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European Journal of Pharmacology 330 (1997) 231–240

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Influence of fatty acid ethanolamides and Δ^9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells

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Received 17 March 1997; revised 6 May 1997; accepted 12 May 1997

Abstract

The effects of arachidonic acid ethanolamide (anandamide), palmitoylethanolamide and Δ^9-tetrahydrocannabinol on the production of tumor necrosis factor-α (TNF-α), interleukin-4, interleukin-6, interleukin-8, interleukin-10, interferon-γ, p55 and p75 TNF-α soluble receptors by stimulated human peripheral blood mononuclear cells as well as [^{3}H]arachidonic acid release by non-stimulated and *N*-formyl-Met–Leu–Phe (fMLP)-stimulated human monocytes were investigated. Anandamide was shown to diminish interleukin-6 and interleukin-8 production at low nanomolar concentrations (3–30 nM) but inhibited the production of TNF-α, interferon-γ, interleukin-4 and p75 TNF-α soluble receptors at higher concentrations (0.3–3 μM). Palmitoylethanolamide inhibited interleukin-4, interleukin-6, interleukin-8 synthesis and the production of p75 TNF-α soluble receptors at concentrations similar to those of anandamide but failed to influence TNF-α and interferon-γ production. The effect of both compounds on interleukin-6 and interleukin-8 production disappeared with an increase in the concentration used. Neither anandamide nor palmitoylethanolamide influenced interleukin-10 synthesis. Δ^9-Tetrahydrocannabinol exerted a biphasic action on pro-inflammatory cytokine production. TNF-α, interleukin-6 and interleukin-8 synthesis was maximally inhibited by 3 nM Δ^9-tetrahydrocannabinol but stimulated by 3 μM Δ^9-tetrahydrocannabinol, as was interleukin-8 and interferon-γ synthesis. The level of interleukin-4, interleukin-10 and p75 TNF-α soluble receptors was diminished by 3 μM Δ^9-tetrahydrocannabinol. [^{3}H]Arachidonate release was stimulated only by high Δ^9-tetrahydrocannabinol and anandamide concentrations (30 μM). These results suggest that the inhibitory properties of anandamide, palmitoylethanolamide and Δ^9-tetrahydrocannabinol are determined by the activation of the peripheral-type cannabinoid receptors, and that various endogenous fatty acid ethanolamides may participate in the regulation of the immune response. © 1997 Elsevier Science B.V.

Keywords: Anandamide; Palmitoylethanolamide; Tetrahydrocannabinol; Cannabinoid receptors; Cytokines; Mononuclear cells; Immune response

1. Introduction

Arachidonic acid ethanolamide, or anandamide, was recently identified as an endogenous ligand for cannabinoid receptors (Devane et al., 1992). Like classical cannabinoids, anandamide causes antinociception, hypothermia, hypomotility and catalepsy (Crawley et al., 1993; Fride and Mechoulam, 1993; Smith et al., 1994; Wiley et al., 1995), and affects hormone levels (Wenger et al., 1995), pregnancy (Fragkakis et al., 1995) and embryo development (Paria et al., 1995). The suppression of the immune response by natural cannabinoids was demonstrated in a series of studies. Cannabinoids diminish general resistance to bacterial or viral infection (Morahan et al., 1979; Klein et al., 1994; Newton et al., 1994), lymphocyte proliferation, antibody production, natural killer activity and macrophage activities (Friedman et al., 1994). At the molecular level, such a modulation is expressed by the inhibition of the production of cytokines such as interferon (Blanchard et al., 1986; Cabral et al., 1986), tumor necrosis factor-α (TNF-α) (Zheng et al., 1992; Fischer-Stenger et al., 1993; Kusher et al., 1994) and interleukin-2 (Nakano et al., 1992). Cannabinoid treatment leads to an increase rather than a decrease of interleukin-1 activity in endotoxin-stimulated macrophages (Zhu et al., 1994) and to an increase of B-cell proliferation (Derocq et al., 1995). All

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PII S0014-2999(97)01007-8

these data suggest that cannabinoid modulation of the immune response is complex (for review see Berdyshev et al., 1996).

The identification of the immune tissue-associated peripheral type of cannabinoid receptors (cannabinoid CB_2 receptors) clarified in part the molecular basis for the action of cannabinoids on immune cells (Munro et al., 1993). The fact that the cannabinoid CB_2 receptor has only 44% identity with the cannabinoid CB_1 receptor (central nervous system type) throughout the whole protein (Munro et al., 1993), the abundance of cannabinoid CB_2 receptor transcripts in immune tissues and cells (Bouaboula et al., 1993; Galiegue et al., 1995), and the discovered preferential sensitivity of cannabinoid CB_2 receptor-expressing mast cells for palmitoylethanolamide, but not to anandamide (Facci et al., 1995), suggest that anandamide as well as other endogenous 'cannabinoids' may participate in the regulation of the immune response.

However, the influence of anandamide and its structural analogs on the immune and inflammatory response is poorly investigated. To date, anandamide has only been shown to inhibit macrophage-mediated killing of TNF-α-sensitive murine L929 fibroblasts (Cabral et al., 1995) and nitric oxide production by mouse peritoneal macrophages activated by lipopolysaccharide (Coffey et al., 1996). Therefore, we investigated the possible effects of anandamide, palmitoylethanolamide and Δ^9-tetrahydrocannabinol on cytokine and arachidonate release by human peripheral blood mononuclear cells.

