

Suppression of Interleukin-2 by the Putative Endogenous Cannabinoid 2-Arachidonyl-Glycerol Is Mediated through Down-regulation of the Nuclear Factor of Activated T Cells

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ABSTRACT

2-Arachidonyl-glycerol (2-Ara-Gl) recently was identified as a putative endogenous ligand for cannabinoid receptor types CB1 and CB2 by competitive binding. More recent immune function assays demonstrated that 2-Ara-Gl possessed immunomodulatory activity. Because several plant-derived cannabinoids inhibit interleukin-2 (IL-2) expression, 2-Ara-Gl was investigated for its ability to modulate this cytokine. The direct addition of 2-Ara-Gl to mouse splenocyte cultures suppressed phorbol-12-myristate-13-acetate plus ionomycin-induced IL-2 secretion and steady state mRNA expression in a dose-dependent manner. 2-Ara-Gl also produced a marked inhibition of IL-2 promoter activity as determined by transient transfection of EL4.IL-2 cells with a pIL-2-CAT construct. 2-Ara-Gl at 5, 10, 20, and 50 μM suppressed phorbol-12-myristate-13-acetate plus ionomycin-induced IL-2 promoter activity by 18%, 28%, 39%, and 54%, respectively. To further characterize the mech-

anism for the transcriptional regulation of IL-2 by 2-Ara-Gl, the DNA-binding activity of transcription factors, nuclear factor of activated T cells (NF-AT), nuclear factor for immunoglobulin κ chain in B cells (NF- κB /Rel), activator protein-1 (AP-1), octamer, and cAMP-response element binding protein was evaluated by electrophoretic mobility shift assay in mouse splenocytes. In addition, a reporter gene expression system for p(NF- κB)₃-CAT, p(NF-AT)₃-CAT, and p(AP-1)₃-CAT was used in transiently transfected EL4.IL-2 cells to determine the effect of 2-Ara-Gl on promoter activity for each of the specific transcription factors. 2-Ara-Gl reduced both the NF-AT-binding and promoter activity in a dose-dependent manner and, to a lesser degree, NF- κB /Rel-binding and promoter activity. No significant effect was observed on octamer- and cAMP-response element-binding activity. AP-1 DNA-binding activity was not inhibited by 2-Ara-Gl, but a modest inhibition of promoter activity was observed.

Two major types of cannabinoid receptors have been identified to date, CB1 (brain receptor) (Matsuda *et al.*, 1990) and CB2 (peripheral receptor) (Munro *et al.*, 1993). CB1 is the predominant form of the cannabinoid receptor found in the brain, but it also has been identified in a number of peripheral tissues, including leukocytes (Matsuda *et al.*, 1990; Kaminski *et al.*, 1992; Schatz *et al.*, 1997). Conversely, CB2 is the predominant form of cannabinoid receptor expressed by leukocytes (Kaminski *et al.*, 1992; Munro *et al.*, 1993; Schatz *et al.*, 1997). One of the cellular mechanisms by which cannabinoid compounds mediate their biological activity is

through binding to CB1 and CB2, both of which couple to pertussis toxin-sensitive G_i/G_o GTP-binding proteins to inhibit adenylate cyclase and, consequently, the cAMP signaling cascade (Howlett *et al.*, 1985; Kaminski *et al.*, 1994). In addition, CB1 agonists can block the N-type Ca²⁺ channel and stimulate arachidonic acid release in neuronal cells (Felder *et al.*, 1993; Howlett, 1995; Shivachar *et al.*, 1996).

A search for endogenous ligands for cannabinoid receptors originally led to the identification of arachidonyl ethanolamide (anandamide). Anandamide was isolated from porcine brain (Devane *et al.*, 1992) and shown, *in vivo* and *in vitro*, to mimic many of the pharmacological actions of Δ^9 -THC, one of the major bioactive components of marijuana (Felder *et al.*, 1993). Shortly after the identification of anandamide, 2-Ara-

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ABBREVIATIONS: CB1, cannabinoid receptor type 1; 2-Ara-Gl, 2-arachidonyl-glycerol; CB2, cannabinoid receptor type 2; IL-2, interleukin-2; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; CBN, cannabinol; NF-AT, nuclear factor of activated T cells; NF- κB , nuclear factor for immunoglobulin κ chain in B cells; I κB , inhibitors of nuclear factor for immunoglobulin κ chain in B cells; AP-1, activator protein-1; AP-1c, activator protein-1 consensus site; AP-1p, activator protein-1 proximal site from the IL-2 promoter; Oct, octamer; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; CAT, chloramphenicol acetyltransferase; p(NF- κB)₃-CAT, CAT plasmid with three copies of nuclear factor for immunoglobulin κ chain in B cells consensus site; p(NF-AT)₃-CAT, CAT plasmid with three copies of NF-AT consensus site; p(AP-1)₃-CAT, CAT plasmid with three copies of activator protein-1 consensus site; PMA, phorbol-12-myristate-13-acetate; Io, ionomycin; CsA, cyclosporin A; NA, naive; VH, vehicle; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay.

GI, a monoglyceride, was isolated from canine gut and found to bind to both CB1- and CB2-transfected Chinese hamster ovary cells (Mechoulam *et al.*, 1995). More recently, 2-Ara-GI was shown to be produced in rat brain (Stella *et al.*, 1997) and the neuroblastoma line N18TG2 (Bisogno *et al.*, 1997). Cannabinoid receptor agonist activity by 2-Ara-GI is supported by the ability of this compound to inhibit forskolin-induced adenylate cyclase activity in mouse splenocytes (Mechoulam *et al.*, 1995) and to cross-desensitize cellular responses with the high affinity cannabinoid receptor ligand WIN 55212-2 in NG108-15 cells. Due to the fact that the gut is a critical lymphoid organ possessing Peyer's patches and gut-associated lymphoid tissue, coupled with the finding that 2-Ara-GI exhibited potential cannabinoid agonist activity in spleen-derived leukocytes, we initially evaluated the ability of 2-Ara-GI to modulate immune responses. A broad-based *in vitro* evaluation of 2-Ara-GI on immune function showed that this compound produced a marked and dose-related inhibition of the mixed lymphocyte response, anti-CD3 monoclonal antibody-induced T cell proliferation and lipopolysaccharide-induced B cell proliferation (Lee *et al.*, 1995). Conversely, *in vitro* IgM antibody-forming cell responses to lipopolysaccharide and sheep red blood cells were enhanced modestly by 2-Ara-GI (Lee *et al.*, 1995). Interestingly, anandamide, at equimolar concentrations, was either less active than 2-Ara-GI or inactive in all of the immune function assays described above (Lee *et al.*, 1995). More recently, several new structurally related putative endogenous cannabinoid receptor ligands have been identified, including homo- γ -linolenylethanolamide, docosatetraenylethanolamide (Barg *et al.*, 1995), mead ethanolamide (Priller *et al.*, 1995), and palmitoylethanolamide (Facci *et al.*, 1995).

