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Suppression of Interleukin-2 Expression by Arachidonylethanolamide is Mediated by Down-regulation of NF-AT

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Abstract

Several plant-derived cannabinoids and endogenous ligands for cannabinoid receptors such as 2-arachidonyl-glycerol have been known to inhibit interleukin-2 (IL-2) expression. In the present study, we utilized arachidonylethanolamide (AEA), a putative endogenous ligand for cannabinoid receptors, to determine whether AEA modulated the expression of IL-2. AEA inhibited phorbol 12-myristate 13-acetate (PMA) plus ionomycin (Io)-induced IL-2 protein secretion and mRNA expression in EL-4 mouse T-cells as determined by ELISA and RT-PCR, respectively. To further characterize the inhibitory mechanism of AEA at the transcriptional level, we performed promoter study for IL-2 gene in PMA/Io-stimulated EL-4 cells. AEA decreased the transcriptional activity of the nuclear factor of activated T-cells (NF-AT) as well as the IL-2 promoter activity. These results suggest that AEA suppresses IL-2 expression and that the inhibition is mediated, at least in part, through the down-regulation of NF-AT.

Keywords: Arachidonylethanolamide, Interleukin-2, Nuclear factor of activated T-cells

Cannabinoids, the active components of *Cannabis sativa* and their derivatives, are well known for their psychoactive and immunomodulatory properties. The identification of two distinct cannabinoid receptors, brain-type CB1 and peripheral-type CB2, has provided important insights for the mechanism of action for cannabinoids[1,2]. Both receptors are negatively coupled to adenylate cyclase via a G_i protein[3], and cannabinoids inhibit the cAMP signaling cascade[4]. With the discovery of cannabinoid receptors, researches on the identification of their endogenous ligands (endocannabinoids) were initiated. The first putative endogenous ligand discovered was arachidonylethanolamide (AEA; termed anandamide)[5], which is a natural eicosanoid exhibiting binding affinity for both CB1 and CB2. Like classical cannabinoids, AEA mediates brain neuromodulation and controlling processes such as peripheral pain, vascular tone, and intraocular pressure[6]. In addition, AEA has been shown to modulate several immune responses such as suppression of general resistance to bacterial or viral infection[7,8], macrophage activity[9,10], antibody production, and lymphocyte proliferation[11]. More recently, studies aimed at elucidating the mechanism responsible for immunosuppressive activity of cannabinoids have focused on changes in cytokine production and on the intracellular events responsible for these changes.

Interleukin-2 (IL-2) is a representative cytokine produced by activated T lymphocytes and plays a pivotal role in initiating and potentiating a variety of immune responses including T-cell proliferation and the regulation of other immune cells such as B-cells, macrophages, and natural killer cells[12,13]. The expression of the IL-2 gene is transiently induced by the combination of immune stimuli provided by the interaction of the TCR/CD3 complex with the antigen/MHC complex, together with accessory signals from the antigen-presenting cell. The physiological stimulating signals can be mimicked by T-cell-specific mitogens, including phytohemagglutinin and concanavalin A, or by the simultaneous presence of phorbol esters and calcium ionophores[14,15]. Although the IL-2 gene is highly regulated and exhibits virtually no basal level of expression in resting cells, it is rapidly but transiently induced upon T-cell activation. Expression of IL-2 gene is controlled primarily at the transcription level via a 5′ promoter region extending ~326 bp upstream of the transcription start site[16]. The IL-2 promoter consists of several functional *cis*-acting elements, including binding sites for the nuclear factor of activated T cells (NF-AT), activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and Oct[16,17].