2. Materials and methods

2.1. Materials

The following reagents and drugs were used: Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), phosphate-buffered saline (PBS), RPMI-1640 medium, glutamine, penicillin and streptomycin (Gibco, Cergy-Pontoise, France), fetal calf serum (Flow Laboratories, Irvine, UK), [^{3}H]arachidonic acid (Amersham, Les Ulis, France), fatty acid free bovine serum albumin, phenylmethylsulfonylfluoride, lipopolysaccharide from *Escherichia coli*, phytohemagglutinin, *N*-formyl-Met–Leu–Phe (fMLP), arachidonic acid ethanolamide (anandamide), Δ^9-tetrahydrocannabinol, 3,3′,5,5′-tetramethylbenzidine–H_2O_2 solution and carbonate buffer for enzyme immunoassay (Sigma, St. Louis, MO, USA). Palmitoylethanolamide (purity > 99% evaluated by gas chromatography) was synthesized at the Institute of Marine Biology (Vladivostok, Russia).

2.2. Preparation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation ($1100 \times g$ at 4°C for 20 min) on Ficoll–Hypaque. The cells recovered at the interface were washed three times by centrifugation at $600 \times g$ at 4°C for 10 min in phosphate-buffered saline without Ca^{2+} and Mg^{2+} and resuspended in a culture medium consisting of RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) (complete RPMI-1640 medium) at a concentration of 2×10^7 cells/ml.

2.3. Cytokine production

To study the effect of Δ^9-tetrahydrocannabinol and fatty acid ethanolamides on lipopolysaccharide (2.5 μg/ml)-induced or phytohemagglutinin (10 μg/ml)-induced cytokine production, peripheral blood mononuclear cell concentration was adjusted to 1.5×10^6 cells/ml (TNF-α, interleukin-6, interleukin-8, interleukin-10, p55 and p75 TNF-α soluble receptor production) or 2.5×10^6 cells/ml (interleukin-4 and interferon-γ production), and the cells were cultured in 24-well plates (Nunc, Napeville, IL, USA) in complete RPMI-1640 medium, at 37°C in a 5% CO_2 and 95% humidity atmosphere, with or without the indicated stimuli. After 24 h for lipopolysaccharide stimulation or 48 h for phytohemagglutinin stimulation, cell-free supernatants were collected, centrifuged, and stored frozen at −30°C prior to cytokine determination by specific ELISA.

TNF-α concentration was determined using DUOSET system (Genzyme diagnostics, Cambridge, MA, USA). Other cytokines and p55 and p75 TNF-α soluble receptors were quantified using commercial kits (Endogen, Cambridge, MA, USA; Genzyme Diagnostics; BIOAdvance, Emerainville, France). Sensitivities of the assays were 3 pg/ml. The supernatant lactate dehydrogenase activity was measured to assess compound cytotoxicity using a commercial kit (Unimate 5, Roche Diagnostic Systems, Basel, Switzerland).

2.4. Arachidonate release

For arachidonic acid incorporation, peripheral blood mononuclear cells (200×10^6 cells in 30 ml complete RPMI-1640 medium per 145-mm diameter Petri dish) were labeled with [^{3}H]arachidonic acid (2 mCi/200×10^6 cells) for 2 h as previously described (Hichami et al., 1995). The supernatant was discarded, and the adherent monocytes were scraped off with a plastic policeman and washed twice with cold (4°C) phosphate-buffered saline without Ca^{2+} and Mg^{2+} supplemented with 0.2% bovine serum albumin. The cells were finally resuspended in RPMI-1640 medium supplemented with 0.2% bovine serum albumin at a concentration of 2×10^7 cells/ml. Aliquots of cell suspension (400 μl, 5×10^6 cells) were distributed in 5 ml polypropylene tubes and incubated at 37°C in an atmosphere of 5% CO_2 and 95% humidity for 30 min. Cells were then treated with 50 μl of various

drugs at appropriate concentrations or vehicle. The cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 30 μM), or vehicle (ethanol, 0.1% final concentration). All compounds, supplied as ethanol solutions, were diluted in RPMI-1640 medium supplemented with 0.5% bovine serum albumin. Cells were stimulated with fMLP (50 μl, 1 μM final concentration) for 10 min, in the presence or absence of test compounds. Samples were then centrifuged for 3 min at $1100 \times g$, 350 μl of supernatant was added to 2 ml of Ultima Gold scintillation cocktail (Packard Instruments, Meriden, CT, USA) in Pico vials (Packard), and samples were counted in a liquid scintillation analyzer (Beckman LS 6500).

2.5. Data analysis

Results for [^{3}H]arachidonate release are expressed as the difference, in %, of the cpm recovered in the supernatants over the control value (cells with vehicle or cells with vehicle plus fMLP). Release of cytokines is expressed as the difference, in %, of pg/ml recovered in the supernatant over the control value (cells stimulated by lipopolysaccharide for TNF-α, interleukin-6, interleukin-8, interleukin-10, p55 and p75 TNF-α soluble receptors, or phytohemagglutinin for interleukin-4 and interferon-γ). Results are expressed as the mean of the difference ± S.E.M. of at least three experiments done in triplicate (arachidonate release) or in duplicate (cytokine release). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test.