In light of the fact that several plant-derived cannabinoids, including Δ^9 -THC and CBN, exhibit the ability to inhibit IL-2 expression, the objective of the current study was to evaluate the effects of 2-Ara-GI on the regulation of this cytokine in primary splenocytes and the murine thymoma EL4.IL-2 (Condie *et al.*, 1996). IL-2 is an autocrine/paracrine factor expressed by activated T lymphocytes that facilitates T cell clonal expansion and helps to regulate the actions of a variety of other cell types, including B cells, macrophages, and natural killer cells (Smith, 1988). Highly regulated, the IL-2 gene exhibits virtually no basal level of expression. On T cell activation, IL-2 gene expression is rapidly but transiently induced. Expression of the IL-2 gene is controlled primarily at the transcriptional level via a 5' promoter region extending ~326 bp upstream of the transcription start site (Jain *et al.*, 1995). The IL-2 promoter consists of several functional *cis*-acting regulatory sequences, including AP-1, NF-AT, NF- κ B/Rel, Oct, and CD28 response element sites, to which transcription factors bind cooperatively to induce maximal IL-2 gene transcription. Here, we report on the ability of 2-Ara-GI to inhibit IL-2 expression in primary splenocytes and in the murine thymoma EL4.IL-2. The mechanism responsible for the inhibition of IL-2 expression by 2-Ara-GI involves changes in the regulation of several transcription factors critical for the activation of IL-2 gene transcription.

Materials and Methods

Animals. Virus-free female B6C3F1 mice (6 weeks old) were purchased from Charles River (Dortage, MI). On arrival, mice were

randomized, transferred to plastic cages containing sawdust bedding (5 mice/cage), and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum* and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were kept at 21–24° and 40–60% relative humidity with a 12-hr light/dark cycle.

Reagents and cell culture. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. 2-Ara-GI (glyceryl-2-monoarachidonin) was purchased from DEVA Biotech (Hatboro, PA). 2-Ara-GI purity was found to be >99% as determined by gas chromatography/mass spectrometry. 2-Ara-GI was reconstituted in absolute ethanol, aliquoted, and stored under nitrogen at –80°. Working solutions were freshly prepared just before addition to culture.

Spleens were isolated aseptically and made into single-cell suspensions as described previously (Kaminski *et al.*, 1994). The splenocytes were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 5% bovine calf serum (Hyclone, Logan, UT). The splenocytes (1×10^6 /ml) were pretreated with 2-Ara-GI (1, 5, 10, 20, and 50 μ M), CsA (1 μ M), VH (0.1% ethanol), or media alone (NA) for 15 min and then stimulated with PMA (40 nM) plus Io (0.5 μ M). The C57BL/6 mouse lymphoma, EL4.IL-2, was obtained from America Type Culture Collection (TIB 181; Rockville, MD) and cultured as described previously (Condie *et al.*, 1996). In all cases, leukocytes were cultured at 37° in 5% CO₂.

Quantitative RT-PCR. All reagents used for RT-PCR were of molecular biological grade and were purchased from Promega (Madison, WI) unless otherwise noted. Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). IL-2 steady state mRNA expression was quantified by quantitative RT-PCR as described previously (Condie *et al.*, 1996) with minor modifications. 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 RT (GIBCO BRL; data not shown). Briefly, known amounts of total RNA and internal standard mRNA were reverse-transcribed simultaneously, in the same reaction tube, into cDNA using oligo(dT)₁₅ as primers. A PCR master mixture consisting of PCR buffer, 4 mM MgCl₂, 6 pmol each of the forward and reverse primers, and 1.25 units of *Taq* DNA polymerase was added to the cDNA samples. Samples were heated to 94° for 4 min and cycled 25 times at 94° for 15 sec, 59° for 30 sec, and 72° for 30 sec, after which an additional extension step at 72° for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. Quantification was performed by assessing the optical density for both of the DNA bands (i.e., internal standard versus target gene) using a Gel Doc 100 video imaging system (BioRad, Melville, NY). The number of transcripts was calculated from a standard curve generated by using the density ratio between the gene of interest and the different internal standard concentrations used (Gilliland *et al.*, 1990).

IL-2 protein quantification. Mouse recombinant IL-2 (as standard), purified rat anti-mouse IL-2 antibody, and biotinylated anti-mouse IL-2 antibody were purchased from PharMingen (San Diego, CA). Splenocytes (1×10^6 /ml) were cultured in triplicate in 48-well cell culture plates (0.8 ml/well; Corning, Corning, NY). The supernatant were collected at the indicated time points and quantified for IL-2 by ELISA as described previously (Ouyang *et al.*, 1995).

EMSAs. Nuclear proteins were prepared as described previously (Francis *et al.*, 1995). Briefly, splenocytes were lysed with buffer (10 mM HEPES, 1.5 mM MgCl₂), and nuclei were pelleted by centrifugation at $6,700 \times g$ for 5 min. Nuclei were lysed using a hypertonic buffer (30 mM HEPES, 1.5 mM MgCl₂, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) that contained 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin, after which the samples were centrifuged at $17,500 \times g$ for 15 min, and the supernatant was retained. Double-stranded deoxyoligo-

nucleotides containing the NF- κ B consensus recognition site (5'-GGGGACTTTCCT-3') (Pierce *et al.*, 1988), the Oct (5'-ATGCAAAT-3'), the NF-AT site (5'-GAGGAAAATTTG-3') from the IL-2 promoter (Jain *et al.*, 1993), AP-1c (5'-TGACTCA-3'), AP-1p site (5'-AGAGTCA-3') from the IL-2 promoter, and CRE (5'-TGACGTCA-3') were synthesized and end-labeled with [γ - 32 P]dATP using Ready-To-Go T₄ polynucleotide kinase (Pharmacia, Piscataway, NJ). Nuclear proteins (5 μ g) were incubated with 1 μ g of poly(dI/dC) in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin and leupeptin) for 20 min on ice and then for 30 min at room temperature for binding after the addition of labeled probe. Protein/DNA complexes were separated from free probe using a 5% acrylamide gel in 0.5 \times Tris/borate/EDTA buffer (1 \times = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). After electrophoresis, the gel was dried and autoradiographed. The intensity of DNA/protein complex bands was measured using a Gel Doc 100 video imaging system (BioRad).

