The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines

J.-M. Derocq*, M. Bouaboula, J. Marchand, M. Rinaldi-Carmona, M. Ségui, P. Casellas

Sanofi Recherche, 371 rue du Professeur Blayac, 34184 Montpellier Cedex 04, France

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Abstract The effect of anandamide, an endogenous ligand for central (CB1) and peripheral (CB2) cannabinoid receptors, was investigated on the growth of the murine IL-6-dependent lymphoid cell line B9 and the murine IL-3-dependent myeloblastic cell line FDC-P1. In conditions of low serum level, anandamide potentiated the growth of both cytokine-dependent cell lines. Comparison with other fatty acid cannabinoid ligands such as (*R*)-methanandamide, a ligand with improved selectivity for the CB1 receptor, or palmitylethanolamide, an endogenous ligand for the CB2 receptor, showed a very similar effect, suggesting that cell growth enhancement by anandamide or its analogs could be mediated through either receptor subtype. However, several lines of evidence indicated that this growth-promoting effect was cannabinoid receptor-independent. First, the potent synthetic cannabinoid agonist CP 55940, which displays high affinity for both receptors, was inactive in this model. Second, SR 141716A and SR 144528, which are potent and specific antagonists of CB1 and CB2 receptors respectively, were unable, alone or in combination, to block the anandamide-induced effect. Third, inactivation of both receptors by pretreatment of cells with pertussis toxin did not affect the potentiation of cell growth by anandamide. These data demonstrated that neither CB1 nor CB2 receptors were involved in the anandamide-induced effect. Moreover, using CB2-transfected Chinese hamster ovary cells, we demonstrated that after complete blockade of the receptors by the specific antagonist SR 144528, anandamide was still able to strongly stimulate a mitogen-activated protein (MAP) kinase activity, clearly indicating that the endogenous cannabinoid can transduce a mitogenic signal in the absence of available receptors. Finally, arachidonic acid, a structurally related compound and an important lipid messenger without known affinity for cannabinoid receptors, was shown to trigger MAP kinase activity and cell growth enhancement similar to those observed with anandamide. These findings provide clear evidence for a functional role of anandamide in activating a signal transduction pathway leading to cell activation and proliferation via a non-cannabinoid receptor-mediated process.

Key words: Cannabinoid receptor; Anandamide; SR 141716A; SR 144528; Hematopoietic cell; Cell growth

1. Introduction

Two G-protein-coupled receptors with seven transmembrane domains have been identified as cannabinoid receptors and are referred to as CB1 and CB2 [1–4]. CB1, initially designated the central cannabinoid receptor, is present not only in the brain but also in peripheral tissues and leukocytes [4–6], whereas CB2 is only present at the periphery and more particularly on cells of hematopoietic origin [7]. Recently, it has been shown that activation of both types of receptors led to activation of the growth-related gene Krox-24 via the mitogen-activated protein (MAP) kinase cascade [8,9]. Thus, the coupling of central and peripheral cannabinoid receptors to a mitogenic pathway allows one to assume that cannabinoid ligands would act as cell growth activators. In line with this observation, we showed for the first time that synthetic cannabinoid ligands such as CP 55940 and WIN 55212-2 behave as CB2-specific growth-activating cofactors for human B-lymphocytes primed via stimulation of B-cell antigen receptor or CD-40 receptor [10,11].

In this study, we focused on the effect of the natural endogenous ligand anandamide [12] on the growth of two cytokine-dependent cell lines of different hematopoietic lineages. Anandamide displays specific binding to CB1 and CB2 receptors and induces pleiotropic effects: anandamide released from neurons is a messenger modulating synaptic functions [13], produced by macrophages it is a mediator of hemorrhagic hypotension [14]; it is also considered a growth stimulator for hematopoietic cells [15] and has been shown to inhibit lymphocyte proliferation and tumor necrosis factor α (TNFα) production [16,17]. Anandamide has been detected in different regions of the central nervous system and also in peripheral tissues such as spleen, heart and skin [18]. The wide distribution of anandamide and its broad range of activity raise the question whether this endogenous mediator exerts its effects only through the two cannabinoid receptors characterized so far. The relatively low affinity of anandamide for both CB1 and CB2 receptors, its presence at significant levels in tissues such as heart known to express few if any cannabinoid receptors [7,18] and the demonstration of cannabinoid receptor-independent effects of anandamide [19] suggest the existence of other binding structures. Moreover, its lipid character and ability to permeate membranes lead one to suspect that direct, non-receptor-mediated effects could occur.

In this study, using the selective antagonists SR 141716 (CB1) and SR 144528 (CB2) [11,20], we demonstrated that anandamide and other fatty acid cannabinoids stimulate cell growth via a cannabinoid receptor-independent process and likely through direct activation of a MAP kinase pathway. This work is a significant example of the dual mechanism of action of endogenous fatty acid cannabinoids which represent a new class of lipid messengers also able to deliver intracellular messages by bypassing their receptors.