3. Results

3.1. The effect of anandamide, palmitoylethanolamide and Δ^9-tetrahydrocannabinol on monocyte-dependent secretion of interleukin-6, interleukin-8, interleukin-10, TNF-α, p55 and p75 TNF-α soluble receptors

We examined the influence of physiologically relevant concentrations of anandamide, palmitoylethanolamide and Δ^9-tetrahydrocannabinol (3 nM to 3 μM) on the secretion of TNF-α, interleukin-6, interleukin-8, interleukin-10, p55 and p75 TNF-α soluble receptors after stimulation of peripheral blood mononuclear cells with lipopolysaccharide for 24 h. As shown in Figs. 1 and 2, Δ^9-tetrahydrocannabinol as well as fatty acid ethanolamides modified the secretion of the pro-inflammatory cytokines measured. The lowest used concentration of Δ^9-tetrahydrocannabinol (3 nM) exerted a maximal inhibitory effect on the secretion of TNF-α (by 18.8%, $P < 0.01$, $n = 6$), interleukin-6 (by 34.1%, $P < 0.05$, $n = 3$) and interleukin-8 (by 28.7%, n.s.). Anandamide diminished the secretion of these three cytokines with a significant difference in potency. The maximal inhibition of TNF-α level (by 41.2%, $P < 0.001$, $n = 6$) was obtained at 3 μM anandamide whereas much lower anandamide concentrations (30 nM and 3 nM) were needed to suppress interleukin-8 (by 36.3%, $P < 0.05$, $n = 3$) and interleukin-6 (by 27.7%, $P < 0.05$, $n = 3$) levels. Palmitoylethanolamide had no significant effect on TNF-α secretion but diminished the supernatant level of interleukin-6 (by 27.7%, $P < 0.05$, $n = 3$) and interleukin-8 (by 37.3%, n.s.) at 300 nM and 30 nM, respectively.

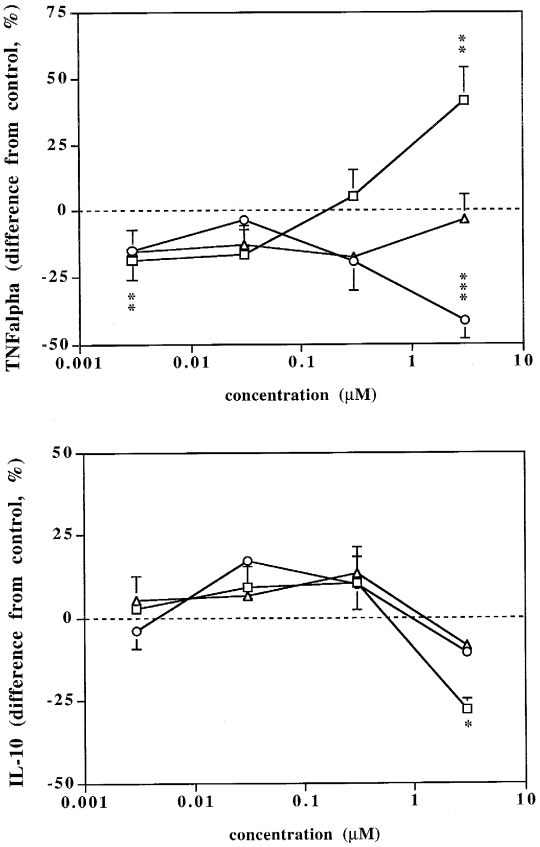

Fig. 1. Effect of Δ^9-tetrahydrocannabinol (□), anandamide (○) and palmitoylethanolamide (△) on TNF-α (TNF-alpha) and interleukin-10 (IL-10) release from lipopolysaccharide-stimulated peripheral blood mononuclear cells. Cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 3 μM), or vehicle (ethanol, 0.1% final concentration) prior to lipopolysaccharide stimulation (2.5 μg/ml). Cells were cultured for 24 h, and then cell-free supernatants were collected, centrifuged, and stored frozen at −30°C prior to cytokine level determination by specific ELISA. Results are expressed as the difference (in %) ± S.E.M of pg/ml recovered in the supernatant over the control value (cells stimulated by lipopolysaccharide). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 3$–6, in duplicate).

The supernatant level of interleukin-10 was influenced only by Δ^9-tetrahydrocannabinol. The effect of Δ^9-tetrahydrocannabinol was inhibitory (by 27.8%, $P < 0.05$, $n = 3$) and seen only at a concentration of 3 μM (Fig. 1).

At micromolar concentrations, Δ^9-tetrahydrocannabinol exerted a complex action on the level of pro-inflammatory cytokines. As shown in Figs. 1 and 2, the supernatant

concentration of TNF-α, interleukin-6 and interleukin-8 was significantly increased rather than decreased by 3 μM Δ^9-tetrahydrocannabinol (by 41.4% for TNF-α, $P < 0.01$, $n = 6$; by 66.7% for interleukin-6 and by 63.7% for interleukin-8). Moreover, an extremely high Δ^9-tetrahydrocannabinol concentration (30 μM) markedly reduced the TNF-α level (by 44.1%) without having a toxic effect, as assessed by measuring lactate dehydrogenase in the supernatant (data not shown). Neither anandamide nor palmitoylethanolamide could augment cytokine secretion above control levels. Nevertheless, the highest concentration of both fatty acid ethanolamides had the lowest inhibitory effect on interleukin-6 and interleukin-8 secretion.

None of the compounds changed the supernatant level of p55 TNF-α soluble receptors but had an inhibitory effect on the secretion of p75 TNF-α soluble receptors (Fig. 3). The inhibition was maximal at 3 μM concentrations of the compounds (by 43.4% for Δ^9-tetrahydrocannabinol, $P < 0.05$, $n = 3$; by 34.9% for palmitoylethanolamide, $P < 0.05$, $n = 3$; by 31.7% for anandamide, $P < 0.05$, $n = 3$). A statistically significant inhibition of the p75 TNF-α soluble receptor secretion was seen also at 3 nM and 30 nM palmitoylethanolamide (by 27.4% and 27.0%, $P < 0.05$, $n = 3$, respectively).