Plasmid construction. Plasmids were constructed as described previously (Han SH, Jeon YJ, Yang KH, and Kaminski NE, submitted for publication). Briefly, a minimal promoter vector containing no enhancer, pCAT-Promoter, was purchased from Promega. To construct p(NF- κ B)₃-CAT, p(NF-AT)₃-CAT, and p(AP-1)₃-CAT, BglII-adhering oligonucleotides containing three copies of each consensus recognition motif (either NF- κ B, NF-AT, or AP-1) were synthesized and cloned into the pCAT-Promoter vector, respectively. Cloning was confirmed by a comparison of *Eco*RI-digested fragments from each recombinant plasmid and pCAT-Promoter vector. pIL-2-CAT (-1890) was kindly provided by Dr. Ellen Rothenberg. The promoters then were purified with use of the Qiagen Plasmid Kit (Qiagen, Chatsworth, CA) and quantified for transient transfection studies.

Transfection and CAT assay. Transient transfections were performed using a general DEAE-dextran method with slight modifications (Pierce *et al.*, 1988). A total of 3×10^7 EL4.IL-2 cells were washed with Tris-buffered saline and incubated in 6 ml of buffer containing 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂ plus 5 μ g of each plasmid and 200 μ g of DEAE-dextran/ml at 37° for 40 min. Cells were washed with HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 6 mM dextrose, and 25 mM HEPES), resuspended in 5% BCS RPMI, and cultured separately in six tissue culture plates at 37° in 5% CO₂. At 23 hr after transfection, cells were treated with the indicated concentrations of 2-Ara-Gl for 15 min and then stimulated with 80 nM PMA plus 1 μ M Io for 18 hr. Cells were harvested, washed with PBS, and freeze-thawed three times in 100 μ l of 0.25 mM Tris-HCl, pH 7.4, using liquid N₂. The supernatants were isolated, and equal amounts of proteins were incubated in the CAT reaction mixture containing 0.1 μ Ci of [14 C]chloramphenicol, 0.7 mM

acetyl-coenzyme A, and 0.14 M Tris-HCl, pH 7.4, at 37° for 5 hr for pIL-2-CAT, 1 hr for p(NF-AT)₃-CAT and p(AP-1)₃-CAT, and 30 min for p(NF- κ B)₃-CAT. The degree of acetylation was assessed by thin layer chromatography and autoradiography. The CAT activity was calculated as the ratio of enzyme activity, and that in the vehicle treatment group was assigned arbitrarily as 100%.

Statistical analysis. The mean \pm standard error was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance, and Dunnett's *t* test was used to compare treatment groups with the vehicle control when significant differences were observed.

Results

Dose-dependent inhibition of IL-2 expression by 2-Ara-Gl. Initially, the effect of 2-Ara-Gl on PMA/Io-induced IL-2 expression was evaluated. Splenocytes were activated with PMA/Io in the presence and absence of 2-Ara-Gl, and supernatants were measured for IL-2 activity at 6 and 24 hr by ELISA. A robust dose-dependent inhibition of IL-2 secretion was observed at both time points compared with the vehicle control (Fig. 1). At 24 hr, 1, 5, 10, and 20 μ M 2-Ara-Gl produced a 34%, 79%, 90%, and 99% inhibition in IL-2 activity. No effect on cell viability was observed at any of the 2-Ara-Gl treatment concentrations compared with the controls at either 6 or 24 hr after PMA/Io activation. CsA was used as a positive control and resulted in almost complete inhibition of IL-2 production. ED₅₀ values for the inhibition of IL-2 secretion were 4 and 2 μ M at 6 and 24 hr, respectively. The effect of 2-Ara-Gl on steady state IL-2 mRNA expression was also evaluated. Because peak steady state IL-2 mRNA expression occurs at 4–8 hr after T cell activation and returns to background levels by ~24 hr (Jain *et al.*, 1995), splenocytes were harvested and mRNA was isolated at 6 hr. The magnitude of IL-2 mRNA expression was quantified by competitive RT-PCR (Fig. 2). 2-Ara-Gl inhibited steady state IL-2 mRNA levels in a dose-dependent fashion by 21%, 45%, and 95% at 5, 10, and 20 μ M, respectively, compared with the vehicle control. The calculated ED₅₀ value for the inhibition of IL-2 steady state mRNA expression by 2-Ara-Gl was 9 μ M. At 50 μ M 2-Ara-Gl and 1 μ M CsA, IL-2 mRNA was below the level of quantification. Overall, 2-Ara-Gl exhibited a similar magnitude of potency in its ability to inhibit IL-2 steady state mRNA expression and IL-2 protein secretion, suggesting that the two effects are related mechanistically.

