesized de novo from membrane components in the locale of their targets. In the case of 2-AG, this is supported by studies showing that diacylglycerol (DAG) is efficiently converted to 2-AG, presumably by a DAG lipase (231). Given the high concentrations of DAG produced in T cells as well as other immune cell types upon activation, it seems likely that the local concentrations of newly synthesized 2-AG are quite high within the local environment of the target in many activated immune cell types.

II. Role of the cannabinoid receptors in the suppression of IL-2 by 2-AG, 2-AG ether and AEA

While a variety of immune effects have been attributed to the cannabinoid receptors, the majority of these have relied solely upon cannabinoid receptor antagonists to ascertain the roles of CB1 and CB2. Studies from our laboratory have demonstrated that Δ^9-THC-mediated calcium influxes can be attenuated with both SR141716A and SR144528 in splenocytes derived from both wild-type and CB1/CB2 null mice, demonstrating that SR141716A and SR144528 can act at targets other than CB1 and CB2 (unpublished observations). Consequently, studies that report effects of CB1 and/or CB2 solely through the use of SR141716A and SR144528 may need to be reevaluated.

Although CB2 is expressed at higher levels than CB1 in immune cells, several immune effects have been correlated with CB1 activation in myeloid cells. Similarly, the majority of immune effects associated with CB2 activation have also been in myeloid cells. Nonetheless, there have been a few reports of CB2-mediated effects in lymphoid cells, including increased proliferation of CD40-activated B cells, decreased $CD8^+$ T cell migration, induction of TGFβ in human peripheral blood lymphocytes, and suppression

of macrophage-dependent T cell activation (113, 116, 123, 297). The involvement of CB2 in all of the aforementioned effects, with the exception of the inhibition of macrophage-dependent T cell activation, was determined through the use of the CB2 antagonist, SR144528, or the CB2-specific agonist, JWH133.

While it has been demonstrated that the cannabinoid-mediated enhancement of CD40-activated B cell proliferation is blocked with SR144528, the magnitude of the effect is modest (approximately 15% and 25% increase in virgin B cells and germinal center B cells, respectively) and as such, it is unclear if the observed effects are physiologically relevant (113). In human $CD8^+$ T cells, migration induced by stromal cell-derived factor 1 (SDF-1) is inhibited 50% by AEA (40 nM) treatment (297). Likewise, the CB2-specific agonist, JWH133 (10 nM) produced a similar level of inhibition, which led the authors to believe that the effect was mediated by CB2. This assertion would be greatly strengthened through the use of CB2 null mice or multiple CB2 antagonists.

Although the role of CB2 in CD8+ T cell migration is not yet entirely clear, evidence for the role of CB2 in the induction of TGFβ production is somewhat stronger. It has been demonstrated that Δ^9-THC induces TGFβ production in human peripheral blood lymphocytes, an effect that is completely blocked with SR144528 (10 nM) (123). Although SR144528 was the sole tool used to ascertain CB2 involvement, the concentrations were sufficiently low to support the supposition that SR144528 is blocking CB2 specifically. Nonetheless, the role of CB2 in the induction of TGFβ production by Δ^9-THC should be confirmed through alternative approaches. Additionally, the impaired macrophage-dependent T cell activation by Δ^9-THC has been

attributed to CB2 as determined through the use of CB2 null mice (116). As reported by McCoy et al., the impaired macrophage-dependent T cell activation was determined to be due to altered antigen processing by macrophages rather than the direct inhibition of T cells by Δ^9-THC (115). Curiously, Δ^9-THC-mediated inhibition of macrophage-dependent T cell activation was not concentration-dependent, such that low nanomolar concentrations of Δ^9-THC suppressed T cell activation, whereas higher concentrations had no effect. In contrast to the aforementioned findings, studies from this laboratory and others have determined that IL-2 production by splenic T cells and the EL4 T cell line is suppressed by Δ^9-THC, independent of macrophage antigen processing (292, 382). There are a number of differences between the two studies, however, including the concentrations of Δ^9-THC and the models used. Additionally, the T cells in the study by McCoy et al. were suboptimally activated, which is significant in that suboptimally activated T cells have been shown to exhibit enhanced IL-2 production with cannabinoid treatment (332, 333).

CB2 activation also produces effects in mixed immune cell populations. It has been demonstrated by McKallip et al. that apoptosis induced by Δ^9-THC treatment can be blocked with the CB2 antagonist, SR144528, in mixed cell populations, such as splenocytes and thymocytes (114). This is in contrast to the findings of this laboratory, in which no effects upon viability were observed with Δ^9-THC-treated splenocytes. A number of factors could account for these seemingly contradictory observations, including differences in when various responses were measured in experiments, the assays used to assess viability and apoptosis, and the strains and/or stocks of mice used. Importantly, the mode of activation typically used by this laboratory (PMA/Io) induces a

particularly robust activation of T cells, which may not necessarily be comparable to the milder modes of T cell activation employed by McKallip et al. Robust activation likely provides strong growth signals, which may make T cells more refractory to apoptosis. Furthermore, because the involvement of CB2 was ascertained solely with SR144528, used in the micromolar range, it is unclear whether the apoptotic effects of Δ^9-THC observed by McKallip et al. are the result of the activation of CB2 or some other target.

Given the ambiguity of the role of CB1 and CB2 in cannabinoid-mediated immune effects, a major objective of the current studies was to examine rigorously the role of CB1 and CB2 in 2-AG/AEA-mediated suppression of IL-2, a critical cytokine for T cell growth and development. The suppression of IL-2 by 2-AG and AEA in splenocytes derived from CB1/CB2 null mice coupled with the failure of the CB1/CB2 antagonists to block the decrease in IL-2 by 2-AG and AEA demonstrates that the cannabinoid receptors are not involved. Likewise, the decrease in IL-2 secretion by 2-AG ether is also independent of CB1/CB2, as determined by splenocytes derived from CB1/CB2 null mice.