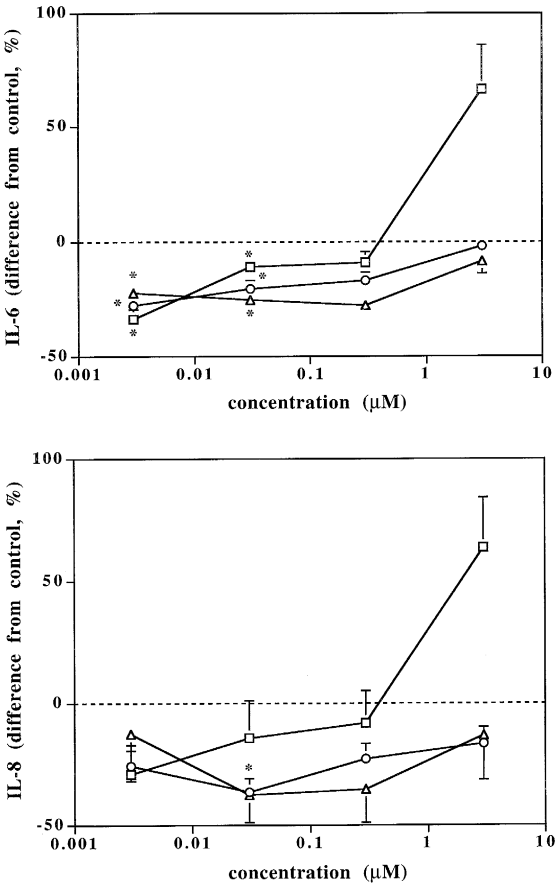

Fig. 2. Effect of Δ^9-tetrahydrocannabinol (□), anandamide (○) and palmitoylethanolamide (△) on interleukin-6 (IL-6) and interleukin-8 (IL-8) release from lipopolysaccharide-stimulated peripheral blood mononuclear cells. Cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 3 μM), or vehicle (ethanol, 0.1% final concentration) prior to lipopolysaccharide stimulation (2.5 μg/ml). Cells were cultured for 24 h, and then cell-free supernatants were collected, centrifuged, and stored frozen at −30°C prior to cytokine level determination by specific ELISA. Results are expressed as the difference (in %) ±S.E.M of pg/ml recovered in the supernatant over the control value (cells stimulated by lipopolysaccharide). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. * $P < 0.05$ ($n = 3$, in duplicate).

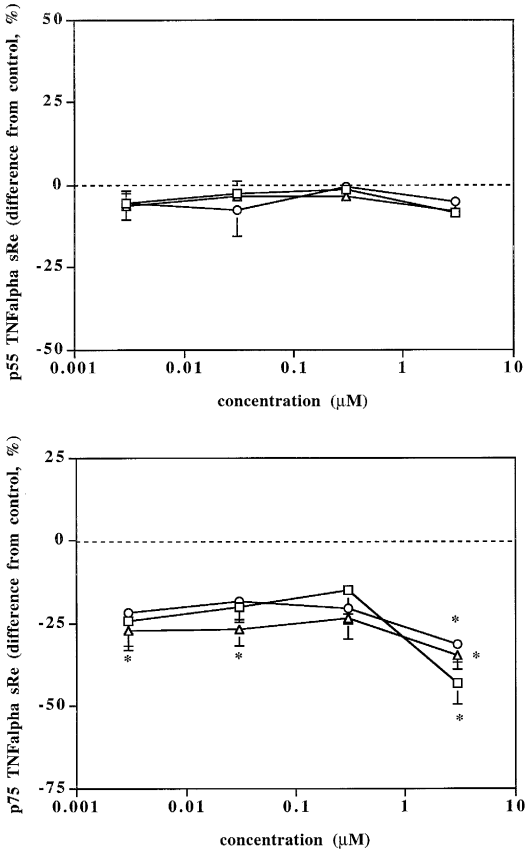

Fig. 3. Effect of Δ^9-tetrahydrocannabinol (□), anandamide (○) and palmitoylethanolamide (△) on the release of p55 TNF-α soluble receptors (p55 TNF-α sRe) and p75 TNF-α soluble receptors (p75 TNF-α sRe) from lipopolysaccharide-stimulated peripheral blood mononuclear cells. Cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 3 μM), or vehicle (ethanol, 0.1% final concentration) prior to lipopolysaccharide stimulation (2.5 μg/ml). Cells were cultured for 24 h, and then cell-free supernatants were collected, centrifuged, and stored frozen at −30°C prior to cytokine level determination by specific ELISA. Results are expressed as the difference (in %) ±S.E.M of pg/ml recovered in the supernatant over the control value (cells stimulated by lipopolysaccharide). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. * $P < 0.05$ ($n = 3$, in duplicate).

3.2. Modulation of lymphocyte-derived interleukin-4 and interferon-γ secretion by fatty acid ethanolamides and Δ^9-tetrahydrocannabinol

To determine the effect of anandamide, palmitoylethanolamide and Δ^9-tetrahydrocannabinol on lympho-

