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Anadamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis

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Abstract

This study examined the immunoregulatory effects of anadamide, the recently identified first endogenous cannabinoid receptor ligand. Anadamide caused dose-dependent inhibition of mitogen-induced T and B lymphocyte proliferation. Its potency was 3- and 10-fold less than that of the synthetic cannabinoids Δ^8-tetrahydrocannabinol (Δ^8-THC) and CP55940, respectively. Anadamide effects on DNA synthesis in T and B lymphocytes occurred rapidly as exposure of the cells during the final 4 h of culture was sufficient to achieve > 40% inhibition. Low doses of anadamide which caused significant inhibition of lymphocyte proliferation caused DNA fragmentation as demonstrated by immunohistochemistry, FACS analysis and Southern blotting. Apoptosis was also induced by high concentrations of Δ^8-THC, but not by CP55940. Brain and peripheral cannabinoid receptor mRNA was expressed in PBMC with varying levels between individual donors. In summary, these findings demonstrate immunosuppressive effects of anadamide which are associated with inhibition of lymphocyte proliferation and the induction of cell death by apoptosis.

Keywords: Anadamide; Cannabinoids; Immunosuppression; Apoptosis

1. Introduction

Cannabinoids are exogenous psychoactive compounds which regulate diverse behavioral responses (Johnson, 1990) and this correlates with the presence of specific binding sites in the central nervous system (CNS) (Devane et al., 1992a; Jansen et al., 1992; Mailleux and Vanderhaeghen, 1992). Recent cloning of the genes encoding the rat (Matsuda et al., 1990) and human (Mailleux et al., 1992; Evans et al., 1992) Cannabinoid receptor (CB) confirmed that it is a G-protein coupled receptor (Houston and Howlett, 1993). Cannabinoids inhibit adenylate cyclase and the accumulation of cAMP (Howlett et al., 1990; Felder et al., 1992) and regulate calcium channels (Mackie and Hille, 1992), effects which are receptor-mediated. In contrast, cannabinoid stimulation of arachidonic acid release by activation of phospholipase C and the release of calcium from intracellular stores are effects which usually occur at higher doses and are not mediated by the cannabinoid receptors (Felder et al., 1993). In addition to psychotropic effects, cannabinoids also influence the reproductive system (Murphy et al., 1990; Patra and Wadsworth, 1991; Schuel et al., 1991) and have inhibitory effects on immune function (Hollister, 1992). Marijuana users may have signs of impaired cellular immunity (Nahas et al., 1974) and treatment of experimental animals with high doses of cannabinoids reduced immune responses (Nahas and Latour, 1992). In vitro studies showed that cannabinoids reduce proliferative responses of T lymphocytes (Luo et al., 1992), cytotoxic T cell activity (Klein et al., 1991), antibody synthesis, microbiocidal activity of macrophages (Arata et al., 1991) and augment immunosuppression induced by murine retrovirus infection (Specter et al., 1991). These immunosuppressive effects of cannabinoids may be therapeutically useful as suggested by studies on experimental autoimmune encephalomyelitis (Lyman et al., 1989). In vitro effects of cannabinoids on im-

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mune function are also stereoselective and associated with inhibition of cAMP accumulation (Kaminski et al., 1992; Schatz et al., 1992). Non-psychoactive effects of cannabinoids could either be mediated centrally or by interactions with receptors on peripheral tissues such as lymphocytes or monocytes. This latter notion is supported by the demonstration of expression of the first CB on lymphocytes and the more recent cloning of a second G-protein coupled cannabinoid receptor which is expressed in rat splenic macrophages but not in the brain (Munro et al., 1993).

The potential physiological and pathogenic significance of cannabinoid-mediated immunomodulation was further supported by the recent identification of an endogenous CB ligand. The arachidonic acid derivative arachidonylethanolamide, termed anadamide, has been identified as a natural ligand that binds cannabinoid receptors on synaptosomal membranes (Devane et al., 1992b) as well as the cloned cannabinoid receptor (Felder et al., 1993; Vogel et al., 1993). Anadamide has cannabimimetic activity as it inhibits adenylate cyclase activation (Felder et al., 1993) and calcium currents (Mackie et al., 1993) and showed the characteristic psychotropic cannabinoid effects when administered to experimental animals (Fride and Mechoulam, 1993).

The objective of the present study was to investigate the effects of anadamide on immune function, to compare its potency to that of synthetic cannabinoids and examine mechanisms involved with its immunomodulatory effects.