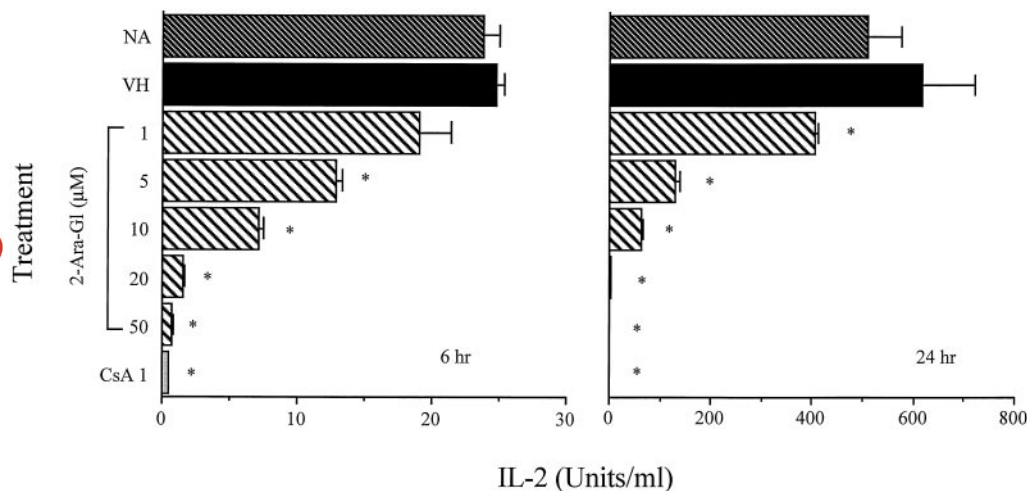


Fig. 1. The effects of 2-Ara-Gl on IL-2 secretion in murine primary splenocytes. Splenocytes (1×10^6 /ml) were pretreated with 2-Ara-Gl (1, 5, 10, 20, and 50 μ M), CsA (1 μ M), VH (0.1% ethanol), or the media (NA) for 15 min and then stimulated with PMA (40 nM) plus Io (0.5 μ M) for 6 or 24 hr at 37°. IL-2 was determined by ELISA. The data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$, significantly different from the vehicle. Results are representative of two separate experiments.

Effect of 2-Ara-Gl on NF-AT-, NF- κ B-, AP-1c-, AP-1p-, Oct-, and CRE-binding activity. Transcription factors NF-AT, NF- κ B/Rel, AP-1, Oct, and CREB have been widely established to be responsible for the regulation of IL-2 transcription (Jain *et al.*, 1995; Barton *et al.*, 1996). To characterize further the mechanism by which 2-Ara-Gl inhibits IL-2 expression, the DNA-binding activity of NF-AT, NF- κ B/Rel, AP-1c, AP-1p, Oct, and CREB were assessed by EMSAs. For this study, nuclear proteins were isolated from splenocytes pretreated for 15 min with 2-Ara-Gl and then stimulated for 90 min with PMA/Io. The rationale for selecting the 90-min time point was based on several reasons. First, we have shown previously that plant-derived cannabinoids alter an early activation event (i.e., within 120 min of activation) in leukocytes (Schatz *et al.*, 1992). Second, we have observed that plant-derived cannabinoids inhibit DNA binding activity of several transcription factors known to regulate IL-2, including NF- κ B/Rel, AP-1p, and CREB. Their inhibition was readily observed within 90 min after T cell activation. Third, as demonstrated in the current study, with the exception of Oct-binding activity, which is constitutive, PMA/Io induces DNA binding by NF-AT, NF- κ B/Rel, AP-1c, AP-1p, and CREB by 90 min compared with unstimulated splenocytes. Interestingly, splenocytes pretreated with 2-Ara-Gl exhibited decreased PMA/Io-induced NF-AT binding activity, which was dose related (Fig. 3). As anticipated, CsA, which was used as a positive control, likewise dramatically suppressed the NF-AT binding activity. 2-Ara-Gl also modestly repressed the binding activity of NF- κ B/Rel (Fig. 4). Conversely, no significant effect by 2-Ara-Gl was observed on binding activity at either the AP-1c or AP-1p (Fig. 5, A and B), Oct (Fig. 6), or CRE (Fig. 7) site. DNA-binding specificity was verified in all of the experiments

using unlabeled competitors (excess 32 P-unlabeled probe; Figs. 3–7, lane 9).

Effect of 2-Ara-Gl on IL-2 promotor activity. IL-2 expression is regulated mainly at the transcription level. To investigate further the underlying mechanism for the 2-Ara-Gl-induced suppression of IL-2, IL-2 promotor activity was assessed by transient transfection of EL4.IL-2 cells with a pIL-2-CAT (–1890 bp). EL4.IL-2 cells were selected as a model for this study because (1) this cell line has been used widely as a model for the elucidation of IL-2-associated regulation, (2) we have used RT-PCR analysis to demonstrate that EL4.IL-2 cells express CB2 but not CB1 receptors (Schatz *et al.*, 1997), and (3) treatment of EL4.IL-2 cells with plant-derived cannabinoids (i.e., Δ^9 -THC or CBN) readily inhibited IL-2 expression (Condie *et al.*, 1996). Consistent with the effects observed in splenocytes, 2-Ara-Gl suppressed IL-2 promotor activity in a dose-dependent manner over a comparable dose range as observed for inhibition of IL-2 expression in primary cells (Fig. 8). IL-2 promotor activity was only 82%, 72%, 61%, and 46% of the control (PMA/Io alone) in the presence of 5, 10, 20, and 50 μ M 2-Ara-Gl, respectively.

Effects of 2-Ara-Gl on NF-AT, NF- κ B, and AP-1 promoter activity. To evaluate further the significance of the decrease in DNA binding by transcription factors required for IL-2 gene regulation, the effect of 2-Ara-Gl was evaluated on promoter activity using the constructs p(NF- κ B)₃-CAT, p(NF-AT)₃-CAT, and p(AP-1)₃-CAT. Concordant with the NF-AT EMSA results, 2-Ara-Gl inhibited NF-AT promoter activity in a dose-dependent manner (Fig. 9). 2-Ara-Gl produced only a modest inhibition of NF- κ B promoter activity at 20 and 50 μ M (Fig. 10). Likewise, inhibition was detected on AP-1 promoter activity with 10 and 20 μ M 2-Ara-Gl (Fig. 11).