III. Role of the vanilloid receptor, VR1, in the suppression of IL-2 by AEA

Although there have been a number of effects by 2-AG and AEA attributed to CB1/CB2, there are also a growing number of reports of endocannabinoid activities which are independent of CB1/CB2 in keeping with the studies reported here (206, 214). One potential mechanism for cannabinoid receptor-independent activity is the vanilloid receptor, VR1. VR1 is a ligand-gated cation channel, which is activated by heat, low pH, or capsaicin and other agonists (383, 384). While the efficacy of AEA for VR1 is

currently the focus of much debate, it is clear that AEA is significantly less potent than capsaicin in the activation of VR1 (214, 215, 385, 386, 387). Although VR1 is highly expressed in central and peripheral neurons, it is also expressed in many other cell types, including T cells (388). It is notable that the VR1 antagonist, capsazepine, at concentrations up to 1 μM did not attenuate AEA-mediated IL-2 suppression in splenocytes. Higher concentrations of capsazepine suppress IL-2 secretion in the absence of AEA and therefore were not used. While the majority of published studies report IC_{50} values of capsazepine for capsaicin-induced calcium influx from 0.04 – 1 μM, there have been reports of higher IC_{50} values (3-4 μM), which necessitated the investigation of the effects of the prototypical VR1 agonist, capsaicin, upon IL-2 secretion (389-393). Despite a published report which demonstrates that capsaicin suppresses IL-2 in activated T cells in a concentration-dependent manner, capsaicin does not decrease IL-2 production in splenocytes under the experimental conditions reported here (394). In fact the two studies are not entirely contradictory as capsaicin was not reported to suppress IL-2 production at concentrations up to 20 μM, such that the effects upon IL-2 were only observed at concentrations of 50 μM and higher. The authors assume that the suppression of IL-2 by capsaicin is not mediated by VR1 because of the high concentrations of capsaicin required as well as the lack of antagonism by capsazepine (394).

There have been reports of other cellular targets for AEA, including the putative endothelial AEA receptor (also called the abnormal cannabidiol receptor), L-type calcium channels, TASK-1 channels, and a G-protein coupled receptor in mouse brain that has not yet been characterized (84, 206, 395-398). While the endothelial AEA receptor has not

been cloned and therefore its distribution is unknown, this receptor has only been characterized pharmacologically in endothelial cells and therefore has not been described in lymphoid tissue thus far. In addition to AEA, abnormal cannabidiol, a synthetic cannabidiol analogue, has also been identified as an agonist of the putative endothelial AEA receptor, whereas cannabidiol acts as a partial agonist/antagonist (84, 228, 229, 399). Studies from this laboratory have demonstrated that cannabidiol is more potent in the suppression of IL-2 secretion than AEA, suggesting that the endothelial AEA receptor is not involved (28). Moreover, WIN55212-2, 2-AG and Δ^9-THC also suppress IL-2 secretion, but are not agonists of the putative endothelial AEA receptor (28, 229, 292, 303).

IV. Effects of AEA and 2-AG upon calcium influx

Activation of T cells requires the induction of a number of different signal transduction pathways, including MAP kinase, NFκB, and NFAT through a rise in intracellular calcium. The elevation of intracellular calcium levels is the result of the release of internal stores followed by the opening of calcium release-activated channels (CRAC) (400). The rise in intracellular calcium is oscillatory in nature and must be sustained for 30 to 45 min for commitment of the T cell to cellular activation, as evidenced by transcription of IL-2 mRNA (401-404). The induction of IL-2 transcription is achieved by calcium-calmodulin activation of calcineurin, which subsequently dephosphorylates NFAT (404). In the dephosphorylated state, NFAT translocates across the nuclear membrane and binds to the IL-2 promoter (310). Immunosuppressive drugs, such as cyclosporin, inhibit calcineurin activation and thus suppress the activation and translocation of NFAT (309, 405, 406). The kinetics of the calcium signal in relation to

the activation of MAP kinase and other signal transduction pathways through the T cell receptor are critical to cell fate. Elevation of intracellular calcium levels in the absence of the full array of signaling pathways necessary for T cell activation results in the induction of an anergic state (375). Anergy is defined as a state characterized by the inability of the T cells to become activated, regardless of antigen presentation and costimulation (407). Conversely, T cell activation also cannot occur in the absence of a sustained rise in intracellular calcium (408-410). Because it has been demonstrated that both 2-AG and AEA produce calcium influx in a variety of different cell types, including leukocytes, the effect of 2-AG and AEA upon intracellular calcium levels in splenocytes was investigated (23, 25, 27). Unlike other cannabinoids, neither 2-AG nor AEA induce a rise in intracellular calcium in splenocytes.

Previously published studies from this laboratory have determined that suppression of IL-2 transcription by 2-AG is the result of the inhibition of NFAT binding to the IL-2 promoter (303). Additionally, it has been demonstrated that 2-AG is able to inhibit certain ion channels, such as voltage-gated calcium channels (249). Consequently, the effects of 2-AG upon intracellular calcium were investigated in activated splenocytes. 2-AG had little effect upon calcium influx mediated by ionomycin. Because ionomycin induces elevated calcium levels through a variety of different mechanisms, including disruption of the plasma membrane, the effects of 2-AG upon concanavalin A-mediated calcium rise were also studied. The intracellular calcium rise produced by concanavalin A is modest, but adequate for full activation of T cells. While it may appear that 2-AG has a modest suppressive effect upon the initial phase of calcium influx induced by concanavalin A, this observation was not consistent in

subsequent experiments. Collectively, this suggests 2-AG does not inhibit calcium rise produced by either ionomycin or concanavalin A and that 2-AG-mediated inhibition of NFAT occurs downstream of the initial calcium influx produced from T cell activation.

V. Role of hydrolysis in the suppression of IL-2 by AEA and 2-AG

Because arachidonic acid inhibits IL-2 production with comparable potency to that of AEA and 2-AG, the role of hydrolysis in the suppression of IL-2 by 2-AG and AEA was investigated. The hydrolysis of AEA is believed to occur through the combined actions of the putative AMT and FAAH, therefore the individual roles of both were considered (143, 152, 172). Likewise, it has also been demonstrated that the AMT may also play a role in the transport of 2-AG across the membrane (244). The inability of inhibitors of the putative AMT to affect the suppression of IL-2 by AEA or 2-AG suggests that the AMT represents neither a mode of activation nor inactivation in this particular system. Likewise, 2-AG/AEA-mediated suppression of IL-2 is unaffected by pretreatment with MAFP, an inhibitor of all enzymes identified thus far which hydrolyze AEA and 2-AG. Furthermore, 2-AG ether, the non-hydrolyzable analogue of 2-AG, produces a concentration-dependent suppression of IL-2, which is similar to that observed with 2-AG. Moreover, studies from this laboratory have also demonstrated that fluoro-methanandamide, an analogue of AEA that is resistant to hydrolysis, also results in decreased IL-2 secretion similar to that observed with AEA (411). Collectively, these results suggest that hydrolysis is not necessary for the suppression of IL-2 by 2-AG and AEA.

There have been a number of reports that AEA mediates arachidonic acid release from various cell types, including human peripheral blood mononuclear cells (296, 412). Therefore, an alternative explanation for the observations of the current investigation is that AEA causes release of arachidonic acid from primary splenocytes. The released arachidonic acid is then subsequently metabolized into an eicosanoid, which mediates the inhibition of IL-2 secretion. While the current studies do not negate the possibility that AEA causes arachidonic acid release, the striking similarity in the concentration responses of AEA and arachidonic acid in addition to their IC_{50} values (11.4 μM and 10.3 μM, respectively) suggests that the same moiety is responsible for the activity of both.