2. Materials and methods

2.1. Isolation and culture of lymphocytes

Human peripheral blood mononuclear cells (PBMC), T and B lymphocytes were prepared as previously described (Kuis et al., 1991; Villiger et al., 1991). In brief, PBMC were isolated from heparinized peripheral blood of healthy volunteers, monocytes were isolated on Percoll gradients and lymphocytes separated into T and B cells by E-rosetting. Purity of the cell preparations was > 80% for B lymphocytes and > 90% for T lymphocytes as determined by staining with antibodies to CD3 and CD19 and fluoresence-activated cell sorting (FACS) analysis. Proliferation assays were performed with PBMC stimulated with OKT3, a mitogenic antibody to CD3 or PHA as previously described (Lotz et al., 1988). B cell proliferation studies were performed as described (Lotz et al., 1994) in 96-well flat-bottom plates with 200 000 cells per well in RPMI 1640 supplemented with 5% FBS, L-glutamine and antibiotics. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) during the final 4 h of a 3-day culture and collected on an automated cell harvester. Radioactivity incorporated by the cells was quantified by liquid scintillation counting. All conditions were tested in triplicate.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

RNA (up to 5 μg) was reverse-transcribed in a 20-μl volume containing 4 μl 5 × RT-buffer (BRL), 10 mM DTT, 500 μM dNTPs, 1 μl random hexanucleotides (2 mg/ml) (Pharmacia), 200 U MoMLV-RT (BRL) and 20 U RNasin (Promega) for 60–120 min at 37°C.

PCR was performed with 2 μl of the RT reaction product in 25 μl volume with 1 U Taq DNA Polymerase (Boehringer, Indianapolis, IN), 140 μM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl and 10 pM of each primer. After a 5-min denaturation step at 94°C, the reaction proceeded in 35 cycles of 30″ at 94°C, 30″ at 55°C and 70″ at 72°C, followed by 5 min at 72°C. For glyceraldehyde 3-phosphate dehydrogenase (G3PDH) 29 cycles were run at 30″ extension time at 72°C.

CB1 primers:

sense:	5′ ATG AGG AGA ACA TCC AGT GTG
antisense:	5′ GCA GAA TTC TCA CAG AGC CTC GGC AGA CGT

CB2 primers:

sense:	5′ GCC AAG CTT ATG GAG GAA TGC TGG GTG
antisense:	5′ AAG GCG GCC GCT CAG CAA TCA GAG AGG TC

G3PDH primers:

sense:	5′ TGG TAT CGT GGA AGG ACT CAT GAC
antisense:	5′ ATG CCA GTG AGC TTC CCG TTC AGC

2.3. DNA isolation and Southern blotting

Cells were harvested by a 5-min centrifugation at 2000 rpm and resuspended in 10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, pH 8.0 (10^6 cells per 50 μl buffer). Extracts were incubated on ice for 20 min and briefly vortexed every 5 min. Cell debris and high molecular mass DNA were removed by a 10-min centrifugation at 14 000 rpm at 4°C. Supernatants were extracted with phenol and phenol/chloroform and precipitated with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol for 2 h at −80°C.

DNA was separated on 2% agarose gels in TAE buffer at 4°C and 3 V/cm, blotted onto a Hybond (Amersham) membrane and crosslinked by UV light

Hybridization was performed with an ^{32}P-labeled oligonucleotide specific for the repetitive human Alu DNA sequence, dGGCACTTTGGGAGGCCAAGG, as described (Kobayashi et al., 1993).

2.4. Histochemical detection of DNA fragmentation

Cells were harvested, centrifuged onto glass slides, fixed with 0.25% glutaraldehyde for 1 min, washed