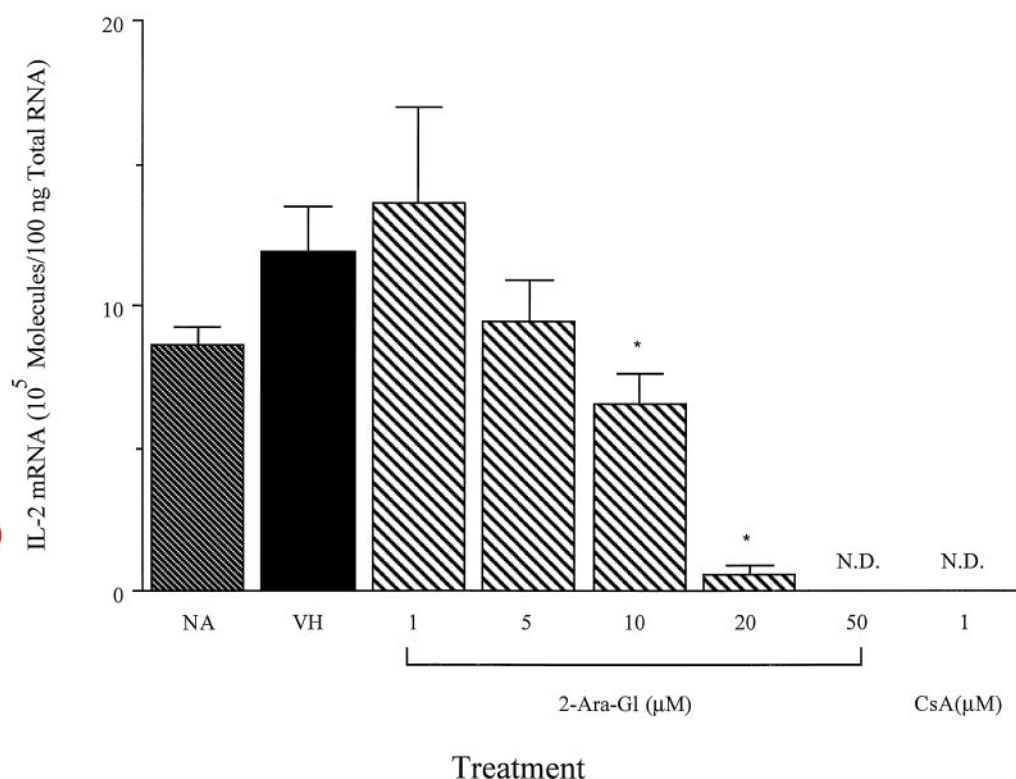


Fig. 2. The effects of 2-Ara-Gl on IL-2 mRNA expression in murine primary splenocytes. Splenocytes (1×10^6 /ml) were pretreated with 2-Ara-Gl (1, 5, 10, 20, and 50 μ M), CsA (1 μ M), VH (0.1% ethanol), or media (NA) for 15 min and then stimulated with PMA (40 nM) and Io (0.5 μ M) for 6 hr at 37°. The cells were harvested, and IL-2 mRNA was analyzed by quantitative RT-PCR. The data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$, values that are significantly different from the vehicle. N.D., IL-2 mRNA was below the level of quantification. The results are representative of three separate experiments.

Discussion

In the current study, we demonstrated that 2-Ara-Gl, a putative endogenous ligand for cannabinoid receptors CB1 and CB2, strongly inhibited IL-2 expression in murine splenocytes and EL4.IL-2 cells. The inhibition of IL-2 secretion by 2-Ara-Gl occurred over a comparable dose range as demonstrated previously in the same model systems with both CBN and Δ^9 -THC (Condie *et al.*, 1996). Similar to the previous studies with plant-derived cannabinoids, the decrease in IL-2 secretion by 2-Ara-Gl was paralleled by a concomitant decrease in steady state IL-2 mRNA expression. In the current study, we used several approaches that focused on the transcriptional regulation of IL-2 to provide insights into the mechanism of immune modulation by 2-Ara-Gl. Transient transfection of EL4.IL-2 cells with a pIL-2-CAT

promoter construct demonstrated that 2-Ara-Gl induced an inhibition of PMA/Io-mediated IL-2 promoter activity that was comparable in magnitude to the inhibition of IL-2 production and IL-2 steady state mRNA expression in mouse splenocytes. These findings suggest that the inhibition of IL-2 expression by 2-Ara-Gl occurs at the level of gene regulation.

The specific signal transduction events induced by 2-Ara-Gl are largely unknown; however, four major mechanisms of signal transduction have been proposed for cannabinoids: inhibition of cAMP signaling cascade (for reviews, see Howlett, 1995; Kaminski, 1996), inhibition of N-type Ca^{2+} channels (Felder *et al.*, 1993; Howlett, 1995), modulation of mitogen-activated protein kinase signaling (Wartmann *et al.*, 1995), and increased production of eicosanoids (Howlett, 1995; Wartmann *et al.*, 1995). To explore further the effects of 2-Ara-Gl on signaling events leading to the regulation of IL-2 gene transcription, a series of EMSA and transient transfection studies were performed to evaluate DNA binding and transcriptional activity of transcription factors known to regulate the IL-2 gene. Interestingly, 2-Ara-Gl primarily affected PMA/Io-induced NF-AT and NF- κ B/Rel DNA binding and promoter activity. Both families of transcription factors are critical for the regulation of IL-2 gene transcription (Jain *et al.*, 1995). NF-AT is regulated primarily by the Ca^{2+} - and calmodulin-dependent phosphatase calcineurin, which is the major target of the immunosuppressive drugs CsA and FK506 (Crabtree and Clipstone, 1994). Increased intracellular Ca^{2+} during leukocyte activation, as occurred during our study as a result of PMA/Io treatment, engages calcineurin. In turn, calcineurin initiates a phosphatase cascade that leads to dephosphorylation of NF-AT, which then translocates to the nucleus. Both the human and murine IL-2 enhancers contain two NF-AT sites (5'-GGAAAA-3'). Consistent with alterations on Ca^{2+} regulation, cannabinoids have been demonstrated to inhibit N-type Ca^{2+} channels in neuronal cell preparation after agonist binding to CB1. However, N-type Ca^{2+} channels are not present in leukocytes and therefore not an intracellular target in the current study. In addition, CB2 has been shown, at least in transfected Chinese hamster ovary cells, not to induce changes in intracellular Ca^{2+} mobilization after agonist binding (Felder *et al.*, 1995). This observation is important in light of the fact that EL4.IL-2 cells, which were used for the CAT promoter studies, express only CB2 mRNA and therefore most likely express only CB2, and not CB1, receptors. Nevertheless, it is notable that to date, Ca^{2+} regulation by cannabinoids has not been investigated extensively in leukocytes and cannot be ruled out as a potential pathway modulated by cannabinimimetic agents. One exception is a study by Yebra *et al.* (1992), which was performed using thymocytes and showed that Δ^9 -THC suppressed concanavalin A-induced increases in cytoplasmic Ca^{2+} . The absence of N-type Ca^{2+} channels in leukocytes does not rule out the possibility of intracellular Ca^{2+} stores being modulated by cannabinoids. It also is noteworthy that in contrast to the inhibitory effect that classic cannabinoids exhibit on intracellular Ca^{2+} , it has been reported recently that 2-Ara-Gl induced a rapid, transient elevation of intracellular free Ca^{2+} in NG108-15 neuroblastoma glioma hybrid cells (Sugiura *et al.*, 1996). In the absence of additional data, it is difficult to relate the above results with 2-Ara-Gl observed in NG108-15 cells to our own stud-