VI. Role of COX in the suppression of IL-2 by AEA and 2-AG

While the vast majority of research on AEA and 2-AG metabolism has focused upon hydrolysis, there has also been considerable interest in the role of the COX enzymes. There are two isoforms of the COX enzyme, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types, whereas COX-2 expression is induced in response to a stimulus in certain cell types (336). Both COX enzymes have been found in most cell types within the immune system, including T cells (334). It has been reported that AEA and 2-AG can bind directly to COX-2 and be metabolized into PGE_2-glyceryl ester or PGE_2-ethanolamine as well as a wide array of other COX products (176, 177, 245). The same studies have also demonstrated, however, that AEA and 2-AG neither bind directly to, nor are oxygenated by, COX-1.

The attenuation of AEA-mediated IL-2 suppression by the nonspecific COX inhibitors, flurbiprofen and piroxicam, implicates COX metabolism in the observed

effects of AEA upon IL-2. Unlike the COX-1-specific inhibitors, SC560 and FR122047, the COX-2-specific inhibitor, NS398, attenuates the inhibitory effects of AEA upon IL-2 secretion in splenocytes. Likewise, flurbiprofen and NS398, but not the COX-1 specific inhibitors, are effective in blocking the suppression of IL-2 by 2-AG in Jurkat T cells. Collectively, this suggests that COX-2 metabolites of 2-AG and AEA rather than the parent molecules themselves, are responsible for the observed effects upon IL-2 production in activated T cells.

Interestingly, the COX-1 selective inhibitor, piroxicam, attenuates the suppression of IL-2 by AEA and arachidonic acid in murine splenocytes, but is not particularly effective in blocking the suppression of IL-2 by 2-AG in Jurkat T cells. This is likely due to species differences in the COX-2 protein as other laboratories have demonstrated that piroxicam is markedly less effective than other COX inhibitors in the inhibition of human COX-2. It is curious, however, that piroxicam is a potent and effective analgesic and anti-inflammatory agent in humans. While the reason for the discrepancy is unknown, it may be that piroxicam also acts at other targets in addition to the COX enzymes.

There is evidence to support that a number of different prostanoids can inhibit IL-2 secretion, including PGE_2, 15-deoxy-$\Delta^{12,14}$ PGJ_2, and PGI_2 (338-340). Of the aforementioned eicosanoids, 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2) is the most recent to be recognized to suppress IL-2 secretion. There has been growing interest in 15d-PGJ_2 in a number of different research areas due to its identification as one of the most potent endogenous ligands of PPARγ (341, 413). While the lack of IL-2 suppression by PGE_2-ethanolamine eliminates it as the AEA metabolite responsible for the effects upon IL-2 secretion, the potent immunosuppressive effects of 15d- PGJ_2 and PGJ_2 upon IL-2 suggest

a possible role for the ethanolamine and glycerol ester analogues of the J-series of prostaglandins in the suppression of IL-2.

15d-PGJ_2 is produced through the sequential metabolism of arachidonic acid by COX and PGD synthase, followed by a series of nonenzymatic transformations (414). Whether 15d-PGJ_2 is produced in vivo has been somewhat of a controversy, however recent studies have identified elevated levels of 15d-PGJ_2 in LPS-stimulated RAW264.7 macrophages (415). Future studies will determine whether 15d-PGJ_2 is produced in other cell types, but it is notable that both enzymes required for 15d-PGJ_2 formation, COX and PGD synthase, are expressed in T cells (334). While 15d-PGJ_2 is one of the most potent activators of PPARγ identified thus far, other COX products are also able to activate PPARγ (341). More studies will be needed to identify the products resulting from the COX-2 metabolism of AEA and 2-AG, which are responsible for the observed decrease in IL-2 production.

While in the majority of cases studied thus far the metabolism of AEA and 2-AG has been associated with the cessation of their physiological activity, the present studies are significant because they show that metabolism of AEA and 2-AG in certain cases may yield biologically active products. It has been demonstrated here that the metabolism of AEA and 2-AG by COX-2 yields products which suppress IL-2 secretion.

VII. Role of PPARγ in the inhibition of IL-2 secretion by 2-AG, 2-AG ether, and AEA

Structurally related to the hormone receptors, PPARs are also members of the nuclear receptor superfamily. Currently, there are three subtypes of PPARs, which have

been identified: PPARα, PPARδ, and PPARγ (413, 416-418). While the function of PPARδ remains unclear, PPARα and PPARγ have been best characterized for their roles in lipid metabolism and have only recently been discovered to be involved with immune regulation as well (419). Although both PPARα and PPARγ are expressed in T cells, only activated PPARγ has been shown to suppress IL-2 secretion (340, 419). Physical association of activated PPARγ with the transcription factor, NFAT, results in transrepression of NFAT binding to the IL-2 promoter, which is thought to be the mechanism for the decrease in IL-2 production by PPARγ agonists (340). Similarly, activated PPARγ has also been shown to cause a decrease in cytokine production in activated macrophages and T cells through direct protein-protein interaction with NFκB, a key transcription factor for a number of different cytokines (344, 345, 362).

Because 2-AG closely resembles the structures of known PPARγ agonists and both 2-AG and activated PPARγ suppress IL-2 secretion through inhibition of NFAT, the role of PPARγ in 2-AG-mediated IL-2 suppression was investigated. The initial studies in this line of investigation were designed to determine whether 2-AG and 2-AG ether activate PPARγ. The induction of adipogenesis and aP2 mRNA transcription suggested that indeed 2-AG/2-AG ether treatment results in PPARγ activation. In order to determine more conclusively that 2-AG and/or 2-AG ether treatment activates PPARγ specifically, subsequent experiments employing a PPARγ trans-acting reporter gene construct were performed. The plasmid construct contains sequence for two components: a fusion protein containing the ligand binding domain (LBD) of PPARγ fused to the DNA binding domain (DBD) of Gal4, as well as the luciferase reporter, which is activated by the binding of the fusion protein to Gal4 response elements. Activation of

the PPARγ-LBD/Gal4-DBD luciferase plasmid demonstrates that treatment of 3T3-L1 cells with 2-AG and/or 2-AG ether results in PPARγ activation.

While 2-AG and 2-AG ether suppress IL-2 in primary splenocytes in the low micromolar range, both compounds appear to be less potent in the 3T3-L1 model. This is likely due to several factors including, the efficient processing of fatty acids in the 3T3-L1 fibroblasts, which is well established, and the labile properties of 2-AG in long-term culture assays (420). Additionally, 2-AG ether is more potent than 2-AG in assays of longer duration, including aP2 induction and adipogenesis, which may be due to its resistance to hydrolysis.