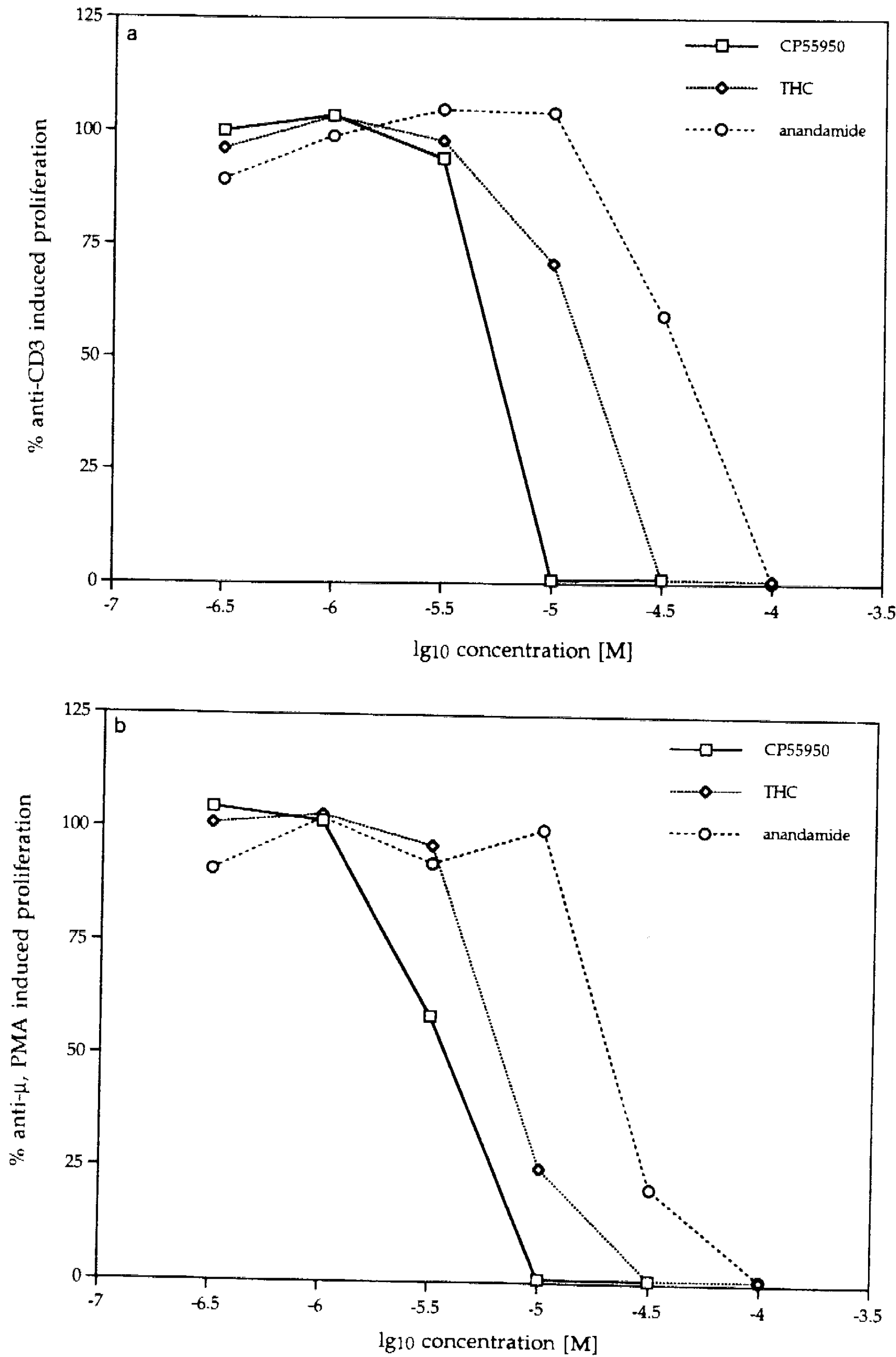

Fig. 1. Inhibition of T and B cell proliferation by anadamide. PBMC were stimulated with optimal doses of T cell specific mitogenic antibodies to CD3 (**A**). Purified peripheral blood B lymphocytes (**B**) were incubated with anti-μ and cannabinoids in microtiter wells for 72 h. Anadamide, Δ^8-THC, CP55940 and anadamide at the indicated concentrations were added at the same time and proliferation was measured by [^{3}H]thymidine incorporation after 72 h. Incorporation of radioactivity was quantified by liquid scintillation counting. Results are expressed as percent proliferation induced by anti-CD3 (**A**) or anti-μ (**B**). The values for [^{3}H]thymidine incorporation for the experiments shown in (**A**) were: media control, 766 cpm; OKT3 alone, 32577 cpm; OKT3 plus anadamide 3×10^{-5} M, 20792 cpm; OKT3 plus THC 3×10^{-5} M, 293 cpm; and OKT3 plus CP55940 3×10^{-5} M, 93 cpm. Each point represents triplicate determinations and results are based on at least three independent experiments. Standard deviation was < 15%.

with water and stained with 1 $\mu g/ml$ 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI) for 10 min at 37°C (Verma et al., 1992). Slides were washed with water, air dried and covered by Vectashield (Vector Labs) cover slides for analysis by microscopy using a chroma triple band filter set (#61000; Chroma Technology, Brattleboro, VT) on an Olympus microscope.

2.5. DNA labeling technique for flow cytometric analysis

Cells were stained with propidium iodide (PI) in a hypotonic staining solution (Nicoletti et al., 1991) and PI fluorescence of individual nuclei was measured on a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) using a 560-nm dichromic mirror and a 600-nm band pass filter. Data are expressed as percent apoptotic (i.e. hypodiploid) nuclei.

2.6. Reagents

Phorbol 12-myristate 13-acetate (PMA) and PHA were purchased from Sigma (St. Louis, MO). Anti-CD3 was used as supernatants from the OKT3 hybridoma (obtained from ATCC) that had been titrated to determine optimal doses for T cell mitogenesis. Goat anti-human IgM F(ab)′2 Ab was purchased from Organon Teknika Corp., Cappel Research Products, Durham, NC).