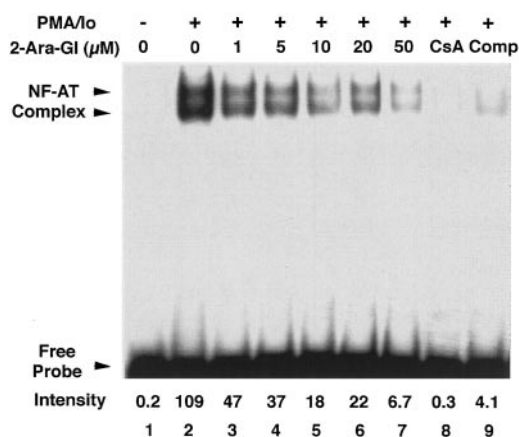


Fig. 3. The effect of 2-Ara-Gl on the DNA-binding activity of NF-AT in murine primary splenocytes. The splenocytes ($1 \times 10^6/\text{ml}$) were pretreated with 2-Ara-Gl (0, 1, 5, 10, 20, and 50 μM) (lanes 2–7) and CsA (1 μM) (lane 8) for 15 min and then stimulated with PMA (40 nM) plus Io (0.5 μM) for 90 min at 37°. In addition, the basal level of NF-AT DNA binding was measured in unstimulated cells (lane 1). The nuclear proteins (5 μg) were resolved by EMSA as described in Materials and Methods. Arrows, DNA/protein complex or free probe, respectively. Lane 9, included 100-fold molar excess of the unlabeled NF-AT oligonucleotide as a competitor using the same protein as loaded in lane 2. The data are representative of two independent experiments.

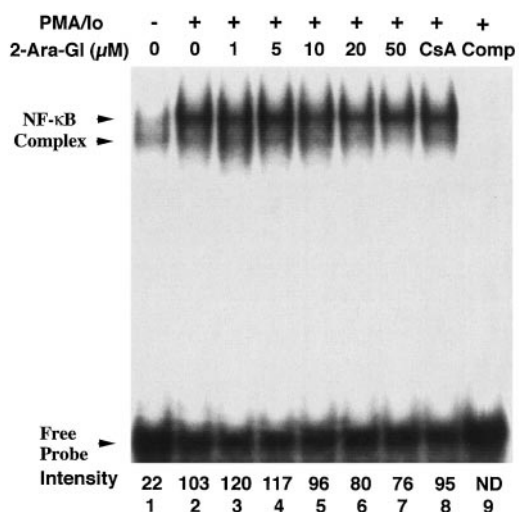


Fig. 4. The effects of 2-Ara-Gl on the DNA-binding activity of NF- κ B in murine primary splenocytes. Experiments were performed as indicated in the legend to Fig. 3. The results are representative of two independent experiments.

ies, which indicate aberrant regulation of NF-AT during leukocyte activation.

NF-AT transcriptional activity also is significantly influenced by a nearby weak AP-1 site (5'-TGTTTCA-3'). AP-1 is obligatory for stable NF-AT binding (Jain *et al.*, 1993) and NF-AT-mediated IL-2 gene transcription (Petrak *et al.*, 1994). Interestingly, although cannabinoids such as CBN and Δ^9 -THC inhibit PMA/Io-induced AP-1 binding (Condie *et al.*, 1996), no effect was observed on DNA-binding activity to either the AP-1c or AP-1p recognition motifs in the presence of 2-Ara-Gl. However, an inhibition of AP-1c promoter activity was observed by 2-Ara-Gl in PMA/Io-activated EL4.IL-2 cells. The difference observed between AP-1 DNA binding activity in the EMSA and AP-1 promoter activity in the transient transfection studies with 2-Ara-Gl may reflect inherent differences in sensitivity between the two assay systems. The more likely explanation for differences between AP-1 DNA binding and promoter activity relates to the marked difference in the duration of cell treatment between the two different assays. In the EMSA studies, cells are treated with PMA/Io and 2-Ara-Gl for 90 min and then harvested for nuclear protein isolation. Conversely, for the promoter studies, the duration of cell treatment with PMA/Io plus 2-Ara-Gl is for 18 hr, at which time promoter activity is determined. Despite the aforementioned differences, with the exception of AP-1, DNA-binding activity and promoter activity were correlated closely for all of the transcription factors examined.

NF- κ B/Rel also is critical in the transcriptional regulation of IL-2 as well as other genes that involve inflammation and immune function (Baeuerle and Henkel, 1994). NF- κ B/Rel is primarily controlled at the post-translational level. In the absence of an appropriate stimulus, a group of inhibitory proteins known as I κ B sequester NF- κ B/Rel in the cytoplasm as inactive precursors. After stimulation of cells with various activators, including cAMP, I κ B phosphorylation, ubiquitination, and subsequent rapid degradation lead to the release of NF- κ B/Rel. Separation from I κ B triggers NF- κ B/Rel nuclear translocation and DNA binding to activate transcription of target genes (Baeuerle and Henkel, 1994). We reported recently that forskolin stimulation of either mouse splenocytes

or thymocytes significantly enhanced NF- κ B/Rel DNA-binding activity (Herring *et al.*, in press). In addition, we showed that in the presence of CBN NF- κ B/Rel, DNA-binding activity was repressed markedly during the first 120 min after forskolin stimulation in mouse leukocyte preparations. A similar but less robust inhibition of NF- κ B/Rel DNA-binding activity was observed by 2-Ara-Gl in PMA/Io-activated mouse splenocytes. The primary difference between the two studies was that PMA/Io, rather than forskolin, was used in the current study and may account for the more modest inhibition by 2-Ara-Gl on NF- κ B/Rel binding activity compared with that induced by CBN. The significance of the current study is that unlike forskolin, PMA/Io is an adequate stimulus to induce not only NF- κ B/Rel binding but, more importantly, IL-2 gene transcription, whereas forskolin is not.