Although the induction of PPARγ-LBD/Gal4-DBD luciferase activity by 2-AG and 2-AG ether suggests that PPARγ is activated, the role of PPARγ in the suppression of IL-2 by 2-AG and 2-AG ether was still unknown. While studies from this laboratory and others have demonstrated that PPARγ agonists, such as ciglitazone and 15d-PGJ_2, suppress IL-2 secretion, the PPARγ antagonist, T0070907, was used to establish causality between activation of PPARγ by 2-AG treatment and suppression of IL-2. Although the PPARγ antagonist, T0070907, attenuates suppression of IL-2 by 2-AG and AEA in primary splenocytes in a concentration-dependent manner, T0070907 was unable to completely abrogate the effects of 2-AG and AEA in this model. The suppression of IL-2 by T0070907 in the absence of 2-AG or AEA is likely the reason why it does not completely block the effects of 2-AG and AEA in primary splenocytes. Unlike murine splenocytes, Jurkat T cells are more refractory to the suppressive effects of T0070907 upon IL-2 secretion. As such, T0070907 pretreatment results in full reversal of the IL-2

suppression by 2-AG and 2-AG ether in Jurkat cells. Collectively, these results suggest a role for PPARγ in the suppression of IL-2 production by 2-AG, 2-AG ether, and AEA.

While PPARγ-specific luciferase induction by ciglitazone, 2-AG, and 2-AG ether may not appear to correlate with the magnitude of IL-2 suppression observed with the same compounds, this is likely due to major differences between the two assays. The inhibition of IL-2 secretion by PPARγ agonists is dependent upon endogenous PPARγ and RXR levels, whereas luciferase induction is dependent upon the expression of the PPARγ/Gal fusion protein from the exogenous plasmid in the transfected cells. Furthermore, endogenous PPARγ competes with the PPARγ/Gal fusion protein for agonists in the luciferase assay. Additionally, other laboratories have shown a comparable level of luciferase activity with a similar type of plasmid (trans-acting PPARγ plasmid) in NIH3T3 cells (247).

Although several studies have emerged which suggest that under certain conditions, activated PPARγ may cause apoptosis in activated T cells, there are also a number of studies suggesting that PPARγ activation may be protective against apoptosis or have no effect upon cell viability (354, 356, 357, 359, 419). While the cause of the differential effects of activated PPARγ upon T cell viability is unclear, it is likely that the concentration of 15d-PGJ_2 or other agonists, the kinetics of cell treatment, and/or the level/mode of activation may be contributing factors. Under the specific conditions used in the present studies, there was no detectable effect upon cell viability. In addition, it is noteworthy that AEA and 2-AG may be metabolized into a number of different products, some of which may be protective against apoptosis.

Early studies examining the role of PPARγ in immune cells indicated that PPARγ agonists cause a variety of immune effects, which tend to be suppressive in nature (419). The initial research on immune effects by PPARγ was generally limited to the use of PPARγ agonists due to the lack of potent PPARγ-specific antagonists at that time as well as to the lack of PPARγ knock-out mice, which were found to be embryonic lethal. The interpretation of the aforementioned early studies has become controversial due to a recent report showing that rosiglitazone and 15-deoxy PGJ_2 have anti-inflammatory effects in macrophages which lack PPARγ (346). As a result, the role of PPARγ in many of the observed inhibitory immune effects by PPARγ agonists has been called into question and may need to be confirmed. As such, the current studies are important because the use of a reporter construct and an antagonist which are both highly specific for PPARγ, provide evidence that the observed effects of 2-AG and 2-AG ether are in fact mediated by PPARγ.

While initial studies of PPARγ centered around lipid metabolism and glucose homeostasis, the breadth of knowledge concerning the role of PPARγ in immune regulation is rapidly increasing. Recently it has been reported that activation of PPARγ can ameliorate autoimmune disease in a number of different animal models, including allergic asthma, experimental crescentic glomerulonephritis, as well as experimental allergic encephalitis, an animal model of multiple sclerosis (363, 364, 367). Furthermore, PPARγ activation causes anti-inflammatory and anti-proliferative effects in T cells derived from human multiple sclerosis patients (371). Similarly, the modulation of a variety of immune responses by 2-AG and AEA, including IL-2 secretion, suggests that 2-AG and AEA may also play an important role in immune regulation. Further evidence

for the regulatory role of 2-AG and AEA in the immune system comes from a number of published studies showing that 2-AG and AEA levels are markedly increased upon the activation of various immune cell types (234-236). Additionally, 2-AG and AEA levels are much higher in the sera of patients suffering from LPS-induced shock as compared to healthy control subjects suggesting that 2-AG and AEA may also play a regulatory role in sepsis (164). Collectively, this suggests that PPARγ may play an important role in the control of exaggerated or inappropriate immune responses, and that activation of PPARγ by 2-AG and AEA may be contribute to the maintenance of immune homeostasis.

VIII. Role of NFAT, NFκB, and AP-1 in the suppression of IL-2 secretion by 2-AG

Although PPARγ is best known to regulate gene expression through binding to PPAR response elements (PPREs) as a heterodimer with the RXR, a number of alternative modes of gene regulation by PPARγ have been described (342). Like other nuclear receptors, activated PPARγ has been shown to coassociate physically with transcription factors and thus to sequester these transcription factors from binding to their response elements in the regulatory regions of target genes. In macrophages, activated PPARγ has been shown to cause a decrease in AP-1, STAT-1, and NFκB DNA-binding, which ultimately results in a repression of gene induction (344). Moreover, two separate laboratories have shown that activated PPARγ physically associates with NFAT and NFκB, which ultimately results in the suppression of IL-2 and IL-4 transcription (340, 345, 362).

Previously published studies from this laboratory have shown that suppression of IL-2 by 2-AG occurs at the transcriptional level through inhibition of NFAT, an essential

transcription factor for IL-2 mRNA production (303). The aforementioned studies showed that 2-AG causes suppression of NFAT DNA-binding and transcriptional activity in splenocytes and EL4 cells. Additionally, NFκB DNA-binding and transcriptional activity were also decreased by 2-AG treatment, albeit to a lesser extent than that of NFAT. The present studies demonstrate that 2-AG produces marked inhibition of NFAT and NFκB, but only modest inhibition of AP-1, in Jurkat T cells. Moreover, the PPARγ antagonist, T0070907, blocks the inhibition of NFAT and NFκB by 2-AG. Collectively, this suggests that the suppression of NFAT and NFκB activation by 2-AG is mediated through activation of PPARγ.