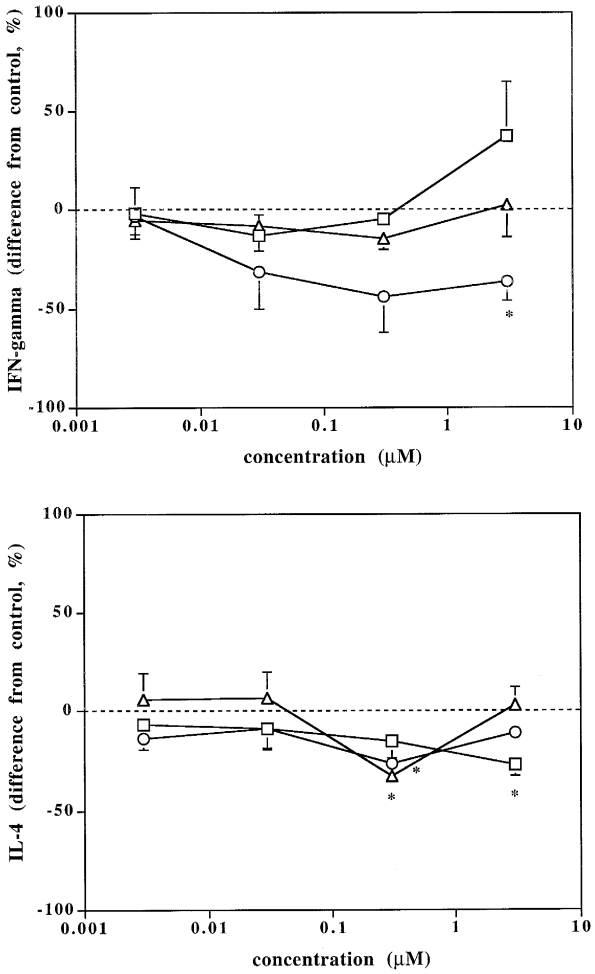

Fig. 4. Effect of Δ^9-tetrahydrocannabinol (□), anandamide (○) and palmitoylethanolamide (△) on interferon-γ (IFN-gamma) and interleukin-4 (IL-4) release from phytohemagglutinin-stimulated peripheral blood mononuclear cells. Cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 3 μM), or vehicle (ethanol, 0.1% final concentration) prior to phytohemagglutinin stimulation (10 μg/ml). Cells were cultured for 48 h, and then cell-free supernatants were collected, centrifuged, and stored frozen at −30°C prior to cytokine level determination by specific ELISA. Results are expressed as the difference (in %) ±S.E.M of pg/ml recovered in the supernatant over the control value (cells stimulated by phytohemagglutinin). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. * $P < 0.05$ ($n = 3$, in duplicate).

cyte functions, peripheral blood mononuclear cells were incubated with compounds or vehicle for 30 min and then activated by phytohemagglutinin, and the supernatant interleukin-4 and interferon-γ levels were measured after 48 h of stimulation. As shown in Fig. 4, anandamide displayed a significant inhibitory effect on the production of interferon-γ in the 0.3–3 μM range of concentrations. Palmitoylethanolamide and Δ^9-tetrahydrocannabinol failed to demonstrate similar properties. As in the case for monocyte-dependent cytokine production, 3 μM Δ^9-tetrahydrocannabinol stimulated rather than inhibited lymphocyte-dependent interferon-γ production.

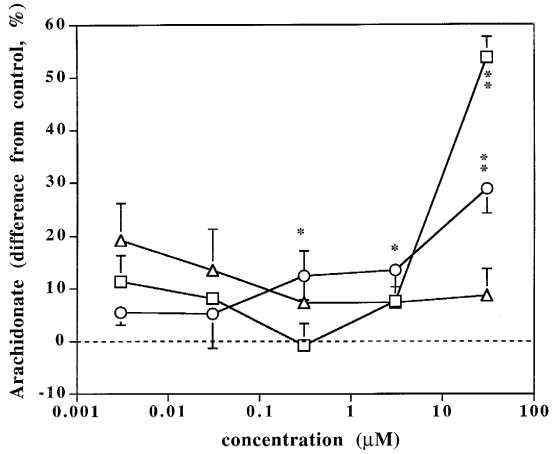

Fig. 5. Effect of Δ^9-tetrahydrocannabinol (□), anandamide (○) and palmitoylethanolamide (△) on [^{3}H]arachidonate release from fMLP-stimulated peripheral blood mononuclear cells. Cells were labeled with [^{3}H]arachidonic acid (2 mCi/200×10^6 cells) for 2 h. The cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 30 μM), or vehicle (ethanol, 0.1% final concentration). Cells were stimulated with fMLP (1 μM). Results are expressed as the difference (in %) ±S.E.M of cpm recovered in the supernatant over the control value (cells stimulated by fMLP). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. * $P < 0.05$; ** $P < 0.01$ ($n = 5$, in triplicate).

The secretion of interleukin-4 was inhibited by all compounds (Fig. 4). The inhibition was seen at 3 μM Δ^9-tetrahydrocannabinol (by 27.0%, $P < 0.05$, $n = 3$), 0.3 μM anandamide and 0.3 μM palmitoylethanolamide (by 26.0%, $P < 0.05$ and by 32.2%, $P < 0.05$, $n = 3$, respectively).

3.3. Influence of Δ^9-tetrahydrocannabinol and fatty acid ethanolamides on [^{3}H]arachidonate release by human monocytes

We compared the ability of a wide concentration range of Δ^9-tetrahydrocannabinol, anandamide and palmi-

Table 1
Effect of Δ^9-tetrahydrocannabinol, anandamide and palmitoylethanolamide on [^{3}H]arachidonate release from non-stimulated peripheral blood mononuclear cells

Concentrations (μM)	[^{3}H] Arachidonate release (the difference from control, in %)		
	Δ^9-tetrahydrocannabinol	anandamide	palmitoylethanolamide
30	26.6±3.8 [b]	20.5±0.9 [c]	4.1±0.9 [a]
3	7.7±0.5 [b]	11.5±2 [c]	6.3±0.4 [a]
0.3	6±4 [a]	9.5±1.8 [c]	nd