Cannabinoids: CP55940 was generously provided by Dr. Saul B. Kadin (Pfizer Inc., Groton, CT), anadamide by Dr. Raphael Mechoulam (Hebrew University of Jerusalem, Jerusalem, Israel) and Δ^8-tetrahydrocannabinol (Δ^8-THC) and Δ^9-THC by the National Institute on Drug Abuse. The drugs were kept in 100% ethanol at −80°C.

3. Results

3.1. Anadamide inhibits mitogen-induced proliferation of T and B lymphocytes

In the first set of experiments we determined the effects of anadamide on mitogen-induced lymphocyte proliferation. The results showed dose-dependent inhibition of T cell proliferation induced by anti-CD3 (Fig. 1A) with complete inhibition at 10^{-4} M anadamide. Partial inhibition of proliferation was obtained at 3×10^{-5} M anadamide. The two synthetic cannabinoids, Δ^8-THC and CP55940 showed similar effects and completely inhibited T cell proliferation at concentrations of 3×10^{-5} and 10^{-5} M, respectively. Δ^9-THC was of equal potency as Δ^8-THC in inhibiting proliferation of OKT3-stimulated PBMC.

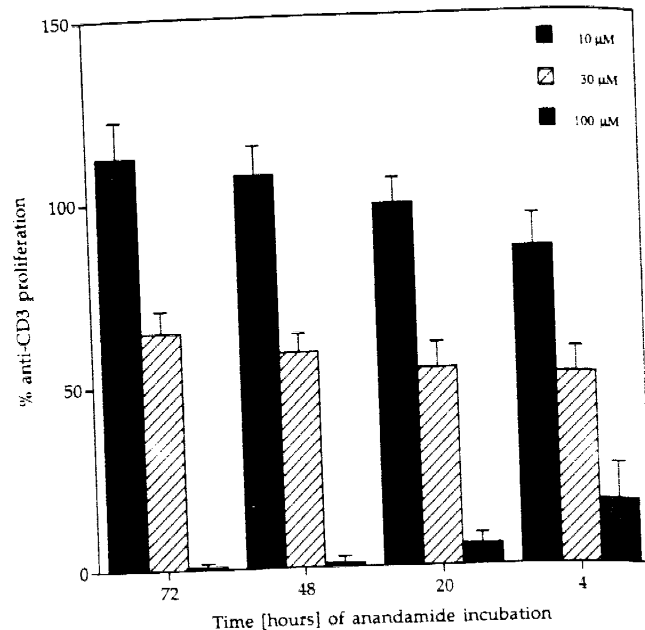

Fig. 2. Kinetics of anadamide inhibition of T lymphocyte proliferation. PBMC were activated with OKT3 and proliferation was measured after 72 h following a 4-h pulse with 1 μCi [^{3}H]thymidine. Anadamide was added at indicated concentrations and times before cell harvesting. Each point represents triplicate determinations and results are based on three independent experiments.

B lymphocytes were purified from PBMC and activated with antibody to cell surface immunoglobulin M (anti-μ) and PMA which induced the expected increases in proliferation. This was inhibited by the three CB ligands in a similar pattern as observed with OKT3-induced T cell proliferation (Fig. 1B).

These results show that anadamide inhibits accessory cell-dependent T cell proliferation and accessory cell-independent proliferation of B lymphocytes. The effects of anadamide on T and B cell proliferation were tested with cells isolated from 25 and 4 different donors, respectively. In all isolates 10^{-5} M anadamide had no effect on proliferation. Anadamide at 10^{-4} M invariably blocked proliferation completely. The inhibitory effects of 3×10^{-5} M anadamide ranged from 30 to 80% in cells from different donors.

We then studied the kinetics of the cannabinoic effects on proliferation of anti-CD3 activated PBMC Addition of anadamide, CP55940 or Δ^8-THC at 72, 48 or 20 h before [^{3}H]thymidine labeling caused simila inhibition of lymphocyte proliferation. Anadamide (10 μM) completely inhibited proliferation when it wa present for 72, 48 and 20 h. Its inhibitory effect decreased moderately when it was present for only 4 h However, anadamide (at 30 μM) was still capable c significantly reducing lymphocyte proliferation when was present only during the final 4 h of the assay (Fi 2). Similar results were obtained in studies on PM

plus anti-μ activated B lymphocytes and this time course was identical in PBMC from three different donors (data not shown).