IX. Summary

The studies reported in this dissertation demonstrate that 2-AG, 2-AG ether, and AEA suppress IL-2 secretion in both activated murine splenocytes and human Jurkat T cells. The decrease in IL-2 production by 2-AG and AEA in splenocytes derived from CB1/CB2 null mice coupled with the failure of the CB1/CB2 antagonists to block inhibition of IL-2 secretion by 2-AG and AEA demonstrates that the cannabinoid receptors are not involved. Likewise, 2-AG ether also suppresses IL-2 secretion independent of CB1/CB2, as determined by splenocytes derived from CB1/CB2 null mice. With the elimination of the cannabinoid receptors as the mediators of the suppression of IL-2 by 2-AG, 2-AG ether, and AEA, other targets were considered. The inability of capsazepine to block AEA-mediated IL-2 suppression coupled with the lack of effect by capsaicin upon IL-2 secretion, suggests that VR1 is not involved. Additionally, the inability of 2-AG to modulate calcium influx in resting or activated

splenocytes, suggests that 2-AG does not disrupt the calcium signal initiated upon T cell activation. Similarly, AEA does not induce calcium influx in resting splenocytes.

Because arachidonic acid inhibits IL-2 production with comparable potency to that of AEA and 2-AG, the role of hydrolysis in the suppression of IL-2 by 2-AG and AEA was investigated. Neither inhibitors of the putative AMT nor MAFP blocked suppression of IL-2 by AEA and 2-AG, suggesting that the effects of 2-AG and AEA are not dependent upon uptake and hydrolysis. Conversely, flurbiprofen pretreatment significantly attenuated suppression of IL-2 by AEA and 2-AG, suggesting a role for COX metabolism. Unlike the COX-1 specific inhibitors, SC560 and FR122047, the COX-2 specific inhibitor, NS398, attenuated AEA/2-AG-mediated IL-2 suppression, which implicates COX-2 rather than COX-1 in the metabolism of AEA and 2-AG.

Because a number of COX metabolites are known agonists of PPARγ and it has been demonstrated that activated PPARγ suppresses IL-2 secretion, the role of PPARγ in the suppression of IL-2 by 2-AG and AEA was investigated. Initially it was determined that treatment of 3T3-L1 cells with 2-AG or 2-AG ether results in PPARγ activation as evidenced by induction of adipogenesis, increased aP2 transcription, and induction of PPARγ-LBD/Gal4-DBD luciferase transcriptional activity. Furthermore, the PPARγ antagonist, T0070907, attenuated suppression of IL-2 by 2-AG, 2-AG ether, and AEA. Moreover, T0070907 also blocked inhibition of NFAT and NFκB transcriptional activity by 2-AG. Collectively, the aforementioned observations show that suppression of IL-2 by 2-AG and AEA occurs independently of CB1/CB2, VR1 as well as the putative AMT and hydrolytic enzymes. Moreover, our findings suggest that suppression of IL-2 by

2-AG and AEA is mediated by a COX-2 metabolite, which activates PPARγ resulting in the transrepression of the transcription factors, NFAT and NFκB (Figure 55).

The findings of this dissertation are relevant in that they challenge the pervasive assumption that the immune effects of 2-AG and AEA are mediated through activation of CB2, and to a lesser extent, CB1. While the cannabinoid receptors may mediate some of the immune effects produced by 2-AG and AEA, the present studies demonstrate that other mechanisms are involved in the suppression of IL-2. Additionally, the present studies are also significant in that they demonstrate that the metabolism of 2-AG and AEA does not always result in the cessation of physiological activity, but in certain circumstances may be responsible for the observed biological effects. Furthermore, the current studies are unique in that they suggest a novel mechanism of action in which metabolites of 2-AG/AEA activate PPARγ resulting in the inhibition of NFAT and NFκB and consequently the suppression of IL-2 production. In addition to IL-2 suppression, activation of PPARγ by 2-AG/AEA metabolites may also play a role in other observed physiological effects of 2-AG and AEA that are independent of CB1/CB2. Moreover, it has been demonstrated that the levels of 2-AG and AEA are elevated in a number of different immune cell types upon activation and that both PPARγ and 2-AG/AEA ameliorate the symptoms of a variety of different animal models of autoimmune diseases. Collectively, this suggests that activation of PPARγ by 2-AG and AEA may play an important role in the control of exaggerated or inappropriate immune responses, thereby maintaining immune homeostasis.