Cells were labeled with [^{3}H]arachidonic acid (2 mCi/200×10^6 cells) for 2 h. The cells were incubated for 30 min with anandamide, palmitoylethanolamide, Δ^9-tetrahydrocannabinol (3 nM to 30 μM), or vehicle (ethanol, 0.1% final concentration). Results are expressed as a difference (in %) ±S.E.M of cpm recovered in the supernatant over the control value (incubation of the cells with vehicle). Analysis of statistical significance was done using variance analysis (ANOVA) and Student's *t*-test. [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ ($n = 5$, in triplicate).

toylethanolamide to modulate fMLP-induced [^{3}H]arachidonate release by human monocytes. As shown in Fig. 5, only extremely high concentrations of Δ^9-tetrahydrocannabinol and anandamide (30 μM) were able to augment arachidonate release (by 53.7%, $P < 0.01$, $n = 5$ and by 28.7%, $P < 0.01$, $n = 5$, respectively). Palmitoylethanolamide was inactive at all concentrations tested. Treatment of cells with phenylmethylsulfonylfluoride (50 μM) was without effect on the release of arachidonic acid (data not shown). Without cell activation by fMLP, extremely high concentrations of Δ^9-tetrahydrocannabinol and anandamide (30 μM) stimulated [^{3}H]arachidonate release as well. However, this stimulation did not exceed 30% and became negligible at 3 μM (Table 1).

4. Discussion

The present manuscript reports complex effects of fatty acid ethanolamides and Δ^9-tetrahydrocannabinol on the activity of mononuclear cells in terms of the inflammatory response in vitro.

The first evidence for immunomodulatory properties of fatty acid ethanolamides was presented as early as in 1957 when *N*-palmitoylethanolamine was identified as the active principle of fractions with anti-inflammatory properties obtained from peanut oil, egg yolk and soybean lecithin (Kuehl et al., 1957). Later, the antiviral and antibacterial properties of *N*-palmitoylethanolamine were proposed. Oral administration of this compound to mice decreased mortality caused by *Shigella dysenteriae* toxin, streptolysin O, or live group A *Streptococcus*, as well as the mortality rate resulting from traumatic shock (Ràskovà and Mašek, 1967; Rašková et al., 1972). Several clinical trials have demonstrated the prophylactic efficacy of oral administration of palmitoylethanolamide in upper respiratory tract viral infections (Perlík et al., 1971).

In 1992, the arachidonic acid ethanolamide, named anandamide, was identified as the endogenous ligand for the central nervous system-type (CB_1) cannabinoid receptor (Devane et al., 1992). This finding appeared simultaneously with the identification of the second, peripheral-type (CB_2) cannabinoid receptor (Munro et al., 1993), which is mainly associated with immune tissues and cells (Bouaboula et al., 1993). The subsequent demonstration of high-binding affinities to cannabinoid CB_2 receptors of different cannabinoids and anandamide (Bayewitch et al., 1995; Felder et al., 1995; Slipetz et al., 1995) provided evidence for a specific, receptor-mediated component of the known suppression of immune response by cannabinoids.

The results reported here characterize the complex immunosuppressive properties of anandamide, a compound which has never been thoroughly explored as potential modulator of immune and inflammatory responses. The potency of anandamide was compared to that of palmitoylethanolamide, which is a poor ligand for the central nervous system-type cannabinoid CB_1 receptor (Mechoulam et al., 1994) and to that of the classical cannabinoid Δ^9-tetrahydrocannabinol which can modulate the production of different cytokines (Blanchard et al., 1986; Zheng et al., 1992; Fischer-Stenger et al., 1993; Klein et al., 1994; Shivers et al., 1994; Zhu et al., 1994) and stimulate arachidonic acid release (Burstein et al., 1994; Diaz et al., 1994). In contrast to the majority of studies, we tested a wide range of concentrations of compounds, beginning from the nanomolar ones, which are relevant to binding experiments (Mechoulam et al., 1994), up to physiologically attainable and generally used micromolar concentrations. The use of a mixed population of peripheral blood mononuclear cells permitted us to evaluate the complex cellular response to the treatment.

Our study demonstrated the potency of anandamide to diminish the monocyte-dependent production of TNF-α, interleukin-6 and interleukin-8 as well as the release of p75 TNF-α soluble receptor and the lymphocyte-dependent production of interleukin-4 and interferon-γ by human peripheral blood mononuclear cells in vitro. To date, anandamide has been shown only to inhibit macrophage-mediated killing of TNF-α-sensitive murine fibroblasts after i.p. injection of the high dose of 20 mg/kg anandamide (Cabral et al., 1995) and to inhibit nitric oxide production (3 μM anandamide) by mouse peritoneal macrophages activated by lipopolysaccharide (Coffey et al., 1996). In our experiments, the maximal inhibition of TNF-α production was also achieved at a relatively high anandamide concentration (3 μM). Nearly the same concentration range of the compound (0.3–3 μM) was needed to diminish interleukin-4 and interferon-γ production. The maximal inhibition of interleukin-6 and interleukin-8 synthesis was seen at 3–30 nM anandamide, suggesting a receptor-mediated mode of action of the compound. Surprisingly, increasing the compound's concentration led to a smaller inhibitory effect instead of the expected enhancing effect. Such a phenomenon is likely to reflect the complex nature of the influence of anandamide on cytokine production by human monocytes. Unfortunately, the absence of specific cannabinoid CB_2 receptor antagonists or binding antibodies does not permit us to confirm the receptor-mediated signaling and to separate possible non-specific and cannabinoid CB_2 receptor-independent actions of anandamide. Potent membrane-stabilizing properties of fatty acid ethanolamides have been demonstrated (Ambrosini et al., 1993), and this may influence the activity of membrane-associated and receptor-coupled enzymes. Moreover, the different effect of anandamide on interleukin-6, interleukin-8 and TNF-α production, measured in the same culture supernatants, demonstrates the distinct sensitivity of monocytes to the compound and suggests multiple mechanisms are implicated in the regulation of signal transduction by anandamide.