3.2. Anadamide induces lymphocyte apoptosis

Based on the complete inhibition of lymphocyte proliferation by high doses of CB ligands and previous reports suggesting cytotoxicity of cannabinoids (Lopez-Cepero et al., 1986), we analyzed cell viability. Higher concentrations of anadamide, CP55940 or Δ^8-THC (not shown) reduced cell viability. To examine this more detail, proliferation and cell viability were analyzed in replicates of the same cultures (Fig. 3). At the highest concentration of anadamide (10^{-4} M) there was a 50% reduction in cell viability but a complete inhibition of proliferation. More importantly, the lower doses of the CB ligands which did not affect cell viability caused significant inhibition of cell proliferation. Furthermore, where anadamide was present only during the final 4 h of the experiment and caused significant inhibition of proliferation (Fig. 2) cell viability was similar as in cultures not treated with anadamide.

These results demonstrate that anadamide and the synthetic cannabinoids are capable of inducing cell death when used at high doses and cell death is at least in part responsible for the complete inhibition of cell proliferation observed at high concentrations. However, anadamide at lower doses also has significant antiproliferative effects which are not associated with the induction of cell death.

We then tested whether the reduction in cell viability by anadamide is due to the induction of necrosis or apoptosis. PBMC were treated with 7.5×10^{-5} anadamide, 3×10^{-5} M Δ^8-THC or 3×10^{-5} M CP55940 for different time intervals and analyzed histochemically by staining with 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI) which enters viable as well as dead cells but only binds to fragmented DNA. Fig. 4 shows staining of fragmented DNA in anadamide treated (Fig. 4B) but not in control cells (Fig. 4A). Δ^8-THC-treated but not CP55940-treated cells displayed similar apoptotic changes (not shown). The apoptotic bodies are not seen in untreated PBMC. For quantification of the anadamide-induced apoptotis, FACS analysis was performed. This showed that anadamide increased the percentage of cells with hypodiploid DNA as compared to media control (Table 1).

To confirm whether anadamide induces internucleosomal cleavage of DNA, we performed Southern blotting on DNA from PBMC that had been treated with 7.5×10^{-5} M anadamide for 1.5, 3 and 6 h. This showed a time-dependent induction of DNA fragmentation of the typical 180 base pair multiples giving rise to the 'ladder pattern' (Fig. 5). The anadamide induction or DNA cleavage was rapid and occurred within 1.5 h.

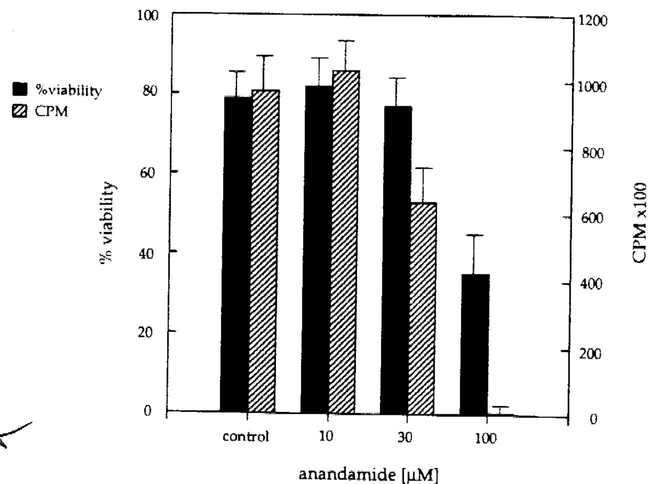

Fig. 3. Effects of CB ligands on lymphocyte viability and proliferation. PBMC were stimulated with anti-CD3 and treated with the indicated doses of anadamide for the entire culture period (72 h). After 72 h, cells were pulsed with [^{3}H]thymidine for 4 h and harvested for scintillation counting. Aliquots of the same cultures that had not been pulsed with [^{3}H]thymidine were stained with Erythrocin red to determine cell viability. Four separate experiments showed similar results.

To analyze the effects of synthetic cannabinoids, PBMC were treated for 3 h with 10^{-5} M and 3×10^{-5} M Δ^8-THC and 3×10^{-6} M, 10^{-5} M and 3×10^{-5} M CP55940 (Fig. 6). Anadamide (7.5×10^{-5} M) was included as a positive control. We observed DNA fragmentation with anadamide and 3×10^{-5} M Δ^8-THC but not with Δ^8-THC at 10^{-5} M. CP55940 did not cause DNA fragmentation at any of the concentrations tested.

Later time points at 4, 8, 16 and 21 h in the same experiment were examined histochemically by DAPI staining of fragmented DNA was seen only in cells treated with 7.5×10^{-5} M anadamide and Δ^8-THC at 3×10^{-5} M. Δ^8-THC at 10^{-5} M did not induce apoptosis even after 21 h. CP55940 also did not induce apoptosis, even at concentrations which are 10 times above those required for the inhibition of proliferation.