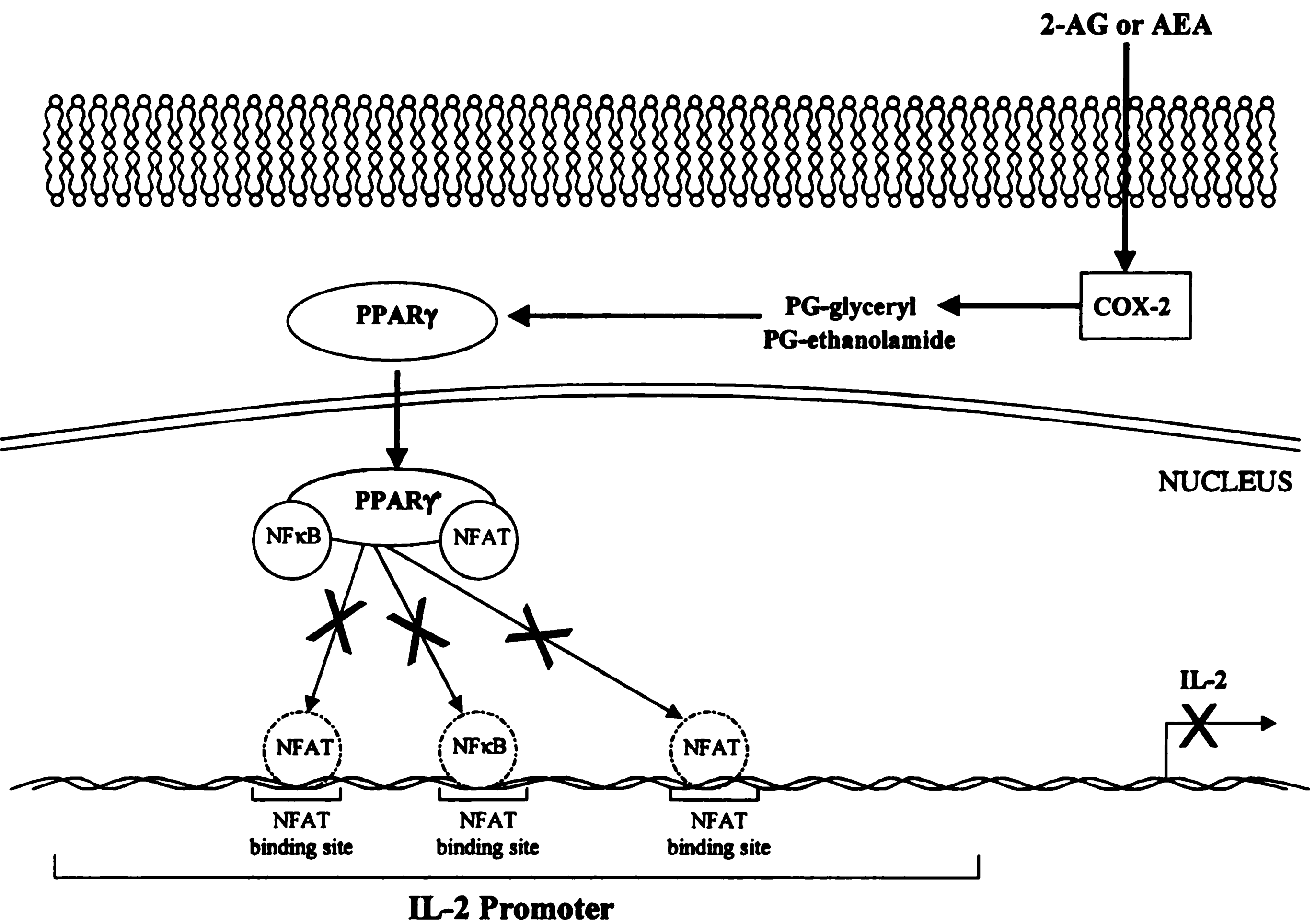


Figure 55. Schematic representation of suppression of IL-2 by 2-AG and AEA in activated T cells.

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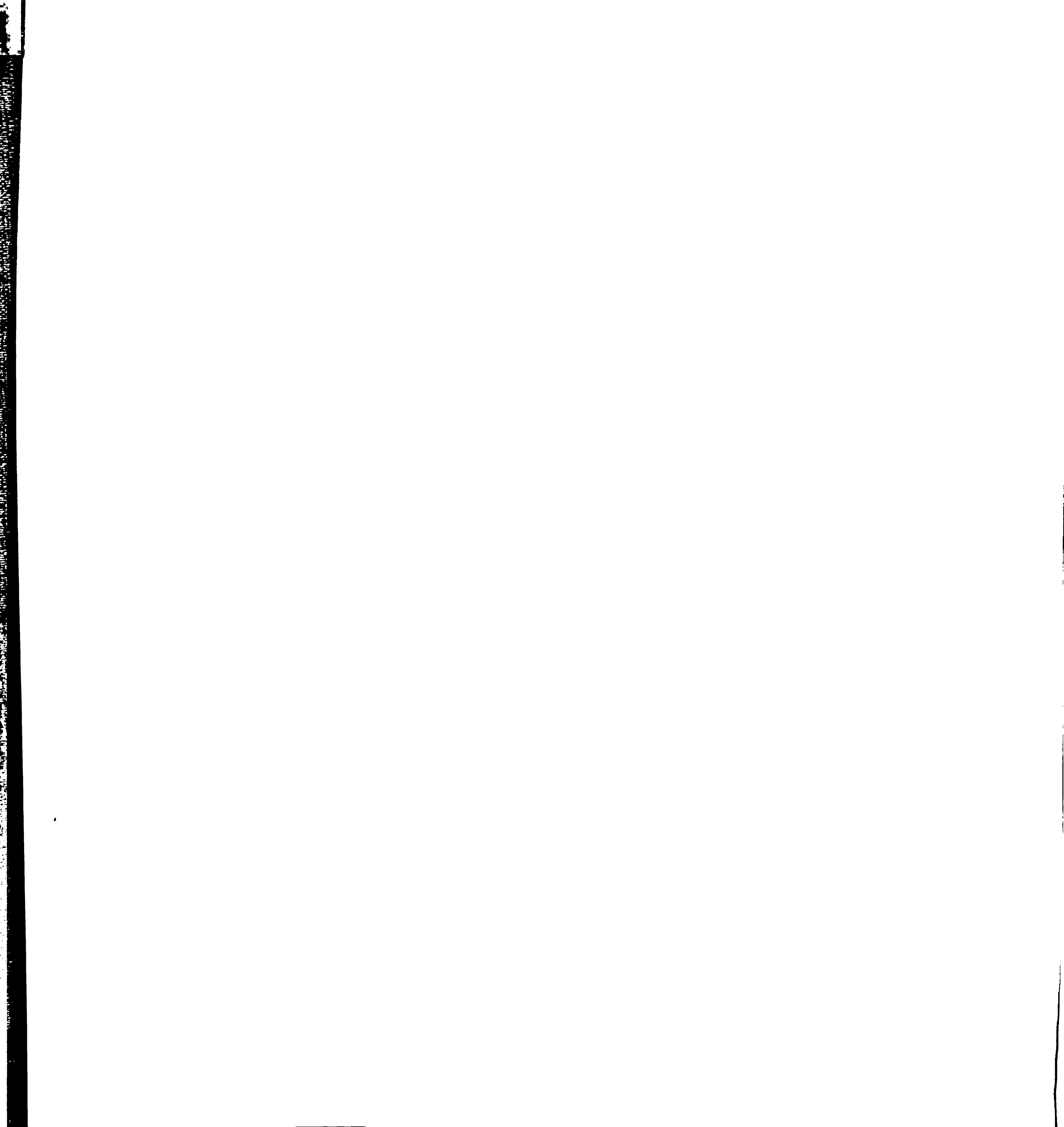