In contrast to the absence of palmitoylethanolamide binding to cannabinoid CB_1 receptors, this fatty acid ethanolamide proved to be a highly potent ligand for cannabinoid CB_2 receptors expressed on RBL-2H3 mast cell membranes (Facci et al., 1995). Moreover, palmitoylethanolamide was more powerful than anandamide and Δ^9-tetrahydrocannabinol in displacing [^{3}H]WIN 55,212-2, a radioactive cannabinoid analog, from mast cell cannabinoid CB_2 receptor. The down-regulation of mast cell activation by palmitoylethanolamide, but not by anandamide, has also been demonstrated (Facci et al., 1995). Similarly, palmitoylethanolamide, in contrast to anandamide, has been shown to protect cultured mouse cerebellar granule cells against glutamate toxicity, possibly through the activation of cannabinoid CB_2 receptors (Skaper et al., 1996). Aloe et al. (1993) have also shown in vivo mast cell down-regulation by short-chain and long-chain saturated fatty acid ethanolamides. The authors suggested that saturated fatty acid ethanolamides, such as palmitoylethanolamide, may behave as local autacoids capable of down-regulating mast cell activation and deleterious cellular processes following pathological events. Our data present additional evidence for the ability of palmitoylethanolamide to suppress immune cell activation. Similar to anandamide and over the same concentration range, palmitoylethanolamide inhibited interleukin-4, interleukin-6 and interleukin-8 production by activated human mononuclear cells. In contrast to anandamide, the compound had no effect on monocyte-dependent TNF-α production and lymphocyte-dependent interferon-γ synthesis. This discrepancy between the regulation by palmitoylethanolamide and anandamide of cytokine synthesis by human immune cells suggests a selective recognition of compounds, possibly at the receptor level, depending on the signaling pathways activated.

As for Δ^9-tetrahydrocannabinol, this cannabinoid displayed a multiform action on the production of cytokines by human peripheral blood mononuclear cells. Three μM Δ^9-tetrahydrocannabinol augmented significantly the monocyte-dependent production of TNF-α, interleukin-6 and interleukin-8, with some variability between different blood samples, and inhibited interleukin-10 production. A decrease in Δ^9-tetrahydrocannabinol concentration led to the inhibition of TNF-α and interleukin-6 synthesis, with maximum suppression occurring at 3 nM Δ^9-tetrahydrocannabinol, and to the disappearance of the inhibition of interleukin-10 synthesis. The compound did not exert statistically significant inhibitory effects on interleukin-8 synthesis. Three μM Δ^9-tetrahydrocannabinol inhibited lymphocyte-dependent interleukin-4 production and augmented slightly the production of interferon-γ. Lowering the Δ^9-tetrahydrocannabinol concentration led to the disappearance of the compound's influence on the synthesis of these two cytokines. It is of interest to note that an increase in Δ^9-tetrahydrocannabinol concentration up to 30 μM (tested only for TNF-α) diminished drastically the culture supernatant TNF-α level without exerting a toxic effect on the cells, as assessed by measurement of the supernatant lactate dehydrogenase activity. This finding may explain the previously observed dual action of Δ^9-tetrahydrocannabinol on TNF-α production (Zheng et al., 1992; Fischer-Stenger et al., 1993; Shivers et al., 1994) and lymphocyte proliferation (Specter et al., 1990; Schwartz et al., 1994; Derocq et al., 1995), as high micromolar concentrations of the compound might exert both types of modulation depending on the cell type and culture conditions.

Cytokine production represents a complex network with positive and negative feedback relationships between different cytokines. Today, it is well-established that interleukin-10 negatively regulates the synthesis of numerous cytokines such as TNF-α, interleukin-6, interleukin-8 (Levitz et al., 1996; Thomassen et al., 1996), and its own production is positively regulated by pro-inflammatory cytokines (Daftarian et al., 1996). It was reasonable to suppose that the influence of fatty acid ethanolamides and Δ^9-tetrahydrocannabinol on TNF-α/interleukin-10 production by monocytes is a dependent process. However, we failed to establish such dependence if one assumes the same mechanism of cell activation by Δ^9-tetrahydrocannabinol and fatty acid ethanolamides. Thus, if the inhibition of interleukin-10 production by 3 μM Δ^9-tetrahydrocannabinol paralleled by an increase in TNF-α production, the absence of an effect of anandamide on interleukin-10 production accompanied by a significant decrease in TNF-α release.

Soluble TNF-α receptors present an important mechanism of regulation of TNF-α bioactivity. Human monocytes express predominantly the p75 TNF-α receptor and release its soluble form upon stimulation (Galvederochemonteix et al., 1996). Indeed, we detected a negligible quantity of p55 TNF-α soluble receptors released by monocytes under our experimental conditions, and much higher level of p75 TNF-α soluble receptors. The elevated level of p75 TNF-α soluble receptors might interfere with TNF-α determination, and it was necessary to estimate the influence of compounds on the release of this anti-inflammatory factor. We found a decline of p75 TNF-α soluble receptor release by stimulated monocytes in the presence of fatty acid ethanolamides as well as Δ^9-tetrahydrocannabinol, a decline that did not correlate with the adverse effect of compounds on TNF-α production. Hart et al. (1996) demonstrated that the stimulation of p75 TNF-α soluble receptor release by human monocytes is under the partial positive control of interleukin-10. In our experiments, fatty acid ethanolamides diminished the release of p75 TNF-α soluble receptors without affecting the synthesis of interleukin-10, while 3 μM Δ^9-tetrahydrocannabinol showed an inhibitory effect on interleukin-10 synthesis.