The most extensively characterized signaling pathway that is modulated by plant-derived cannabinoids is the cAMP cascade. Both CB1 and CB2 agonist binding leads to the inhibition of adenylate cyclase, and subsequently the inhibition of cAMP-mediated signal transduction (for reviews, see Howlett, 1995; Kaminski, 1996). Specifically, cannabinoids inhibit downstream events within the cAMP cascade, including protein kinase A activation and CRE and AP-1p DNA-binding activity (Koh *et al.*, 1997; Condie *et al.*, 1996). In the case of the AP-1p site, both fos/CREB and jun/CREB dimers were identified as part of the DNA-binding complex (Chen and Rothenberg, 1993). Moreover, it is interesting that despite the fact that 2-Ara-Gl inhibits forskolin-induced cAMP accumulation, no effect was observed in the current study on either CRE or AP-1p binding. This result is somewhat paradoxical in light of the decrease in cAMP formation induced by 2-Ara-Gl in splenocytes (Mechoulam *et al.*, 1995). It also is in contrast to the strong inhibition induced by Δ^9 -THC on PMA/Io-induced CRE binding activity in mouse splenocytes (Koh *et al.*, 1997). Although it is presently unclear why CRE- and AP-1p-binding activity was not inhibited by 2-Ara-Gl, it most likely is due to the fact that 2-Ara-Gl is degraded rapidly to arachidonic acid in culture and then likely converted to other biologically active molecules (e.g., prostaglandins and leukotrienes). The instability of 2-Ara-Gl is perhaps best exempli-

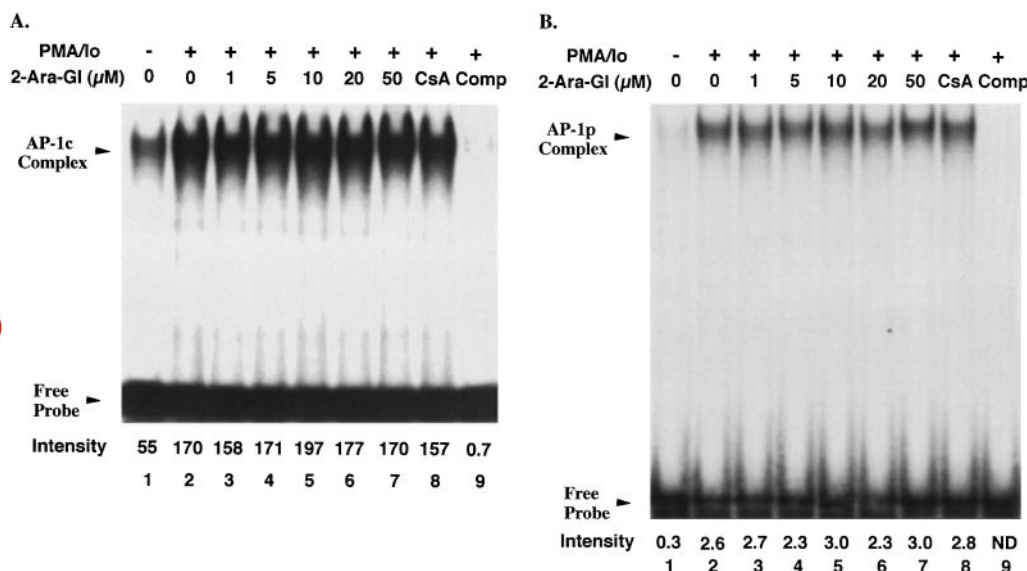


Fig. 5. The effects of 2-Ara-Gl on the DNA-binding activity at the (A) AP-1c and (B) AP-1p from the IL-2 promoter in murine primary splenocytes. Experiments were performed as indicated in the legend to Fig. 3. The results are representative of two independent experiments.

fied by recently reported radioligand binding analyses, which showed that although 2-Ara-Gl competed for cannabinoid receptor binding with the high affinity ligand CP-55940, its binding affinity increased markedly when the assay was carried out in the presence of esterase inhibitors or at 0° (Sugiura *et al.*, 1995). However, the lack of inhibition on CRE and AP-1p binding may be more a function of the formation of other regulatory molecules than due to a loss of agonist binding at cannabinoid receptors by 2-Ara-Gl. It is notable that several prostaglandins, including prostaglandin E2, are potent positive modulators of adenylate cyclase (Phipps *et al.*, 1991). Binding of newly formed prostaglandins potentially could offset the inhibition of adenylate cyclase that is mediated through 2-Ara-Gl binding to cannabinoid receptors.

In summary, we have shown that 2-Ara-Gl, a putative endogenous cannabinoid receptor ligand, strongly inhibited IL-2 expression at both the mRNA and protein levels in

murine splenocytes. Furthermore, we demonstrated that the inhibition of IL-2 by 2-Ara-Gl seems to be mediated, at least in part, at the transcriptional level as demonstrated by an inhibition of pIL-2-CAT promoter activity in transiently transfected EL4.IL-2 cells. Examination of the transcription factors that regulate IL-2 expression revealed an inhibition of NF-AT and, to a lesser degree, NF- κ B/Rel. As discussed in the introduction, we have shown previously that some of the biological actions of 2-Ara-Gl on immune responses mimicked those of plant-derived cannabinoids such as Δ^9 -THC and CBN, whereas other responses were either unaffected or modulated differently. We speculate that these differences are likely due to the rapid degradation of 2-Ara-Gl to arachidonic acid, which may in turn lead to the formation of a number of biologically active molecules. In the event that

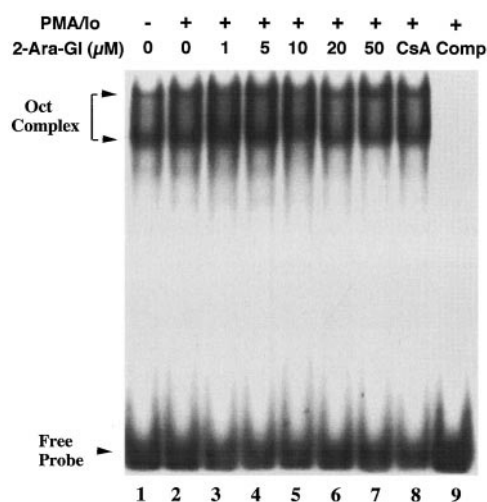


Fig. 6. The effects of 2-Ara-Gl on the DNA-binding activity of Oct in murine primary splenocytes. Experiments were performed as indicated in the legend to Fig. 3. The results are representative of two independent experiments.