In light of the fact that several plant-derived can-

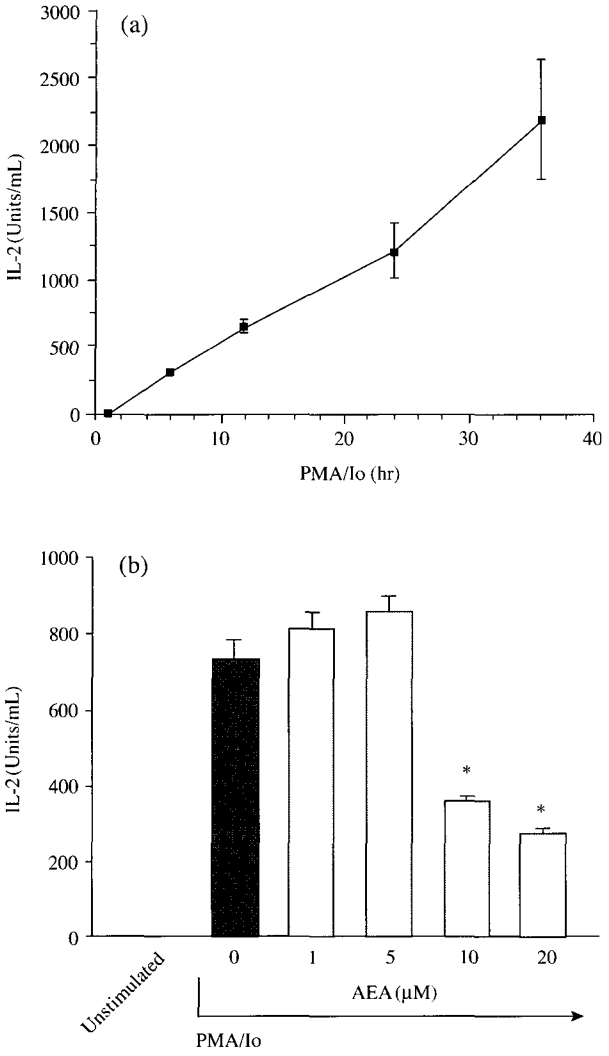

Fig. 1. Inhibition of IL-2 secretion by AEA in PMA/Io-induced EL-4 cells. (a) EL-4 cells (5×10^5 cells/mL) were stimulated with PMA/Io (80 nM/1 μM) for various lengths of time. (b) EL-4 cells (5×10^5 cells/mL) were pretreated with different concentrations of AEA for 30 min and then stimulated with PMA/Io (80 nM/1 μM) for 24 h. The supernatants were collected, and IL-2 secretion was determined by ELISA. The data are expressed as the mean ± S.D. of triplicate cultures. An asterisk denotes any response that is significantly different from the control group as determined by Dunnett's two-tailed *t*-test ($P < 0.05$). The results are representative of two separate experiments.

nabinoids such as Δ^9-tetrahydrocannabinol (THC) and endocannabinoids such as 2-arachidonyl-glycerol (2-Ara-Gl) exhibit the ability to inhibit IL-2 expression[18-21], in the present study we investigate the effect of AEA on the regulation of this cytokine in the murine thymoma EL-4.

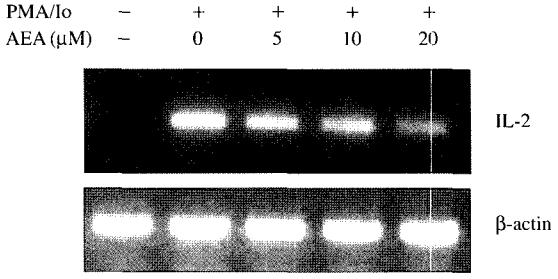

Fig. 2. Inhibition of IL-2 mRNA expression by AEA in PMA/Io-induced EL-4 cells. EL-4 cells (5×10^5 cells/mL) were pretreated with AEA for 30 min and then stimulated with PMA/Io (80 nM/1 μM) for 6 h. Total RNA was isolated, and IL-2 mRNA expression was determined by RT-PCR. PCR products were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining. As an internal control, β-actin was amplified. The results are representative of two separate experiments.

Effect of AEA on IL-2 Protein Secretion and mRNA Expression

Initially, the effect of AEA on IL-2 protein production was determined in EL-4 cells, a CB2-expressing murine thymoma widely used in studies of IL-2 regulation and expression. EL-4 cells were activated with phorbol 12-myristate 13-acetate (PMA, 80 nM) plus ionomycin (Io, 1 μM) in the presence or absence of AEA, and supernatants were measured for IL-2 production at 24 h by enzyme-linked immunosorbent assay (ELISA). Naive EL-4 cells exhibited an undetectable amount of IL-2 production, but treatment with PMA/Io caused a robust increase in the IL-2 secretion (Fig. 1A). AEA treatment suppressed the PMA/Io-induced IL-2 secretion in a concentration-dependent manner (Fig. 1B). No effect on cell viability was observed at any concentration of AEA tested (data not shown). The effect of AEA on IL-2 mRNA expression was also evaluated using RT-PCR. Given that steady-state IL-2 mRNA expression reaches a peak at 4 to 8 h after T-cell activation and returns to background levels by 24 h[16,17], EL-4 cells were harvested and mRNA was isolated at 6 h after stimulation with PMA/Io. Unstimulated EL-4 cells expressed an undetectable amount of IL-2 mRNA, but treatment with PMA/Io caused a robust increase in the IL-2 mRNA expression, which was inhibited by AEA in a dose-dependent manner (Fig. 2).