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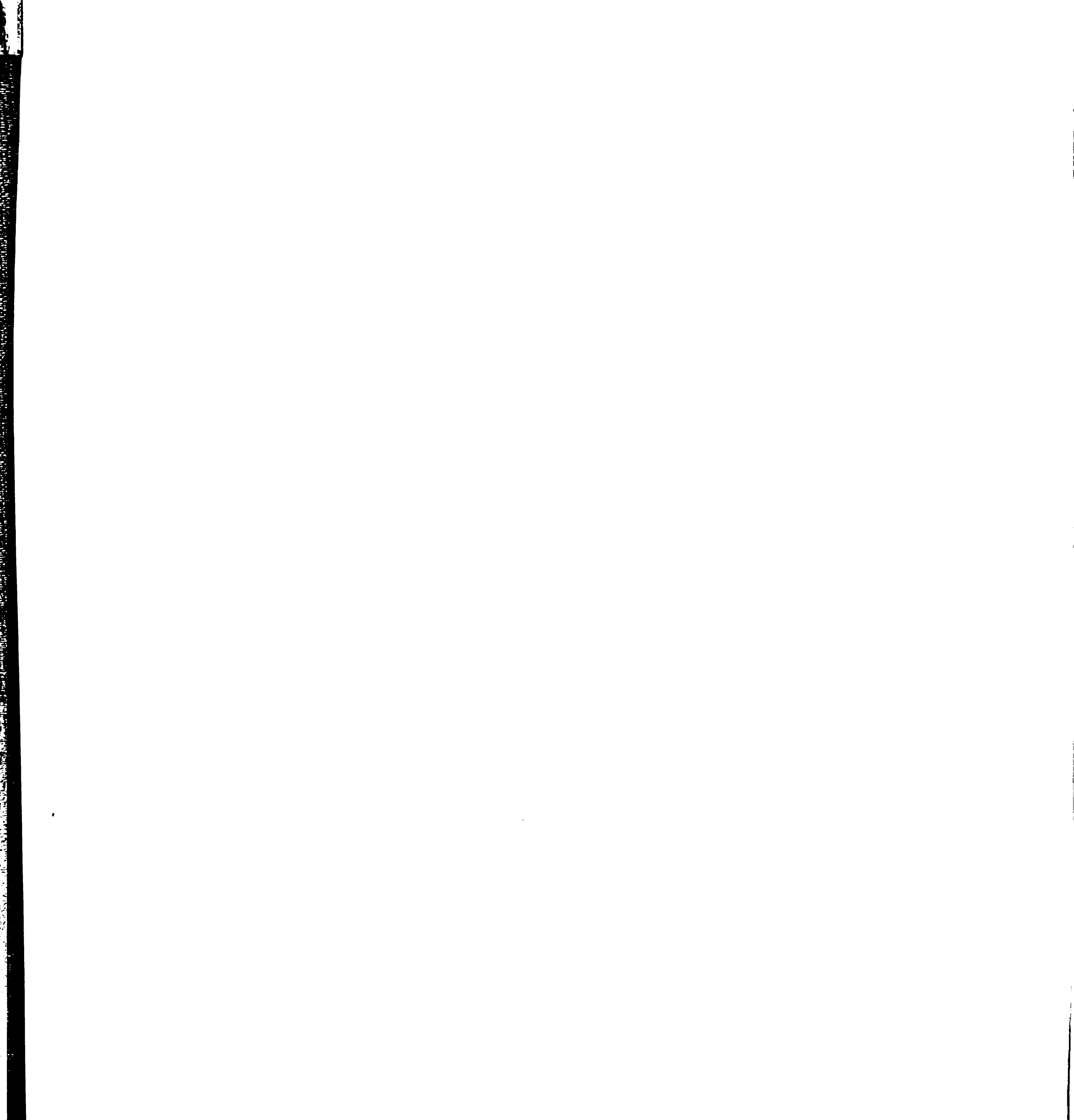

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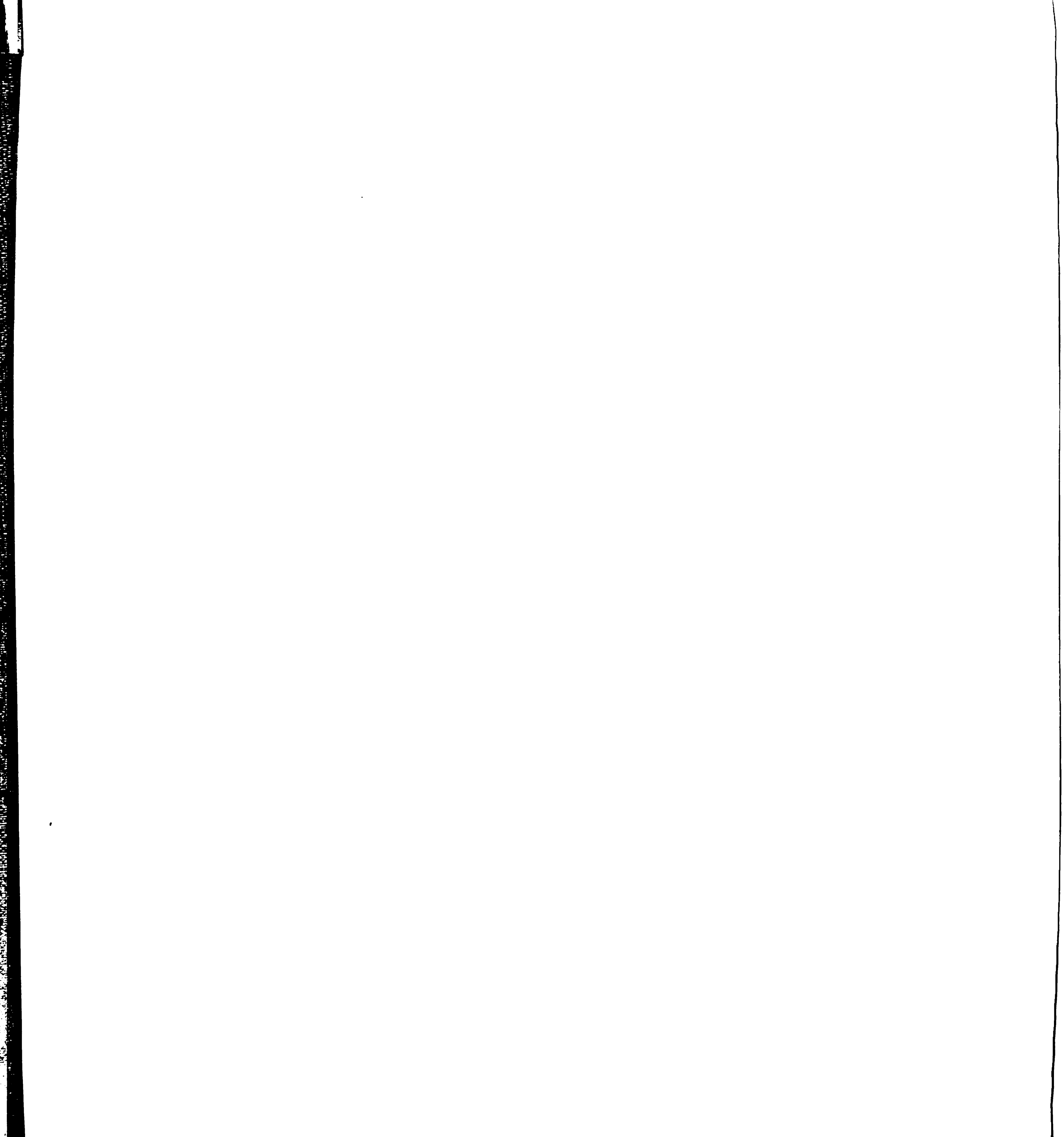