Table 1
Induction of lymphoctye apoptosis by anadamide

	6 h	7 h	9 h
Media control	5	8	7
Anadamide	22	36	32

PBMC were cultured in media alone or in the presence of 7.5×10^{-5} M anadamide for the time periods indicated. Cells were stained with propidium iodide and analyzed by flow cytometry. Results are expressed as percent hypodiploid nuclei.

3.3. CB expression in PBMC

The cannabinoid doses required for the effects on lymphocytes are identical to those where cannabinoids cause non-receptor mediated increases in calcium release arachidonic acid metabolism (Felder et al., 1992) and phospholipase A_2 activation (Felder et al., 1993). The following experiments to determined whether CB expression correlates with the effects on lymphocyte proliferation. Two types of cannabinoid receptors have been identified. The brain type cannabinoid receptor (CB1) is expressed on human T and B lymphocytes and monocytes (Bouaboula et al., 1993). The peripheral cannabinoid receptor (CB2) was identified on macrophages (Munro et al., 1993). Both types of cannabinoid receptors bind labeled cannabinoids and cannot be distinguished in binding studies (Munro et al., 1993). We used RT-PCR to test the levels of CB1 and CB2 mRNA expression in PBMC of individual donors (Fig. 7). Both mRNAs were detectable in un-

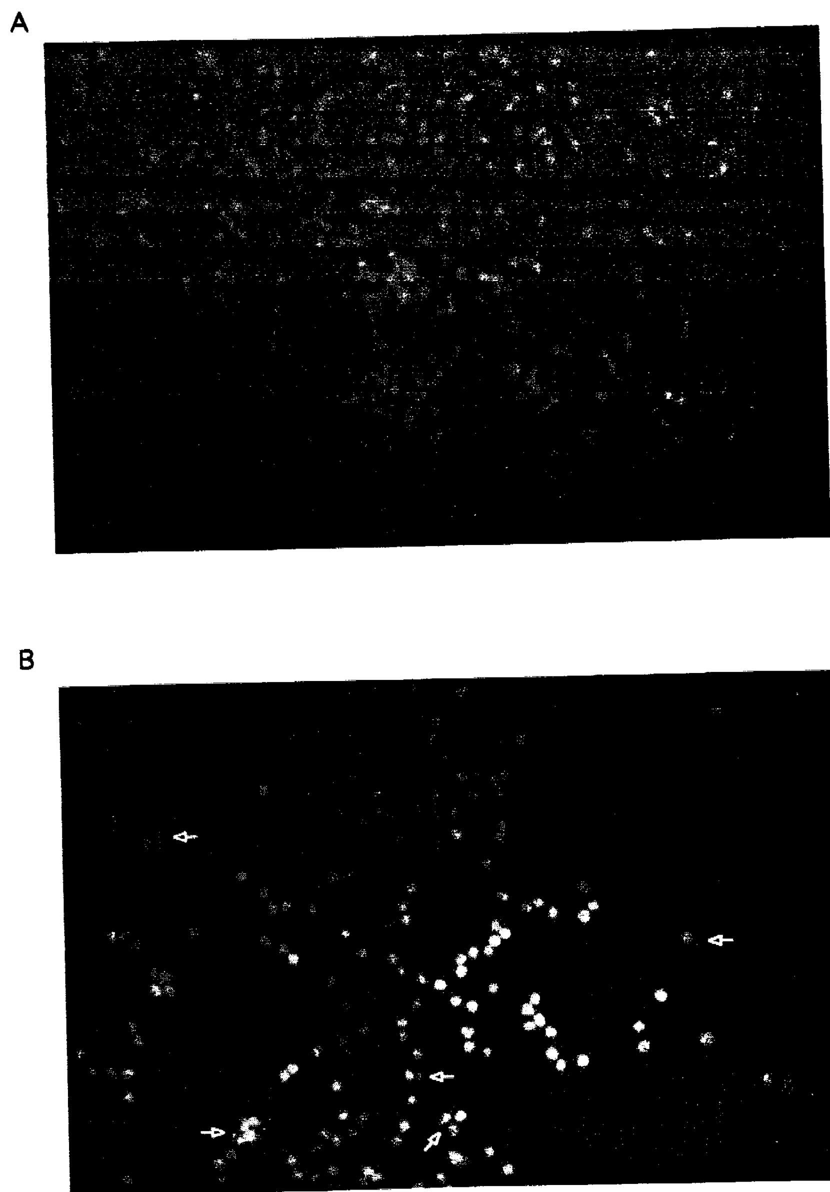

Fig. 4. Anadamide induces DNA fragmentation. PBMC were treated with cannabinoids for 21 h. The cells were collected, fixed and stained with DAPI. Photomicrographs (20 ×) of representative fields are shown. Bright cells are stained with DAPI and are apoptotic. Apoptotic bodies are indicated by arrows. (**A**) Untreated cells. (**B**) 7.5×10^{-5} M anadamide-treated cells.