*Corresponding author. Fax: (33) 467 10 68 83.
E-mail: jean-marie.derocq@tls1.elfsanofi.fr

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2. Materials and methods

2.1. Reagents

CP 55940 was obtained from Pfizer. Anandamide, (*R*)-methanandamide, palmitylethanolamide, arachidonic acid and pertussis toxin were purchased from Biomol Research Laboratories (Plymouth Meeting, USA). The CB1 antagonist SR 141716A [20] and the CB2 antagonist SR 144528 [11] were synthesized at Sanofi Recherche (Montpellier, France). Drugs were dissolved at 10^{-2} M in ethanol and stored at −80°C. Human rIL-6 was produced in *Escherichia coli* at Sanofi Recherche, the specific activity was 5×10^{9} U/mg. Murine recombinant IL-3 was obtained from Genzyme (Cergy, France).

2.2. Cell lines

The murine IL-6-dependent hybridoma cell line B9 [21] was grown in RPMI medium supplemented with 10% fetal calf serum, 5×10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 U/ml IL-6. The murine IL-3-dependent myeloblastic cell line FDC-P1 [22] was grown in IMDM medium supplemented with 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 ng/ml IL-3.

2.3. Detection of CB1 and CB2 receptor mRNAs

The expression of mRNAs for β_2-microglobulin, CB1 and CB2 receptors was examined by reverse transcription coupled to the polymerase chain reaction (RT-PCR) as previously described [23]. mRNA purification from B9 and FDCP1 cells followed by conversion to first strand cDNA was performed with the PolyATract Series 9600 mRNA Isolation System with cDNA Synthesis Reagents (Promega, Charbonières, France) according to the manufacturer's instructions. DNA amplifications were carried out in PCR buffer (Perkin Elmer-Cetus, Saint-Quentin, France) containing 6 μl of cDNA solution with 0.1 mM dNTP (Pharmacia Biotech, Orsay, France), 0.25 μM of each primer and 0.5 U *Taq* polymerase (Perkin Elmer-Cetus) in a final volume of 20 μl. The mixture was amplified with the GeneAmp PCR 9600 Thermal cycler (Perkin Elmer-Cetus). The thermal reaction profile consisted of a denaturation step at 95°C for 20 s, annealing at 60°C for 30 s and an extension step at 75°C for 20 s. Reaction was performed for 35 cycles. After PCR, 5 μl loading buffer (Novex, San Diego, CA, USA) was mixed with 10 μl PCR products and separated by electrophoresis on 3% agarose gel in the presence of ethidium bromide with 123 bp DNA markers (Gibco, Eragny, France) as molecular weight controls.

The specific mouse oligonucleotides used were purchased from Genset (Paris, France). All the primers were made of 18–20-residue oligonucleotides with 50% G+C content and lacking 3′ complementary between primer pairs. β_2-Microglobulin primers: sense 5′-TGACCGGCTTGTATGCTATC-3′; antisense 5′-CAGTGTGAGCCAGGATATAG-3′. CB1 primers: sense 5′-TACCATCACCACAGACCTCC-3′; antisense 5′-TGACACATAGCACCAGCAG-3′. CB2 primers: sense 5′-GCCTGCAACTTTGTCATC-3′, antisense 5′-GGCTTTCCAGAGGACATAC-3′. The expected size of the amplicons were 222 bp for β_2-microglobulin, 383 bp for CB1 receptor and 386 bp for CB2 receptor.

2.4. Treatment of cells, evaluation of DNA synthesis and expression of results

Five thousand B9 cells, in a total volume of 200 μl serum-free medium, were cultured in triplicate in 96-well microtiter trays for 24 h in the presence of the indicated concentrations of IL-6, cannabinoid agonists or antagonists. Ten thousand FDC-P1 cells in medium containing 1% horse serum and the indicated concentrations of IL-3 were similarly treated for 48 h. Cells were used in the exponential phase of growth and were serum-deprived at the beginning of the experiment. DNA synthesis was determined by pulsing the cells with 1 μCi/well of [^{3}H]thymidine for the last 4 h of the culture period. Cells were then harvested on nitrocellulose filters using a Harvester 96 (Tomtec) and radioactivity counted in a microscintillation counter (Microbeta Trilux, Wallac).

Experiments were done at least twice and results were expressed as mean [^{3}H]thymidine uptake (cpm ± S.D.) in most of the experiments. The effect of cannabinoid receptor antagonists or pertussis toxin (Figs. 4 and 5) was evaluated by comparing the range of stimulation induced by anandamide in the presence or in the absence of these agents and expressed as % increase of DNA synthesis

$$\%\ \text{increase} = [\text{cpm anandamide} \pm \text{antagonist or PTX}/\text{cpm control} \pm \text{antagonist or PTX}]\times 100$$

2.5. Expression of CB2 receptors in Chinese hamster ovary (CHO) cells and culture conditions

CB2 cDNA was obtained by screening a cDNA library from the human promonocytic cell line U937 with a horseradish peroxidase-labeled CB2-specific oligonucleotide (5′-CTCACACACTTCTTCCAGTG), followed by detection using the ECL system (Amersham). Human CB2 coding region was amplified by PCR using the primer pair 5′- CCACACAAGCTTGCCACCATGGAGGAATGCTGGGTG and 5′- CCACTCGGATCCTCAGCAATCAGAGAGGTCTAG and inserted into the p658 plasmid, an expression vector derived from p7055 in which the IL-2 coding sequence was replaced by a polylinker [8]. The sequence of the insert in the expression vector was verified before transfection into CHO dihydrofolate-reductase-negative cells by the $Ca_3(PO_4)_2$ precipitation method [9].