Effect of AEA on Promoter Activity of IL-2 and NF-AT

IL-2 expression is regulated mainly at the transcriptional level. To examine whether the decrease in IL-2 mRNA expression and IL-2 secretion by AEA in activated EL-4 cells was due to the inhibition of the

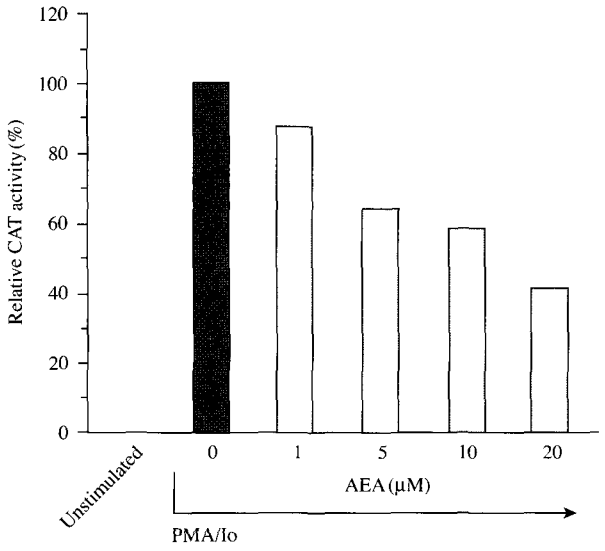

Fig. 3. Effect of AEA on the transcriptional activity of the IL-2 promoter. EL-4 cells (5×10^5 cells/mL) were transiently transfected with a reporter plasmid, pIL-2-CAT (−578 to +50). The transfected cells were pretreated with vehicle (0.1 % ethanol) or AEA for 1 h and then stimulated with PMA/Io (80 nM/1 μM) for 18 h at 37°C. In addition, the basal level of IL-2 promoter activity was measured in unstimulated cells. CAT activity was measured using autoradiography and liquid scintillation counting. The CAT activity in the vehicle control was arbitrarily assigned a value of 100%. All other groups were compared with the control. The result is representative of two independent experiments.

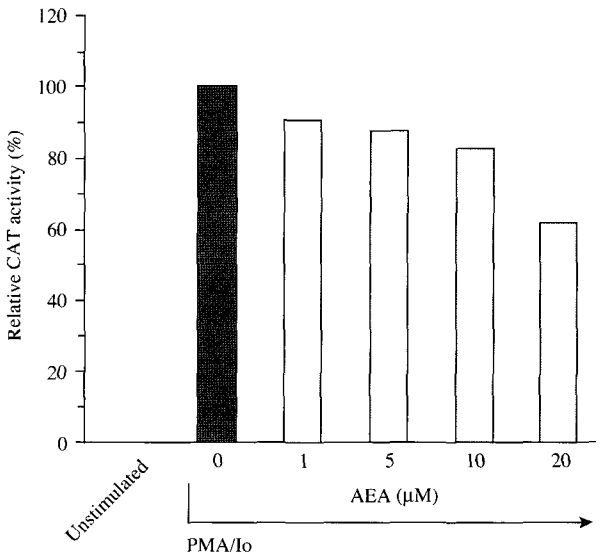

Fig. 4. Effect of AEA on p $(NF\text{-}AT)_3$-CAT promoter activity. EL-4 cells (5×10^5 cells/mL) were transiently transfected with a reporter plasmid, p $(NF\text{-}AT)_3$-CAT. The transfected cells were pretreated with vehicle (0.1% ethanol) or AEA for 1 h and then stimulated with PMA/Io (80 nM/1 μM) for 18 h at 37°C. In addition, the basal level of NF-AT activity was measured in unstimulated cells. CAT activity was measured using autoradiography and liquid scintillation counting. The CAT activity in the vehicle control was arbitrarily assigned a value of 100%. All other groups were compared with the control. The result is representative of two independent experiments.