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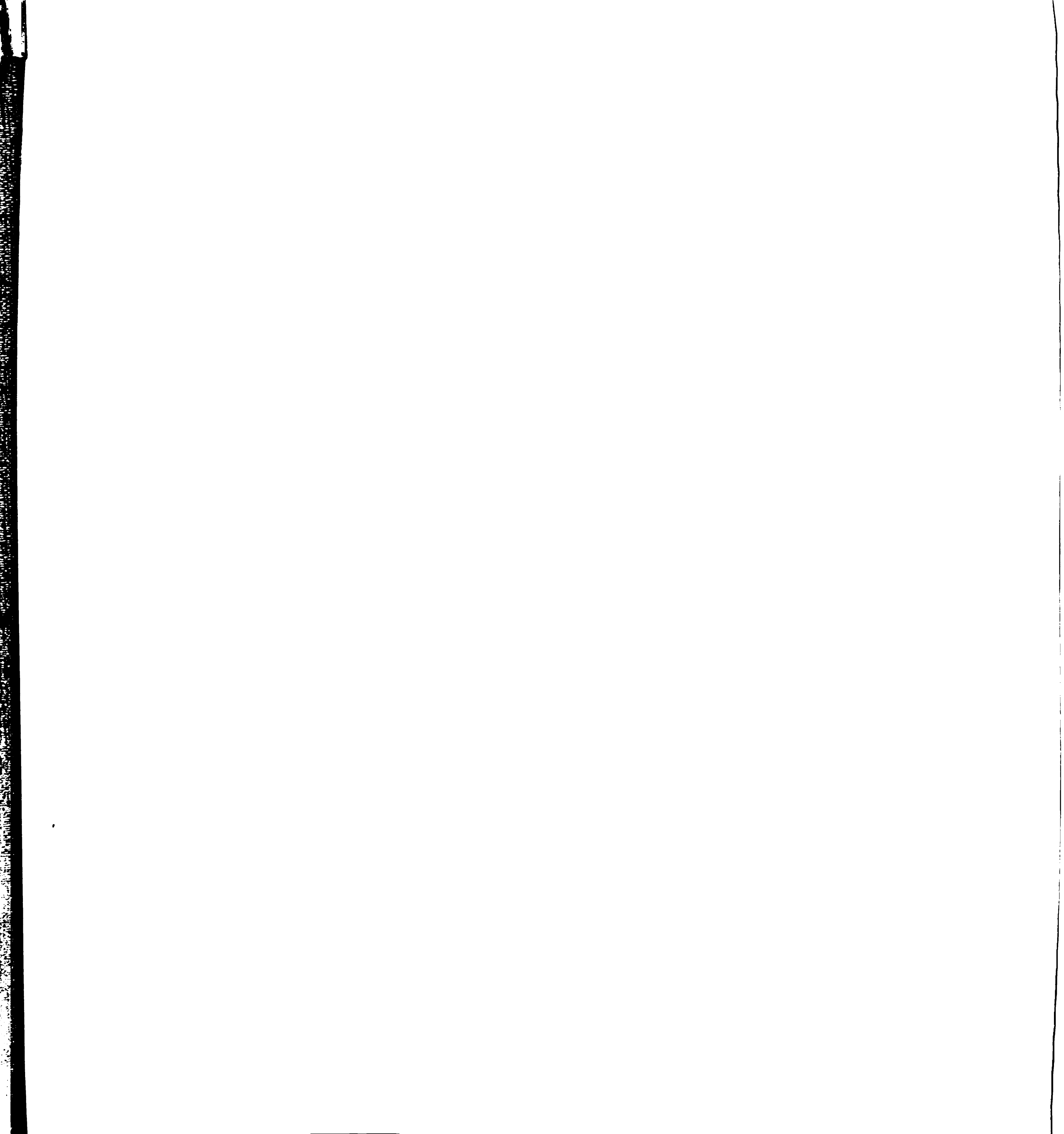

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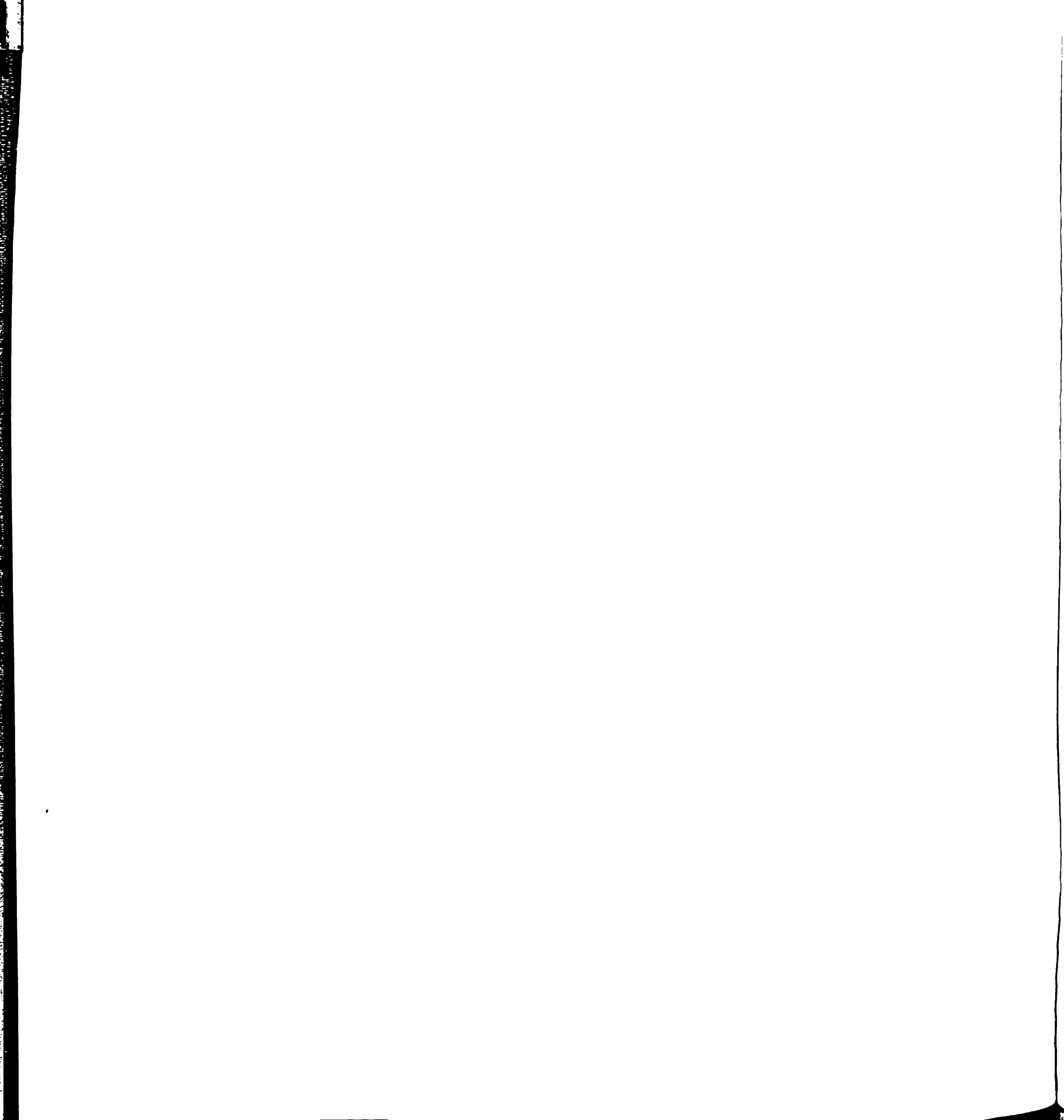

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