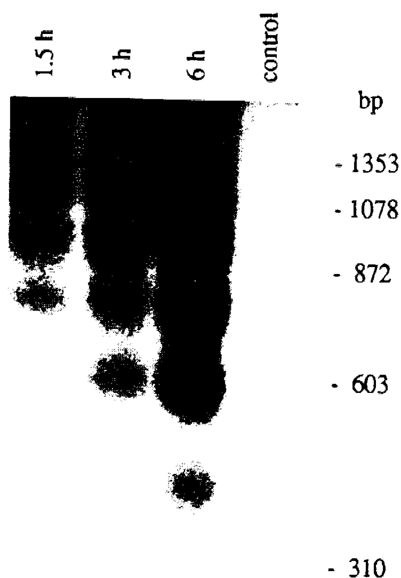

Fig. 5. High concentrations of anadamide cause apoptosis. PBMC were incubated with 7.5×10^{-5} M anadamide for the indicated time intervals. DNA was extracted and analyzed by Southern blotting.

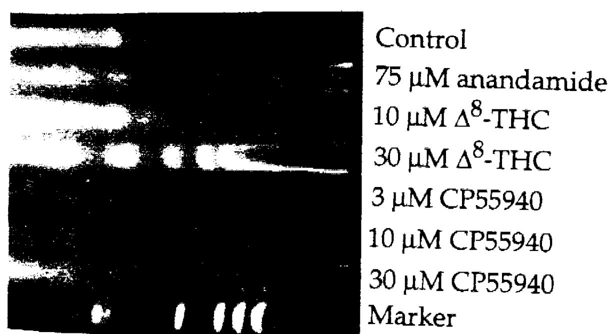

Fig. 6. Anadamide and Δ^8-THC but not CP55940 cause apoptosis. PBMC were incubated for 3 h with various concentrations of cannabinoids. DNA was extracted and analyzed by gel electrophoresis through 2.5% MetaPhor agarose (FMC, Rockland, ME). Marker, PhiX *Hae*III.

stimulated PBMC with variability in their levels among individual donors.

4. Discussion

This study describes the immunomodulatory effects of anadamide, the first identified endogenous cannabinoid receptor (CB) ligand. The results show that anadamide inhibits proliferation of T and B lymphocytes and induces apoptosis.

Cannabinoids had previously been shown to inhibit immune function but the structural basis for these effects and their physiological significance remained unclear. However, two recent advances have renewed interest in the immunoregulatory effects of cannabinoids. First, two CB cDNAs have been cloned. The CB1 cDNA was isolated from rat brain and shown to be a member of the seven-transmembrane-region, G-protein coupled family of receptors (Matsuda et al., 1990; Mailleux et al., 1992; Evans et al., 1992). This CB gene is constitutively expressed by cells of the immune system (Bouaboula et al., 1993). More recently, a second G-protein coupled cannabinoid receptor has been identified as a gene which is expressed in rat splenic