IL-2 gene transcription, the 5′ regulatory region (from +50 to −578 bp) of the IL-2 gene linked to the CAT reporter gene, p (IL-2)-CAT, was used. Specifically, the effect of AEA on CAT expression was measured in PMA/Io-activated EL-4 cells that had been transiently transfected with p (IL-2)-CAT. As shown in Fig. 3, AEA inhibited PMA/Io-induced CAT activity in a concentration-dependent manner. The magnitude of inhibition by AEA closely correlated with observed inhibition in IL-2 mRNA expression. Transcription factor NF-AT has been widely established to be responsible for the regulation of IL-2 transcription[16,17]. To characterize further the mechanism by which AEA inhibits IL-2 promoter activity, the effect of AEA was evaluated on promoter activity using the reporter gene construct p $(NFAT)_3$-CAT. Concordant with the result of IL-2 promoter activity, AEA inhibited NF-AT promoter activity in a dose-dependent manner (Fig. 4).

Discussion

In the present study, we demonstrated that AEA, a naturally occurring eicosanoid and putative endogenous ligand for cannabinoid receptors, inhibited IL-2 expression in EL-4 T-cells and the inhibition of IL-2 expression by AEA is mediated through direct inhibition of IL-2 gene transcription. The suppression of IL -2 secretion and mRNA expression by AEA occurred over a comparable dose range as demonstrated previously in the lymphocyte model systems with plant-derived or endogenous cannabinoids. Based on the knowledge that IL-2 facilitates T-cell proliferation and helps to regulate the actions of a variety of other cell types[12,13], we believe that the inhibition of IL-2 expression is one of the mechanisms responsible for the immune modulation by AEA. Similar to the previously studies with the cannabinoids, the decrease in IL-2 secretion and mRNA expression by AEA was paralleled by a concomitant decrease in the promoter activity of IL-2 gene. This conclusion is supported by transient transfection studies in which AEA inhibited PMA/Io-induced expression of a CAT reporter plasmid (p (IL-2)-CAT) under the control of the minimal essential region (−578 to +50) of the IL-2 promoter. We also disclose in this report that, along with AEA-

mediated inhibition of IL-2 gene transcription, there is inhibition of the signaling pathways mediating the activation of transcription factor NF-AT, which is known to play a critical role in the immune response. Overall, AEA exhibited potency in inhibiting NF-AT transcriptional activity, promoter activity of IL-2 gene, steady state IL-2 mRNA expression, and IL-2 protein secretion, suggesting that the effects are mechanistically related.

Activation of TCR/CD3 complex in T-cells triggers the signal transduction pathways leading to the immediate activation of transcription factors that regulate a variety of activation-associated genes including IL-2. One of the events after antigenic stimulation is the generation of inositol 1,4,5-triphosphate and diacylglycerol, which mediate mobilization of Ca^{2+} from intracellular stores and activation of PKC family members and the Ras-ERK pathway, respectively[22,23]. It is well established that calcium ionophores and phorbol esters mimic the signaling event induced by antigen interactions with the TCR/CD3 complex[14,15]. As a consequence of an increase of intracellular Ca^{2+} levels, Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, is activated and subsequently dephosphorylates NF-AT, allowing its nuclear shuttling and binding to DNA[24,25]. Our result showing AEA-mediated inhibition of NF-AT in PMA/Io-activated EL-4 cells suggests that AEA modulates the Ca^{2+}-mediated signaling pathways. More recently, cannabinoids have been found to also influence additional signal transduction pathways, which may further contribute to the effects cannabinoids exert on leukocyte function. Therefore, the specific upstream signaling events disrupted by AEA that lead to altered regulation of NF-AT remain to be elucidated. In addition to NF-AT, AP-1 and NF-κB are known to be other transcription factors for IL-2 expression as well as T-cell activation. Activated PKC and Ras-ERK pathways primarily play roles in enabling the activation of AP-1 transcription factor[22,23], and IKK and MAPK pathways play important roles in the activation of NF-κB through degradation of I-κB, inhibitory proteins preventing NF-κB from translocation to the nucleus and binding to DNA[26,27]. Therefore further studies on the signaling of AP-1 and NF-κB are needed to elucidate the mechanism of action by AEA. Nevertheless, the present finding that under the present experimental conditions NF-AT is negatively regulated by AEA is significant because the transcription factor plays a critical role in the regulation of a wide range of cytokines, and this observation may help to explain the diverse effects produced by AEA on immune function.