It is known that Δ^9-tetrahydrocannabinol has specific (cannabinoid CB_1–CB_2 receptors) as well as non-specific

binding sites throughout a wide range of tissues and cells. The highly lipophilic nature of cannabinoids (Wing et al., 1985; Makriyannis and Rapaka, 1990) cannot solely explain their non-specific binding, since these compounds were shown to bind equipotently to tissues with high and low fat content (Lynn and Herkenham, 1994). In addition, Fischer-Stenger et al. (1993) have demonstrated that the high 10 μM Δ^9-tetrahydrocannabinol concentration did not interfere with the process of TNF-α secretion by the RAW264.7 macrophage cell line, which is membrane-determined, but diminished the intracellular conversion of the TNF-α 26-kD presecreted form to the 17-kD secreted form without affecting the level of TNF-α messenger RNA. Derocq et al. (1995) described the pertussis toxin-sensitive stimulatory effect of low nanomolar concentrations of Δ^9-tetrahydrocannabinol and other synthetic cannabinoids on lymphocyte proliferation which became inhibitory and pertussis toxin-insensitive at 10 μM concentrations. It is therefore possible that the inhibitory effect of nanomolar concentrations of Δ^9-tetrahydrocannabinol on TNF-α and interleukin-6 production by peripheral blood mononuclear cells in our experiments reflects the receptor-mediated action of the compound which is masked by the dominant non-specific stimulatory effect elicited at higher concentrations of the compound. Possibly, the same non-specific binding sites are responsible for the decrease in the inhibitory effect of micromolar concentrations of anandamide and palmitoylethanolamide on interleukin-6 synthesis by peripheral blood mononuclear cells. Subsequent studies are needed to clarify the nature of cannabinoid non-specific binding sites to understand their role in the modulation of cellular response by classical cannabinoids as well as fatty acid ethanolamides.

Cannabinoids and anandamide have been shown to stimulate the release of arachidonic acid and its metabolites by various cells, including human peripheral blood mononuclear cells (Burstein et al., 1994; Diaz et al., 1994; Felder et al., 1995; Wartmann et al., 1995). The stimulation of arachidonic acid release by cannabinoids is thought to be cannabinoid receptor-independent since it occurs only at micromolar concentrations of the compounds and also appears in the cells which do not express cannabinoid CB_1 or CB_2 receptors (Felder et al., 1993). The involvement of G-proteins in anandamide-induced arachidonate release by WI-38 fibroblasts has also been demonstrated (Wartmann et al., 1995). Our data confirm the ability of anandamide and Δ^9-tetrahydrocannabinol, but not of palmitoylethanolamide, to stimulate arachidonate release by non-activated and fMLP-activated human monocytes. However, this stimulation appears to be important only at extremely high concentrations of the compounds, and suggests a non-specific action on the cells. The effect of the test compounds, at least that of anandamide, on the release of arachidonate by fMLP-stimulated monocytes seems to be additive to the effect of fMLP itself, suggesting the absence of interference with the classical cascade of biochemical reactions induced by the activation of the fMLP receptor. The possible interference of ethanolamide amidase in our experiments was excluded since pretreatment of the cells with phenylmethylsulfonylfluoride did not increase the amount of radioactivity released.

Diaz et al. (1994) reported that Δ^9-tetrahydrocannabinol (32 μM) treatment of human peripheral blood mononuclear cells leads to an increase in arachidonic acid metabolism through the lipoxygenase pathway. Leukotrienes are known to be important mediators of the acute inflammatory response, playing an immuno-enhancing role (Rola-Pleszczynski, 1985). However, the significance of this pathway for the immune regulation by fatty acid ethanolamides is hardly physiologically relevant since large concentrations of anandamide are needed to stimulate the release of arachidonic acid and its metabolites.

Thus, we report the first evidence for the down-regulation of cytokine production by anandamide and palmitoylethanolamide in vitro in human mononuclear cells. Importantly, this down-regulation occurred mainly at low nanomolar concentrations of anandamide and palmitoylethanolamide, suggesting a cannabinoid CB_2 receptor-mediated mode of action for both compounds. The clear difference in the inhibition exerted by these two fatty acid ethanolamides of monocyte-dependent interleukin-6 and interleukin-8 production in comparison to TNF-α release suggests different cannabinoid receptor coupling to the subsequent steps of cell activation, depending on the cytokine synthesized. The ability of anandamide but not of palmitoylethanolamide to decrease interferon-γ synthesis by phytohemagglutinin-stimulated lymphocytes is an additional indication for the specific cell sensitivity to different molecular species of fatty acid ethanolamides, and stresses the importance of fatty acid ethanolamides as possible endogenous autacoids playing a multiple down-regulatory role in the complex network of immune responses. As for Δ^9-tetrahydrocannabinol, the discovered inversion of its inhibitory effect on cytokine production with an increase in concentration suggests the significance of non-specific interactions of this cannabinoid with cells involved in immune and inflammatory processes. Different cells are known to synthesize a wide range of fatty acid ethanolamides concomitantly with anandamide (Di Marzo et al., 1994; Sugiura et al., 1996), and extensive additional studies are needed to clarify the real place of these fatty acid derivatives and cannabinoid receptors in cell interactions.

Acknowledgements

the authors thank Laurent Corcos for his helpful discussion, and Dr. Tardivel and Mrs. Massot (CRTS Rennes) for the supply of buffy coats

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