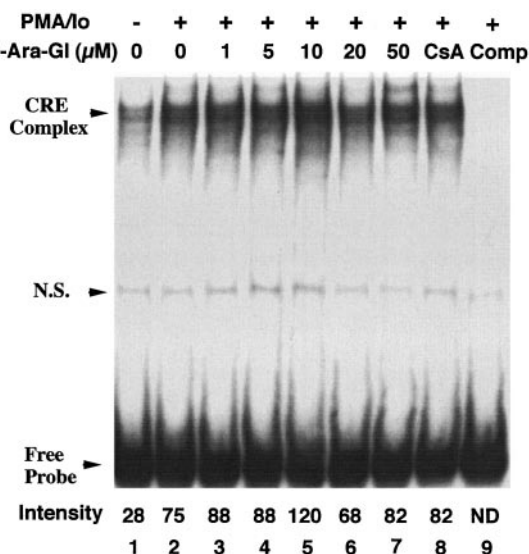


Fig. 7. The effects of 2-Ara-Gl on the DNA-binding activity of CRE in murine primary splenocytes. Experiments were performed as indicated in the legend to Fig. 3. N.S., nonspecific binding. The results are representative of two independent experiments.

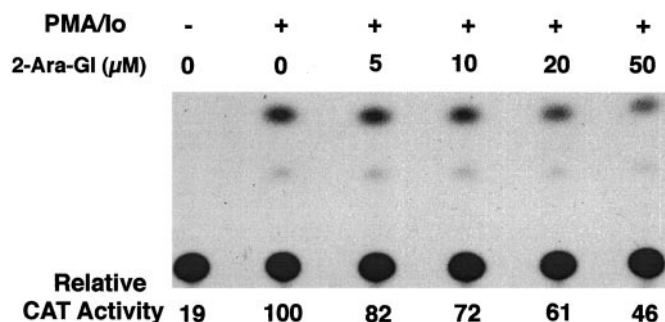


Fig. 8. The effects of 2-Ara-Gl on IL-2 promoter activity in EL-4. IL-2 cells. A reporter plasmid, pIL-2-CAT (-1890), was transfected transiently into EL-4. IL-2 cells (5×10^5 /ml). The transfected cells were pretreated with 2-Ara-Gl (0, 5, 10, 20, and 50 μM) for 15 min and then stimulated with PMA (80 nM) plus Io (1 μM) for 18 hr at 37°. In addition, the basal level of IL-2 promoter activity was measured in unstimulated cells (lane 1). CAT activity was measured as described in Materials and Methods. The CAT activity in the control (lane 2) was arbitrarily assigned a value of 100%. All other groups were compared with the control. The results are representative of two independent experiments.

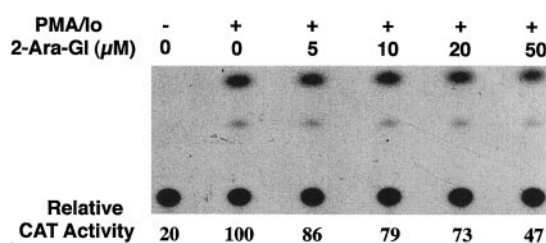


Fig. 9. The effects of 2-Ara-Gl on NF-AT promoter activity in EL-4. IL-2 cells. A reporter plasmid, p(NF-AT)₃-CAT, was transfected transiently into EL-4. IL-2 cells (5×10^5 /ml). Experiments were performed as indicated in the legend to Fig. 8. The results are representative of two independent experiments.

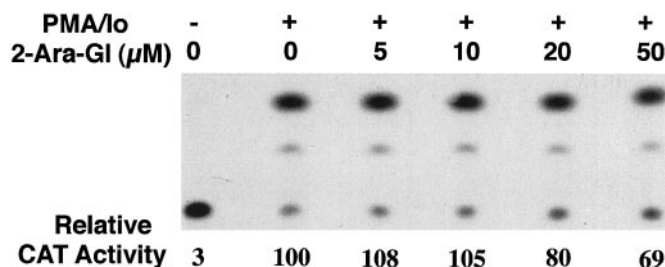


Fig. 10. The effects of 2-Ara-Gl on NF- κ B promoter activity in EL-4. IL-2 cells. A reporter plasmid, p(NF- κ B)₃-CAT, was transfected transiently into EL-4. IL-2 cells (5×10^5 /ml). Experiments were performed as indicated in the legend to Fig. 8. The results are representative of two independent experiments.

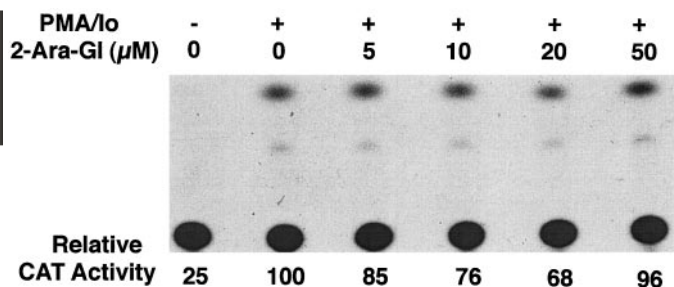


Fig. 11. The effects of 2-Ara-Gl on AP-1 promoter activity in EL-4. IL-2 cells. A reporter plasmid, p(AP-1)₃-CAT, was transfected transiently into EL-4. IL-2 cells (5×10^5 /ml). Experiments were performed as indicated in the legend to Fig. 8. The results are representative of two independent experiments.

2-Ara-Gl is converted to other biologically active molecules, the profile of immune modulation is likely to be highly complex.

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