Methods

Reagents

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. AEA was reconstituted in ethanol, aliquots were taken, and the aliquots were stored at −80°C. Working solutions were prepared freshly prior to AEA addition to cell cultures. Mouse recombinant IL-2 (as a standard), purified rat anti-mouse IL-2 antibody, and biotinylated antimouse IL-2 antibody were purchased from PharMingen (San Diego, CA).

Cell Culture

The C57BL/6 mouse T-cell lymphoma, EL-4, was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and the cells were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 100 units of penicillin/mL, 100 μg of streptomycin/mL, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 5% fetal bovine serum at 37°C in a 5% CO_2-humidified incubator.

IL-2 Protein Quantification

EL-4 cells (5×10^5 cells/mL) were pretreated with vehicle (0.1% ethanol) or AEA for 30 min and then stimulated with PMA/Io (80 nM/1 μM) at 37°C in a 5% CO_2-humidified incubator for 24 h. At the end of incubation, the supernatant was collected and quantified for IL-2 by ELISA as described previously[20].

RNA Preparation and RT-PCR

EL-4 cells (5×10^5 cells/mL) were pretreated with vehicle (0.1% ethanol) or AEA for 30 min and then stimulated with PMA/Io (80 nM/1 μM) at 37°C in a 5% CO_2-humidified incubator for 6 h. At the end of incubation, the cells were harvested, and total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). All isolated RNA samples were confirmed to be free of DNA contamination as determined by the absence of product after PCR amplification in the absence of reverse transcriptase (data not shown). Steady-state mRNA expression was determined by RT-PCR as described previously[19]. Briefly, total RNA was reverse transcribed into cDNA using oligo $(dT)_{15}$ as primers. The following forward and reverse primer sequences were used: for IL-2, 5′-TGCTCCTTGTCAACAGCG-3′ and 5′-TCATCATCGAATTGGCACTC-3′; for β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmole each of IL-2 or β-actin forward and reverse primers, and 2.5 U of *Taq*

DNA polymerase was added to the cDNA samples. Samples were then heated to 94°C for 3 min and cycled 20 times at 94°C for 10 s, 59°C for 30 s, and 72°C for 45 s, after which an additional extension step at 72°C for 5 min was included. PCR products were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining.

Plasmid Construction

Plasmids were constructed as previously described[19]. Briefly, a minimal promoter vector containing no enhancer, pCAT-Promoter, was purchased from Promega (Madison, WI). To construct p $(NF\text{-}AT)_3$-CAT and p $(Oct)_3$-CAT, *Bgl*II-adhering oligonucleotides containing three copies of each consensus recognition motif, either NF-AT or Oct, were synthesized and cloned into the pCAT-Promoter vector, respectively. Cloning was confirmed through a comparison of *Eco*RI-digested fragments from each recombinant plasmid and pCAT-Promoter vector. pIL-2-CAT was kindly provided by Dr. Ellen Rothenberg. The plasmids were then purified with Quiagen Plasmid Kit (Quiagen Inc., Chatsworth, CA) and quantified for transient transfection studies.

Transfection and CAT Assay

EL-4 cells (5×10^5 cells/mL) were transiently transfected with the pIL-2-CAT, p $(NF\text{-}AT)_3$-CAT or p $(Oct)_3$-CAT plasmids using the DEAE-dextran method with slight modifications[19]. After transfection, the cells were plated and incubated for 24 h. The transfectants were pretreated with vehicle (0.1% ethanol) or various concentrations of AEA for 1 h, followed by stimulation with PMA/Io (80 nM/1 μM) for 18 h. Cells were then harvested, washed with PBS, and freeze-thawed three times in 100 mL of 0.25 mM Tris -HCl, pH 7.4, with the use of liquid N_2. The supernatants were isolated, and equal amounts of proteins were incubated in the CAT reaction mixture containing 0.1 μCi of [^{14}C]chloramphenicol, 0.7 mM acetyl-coenzyme A, and 0.14 M Tris-HCl, pH 7.4, at 37°C for 1 h. The degree of acetylation was assessed through the use of thin-layer chromatography, autoradiography, and liquid scintillation counting. The CAT activity was calculated as the ratio of enzyme activity. The CAT activity in the PMA/Io-treated vehicle group was arbitrarily assigned a relative value of 100%.

Statistical Analysis

The mean ± S.D. was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance, and Dunnett's two-tailed *t*-test was used to compare treatment groups to the vehicle control when significant differences were observed[28].

Acknowledgements

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