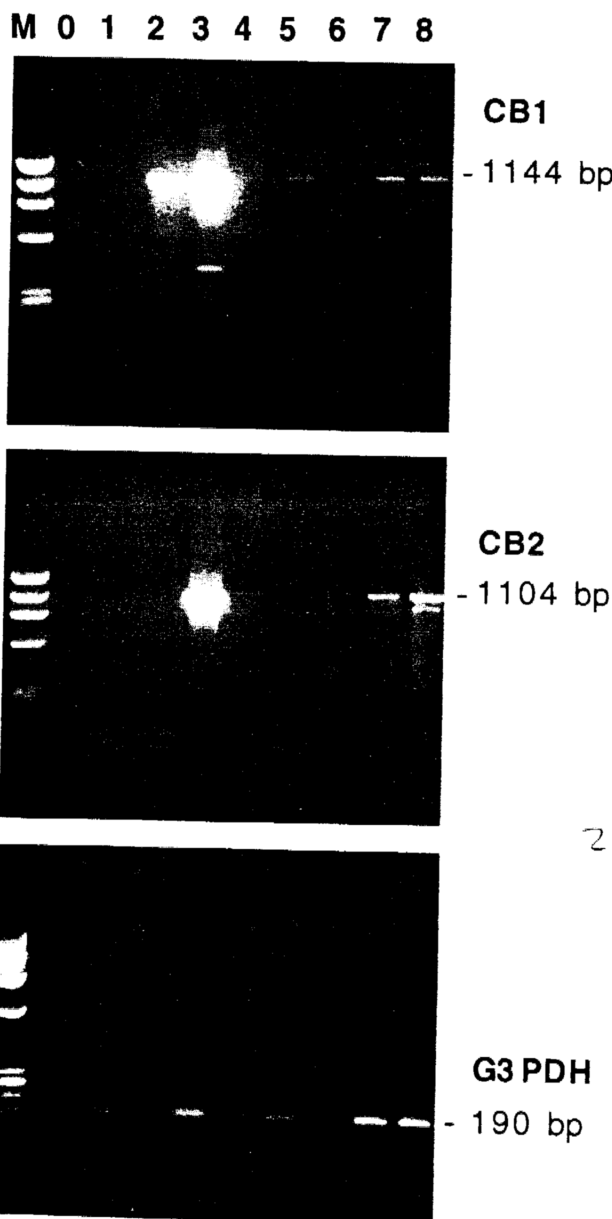

Fig. 7. Donor variability in cannabinoid receptor mRNA expression. PBMC were isolated from eight different donors (nos. 1–8). RNA was extracted and analyzed for CB1 and CB2 expression by RT-PCR. To document similar amounts of RNA and cDNA, the samples were also analyzed for G3PDH. 0 represents the negative PCR control (no template).

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macrophages but not in the brain (Munro et al., 1993). The second advance concerning the immunomodulatory effects of CB ligands was provided by the identification of anadamide as an endogenous CB ligand (Devane et al., 1992b). Anadamide was isolated from porcine brain tissue and showed cannabinoid-like binding to synaptosomal membranes (Devane et al., 1992b) and also bound to the cloned CB1 (Vogel et al., 1993; Felder et al., 1993). Signaling events that are modulated by anadamide include inhibition of forskolin induced cAMP accumulation, inhibition of calcium currents (Fride and Mechoulam, 1993) and stimulation of arachidonic acid and intracellular calcium release (Felder et al., 1993). The latter effects are not mediated by CB and occur at higher doses (1–100 μM) of anadamide or other cannabinoids (Felder et al., 1993). These results raised the possibility that anadamide/CB interactions cause immunosuppression.

The present study shows that anadamide is an inhibitor of T and B lymphocyte proliferation. The anadamide effects were dose-dependent and in a concentration range where it has been shown to regulate neuronal responses. As compared to the effects of two synthetic cannabinoids, anadamide was 3–10-fold less potent. The order of potency of the three cannabinoids in the inhibition of lymphocyte proliferation correlates with the potency of inhibition of forskolin-induced adenylate cyclase activation in CB1-transfected cells (Mackie et al., 1993), suggesting that inhibition of lymphocyte proliferation may be receptor-mediated. However, the relative homogeneity in the inhibitory effects of cannabinoids on proliferation of lymphocytes contrasts with a large variation of CB mRNA expression among individual donors. Definitive information on CB-mediated versus non-CB-mediated mechanisms involved with the antiproliferative effects of cannabinoids will depend on the availability of CB subtype-specific blocking antibodies.

The antiproliferative effects of anadamide were observed with mitogen activation of T or B lymphocytes which represents a much stronger stimulus for lymphocytes as compared to antigens. It is thus conceivable that anadamide is capable of more profoundly or completely inhibiting lymphocyte proliferation in response to antigens. Concentrations of anadamide effective at reducing lymphocyte proliferation did not reduce cell viability or induce apoptosis, suggesting that as part of the biochemical events leading to the induction of apoptosis, DNA synthesis is suppressed prior to the fragmentation of DNA.

Dose–response curves of the different CB ligands were similar in B and T lymphocytes, suggesting comparable numbers of binding sites in these two cell types.

At higher doses anadamide reduced cell viability. This was associated with the induction of apoptosis as indicated by the cleavage of high molecular mass DNA. Δ^8-THC also induced apoptosis at high concentrations but not at the concentrations which were effective at reducing lymphocyte proliferation. CP55940 although 3 and 10 times more effective than Δ^8-THC and anadamide, respectively, in reducing lymphocyte proliferation, did not induce apoptosis, even at concentrations 10 times higher than those required for reduction of lymphocyte proliferation. In a time course analysis over 21 h, where Δ^8-THC at 3×10^{-5} M caused apoptosis within 3 h, the same concentration of CP55940 showed no effect.

In conclusion, these results suggest that immunomodulation via the cannabinoid receptors is not limited to the administration of exogenous ligands but that lymphocytes which express both cannabinoid receptors are sensitive to the antiproliferative effects of the endogenous cannabinoid anadamide. The fact that anadamide is produced in the brain raises the possibility that it participates in neuroimmune interactions. Future studies will determine whether anadamide or other CB ligands are products of hematopoietic cells and whether this mechanism of immunosuppression can be utilized therapeutically.

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