Cells stably transfected with CB2 were grown in minimal essential medium supplemented with 10% dialyzed fetal calf serum, 2 mM glutamine, 40 μg/ml L-proline, 1% anti-PPLO agent, 1 mM sodium pyruvate and 5 μg/ml gentamicin. Wild type (wt) CHO cells were grown in the same medium supplemented with 10% fetal calf serum.

2.6. Analysis of MAP kinase activity

MAP kinase activity was measured as described [9]. Briefly, wt and CB2-CHO cells grown to 80% confluence were maintained in culture medium without serum for 24 h prior to the addition of ligands. Cells were pretreated with the CB2 antagonist SR 144528 (500 nM) for 2 min before treatment with the indicated concentrations of anandamide for another 10 min at 37°C. Cells were then washed at 4°C with 0.5 ml buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*-tetraacetic acid, 1 mM Na_3VO_4) and lysed for 15 min in buffer A supplemented with 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Solubilized cell extracts were then clarified by centrifugation at $14\,000\times g$ for 15 min at 4°C. Aliquots (15 μl) were removed and stored at −80°C until use. Phosphorylation assays were carried out at 30°C for 30 min (linear assay conditions) with γ-^{33}P-ATP using the Biotrack p42/p44 MAP kinase enzyme system (Amersham). The radioactivity incorporated was determined by liquid scintillation counting and expressed as percent increase over basal level. The background radioactivity was less than 10% of the total radioactivity incorporated in non-treated cells.

3. Results

3.1. CB1 and CB2 receptor mRNAs expression in murine hematopoietic cell lines

Using semi-quantitative RT-PCR, we compared the levels of transcripts for CB1 and CB2 receptors in B9 and FDC-P1 cell lines. As shown in Fig. 1A, B9 cells displayed equivalent amounts of CB1 and CB2 mRNAs. B9 is a mouse-rat heterohybrid lymphoid cell line [21] and RT-PCR analysis using specific rat primers instead of mouse primers as in Fig. 1 gave similar results (not shown). In FDC-P1 cells (Fig. 1B), CB2 and CB1 mRNAs were also present at similar levels. Thus, the expression of transcripts for the 'central' and the peripheral cannabinoid receptors was obvious on both cell lines and provided a molecular basis for the evaluation of the potential effects of cannabinoid ligands on the activation of these cells.

3.2. Effect of anandamide on the proliferative response

The effect of anandamide, a natural endogenous ligand of cannabinoid receptors [12], was first examined at optimal concentrations of 0.5–1 μM on B9 and FDC-P1 cells cultured with increasing amounts of IL-6 and IL-3 respectively (Fig. 2A,C). In the absence of any growth factor, anandamide by

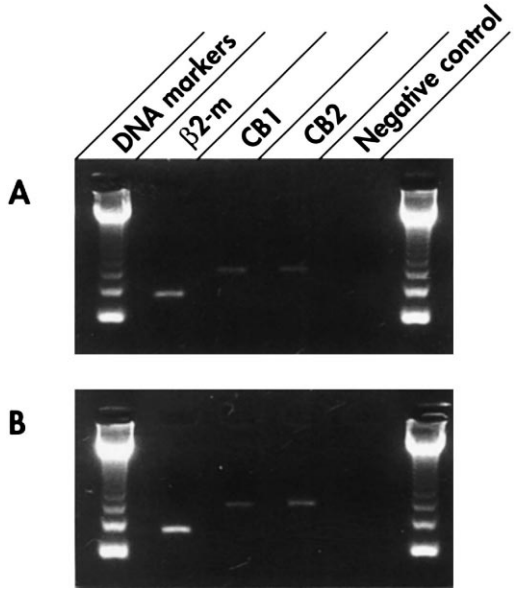

Fig. 1. Cannabinoid receptor gene expression in B9 and FDC-P1 cells. PCR products were obtained after amplification of mRNA-derived cDNA from murine B9 cells (A) and murine FDC-P1 cells (B) using mouse CB1- and CB2-specific primers. Negative control was performed with no cDNA. Internal control was obtained by PCR of β_2-microglobulin gene.

itself did not significantly influence the DNA synthesis of the cytokine-dependent cell lines. However, anandamide greatly enhanced the rate of DNA synthesis of both cell lines, by a factor of 2–3, when the respective growth factors were present in the culture medium. The stimulating effect of anandamide

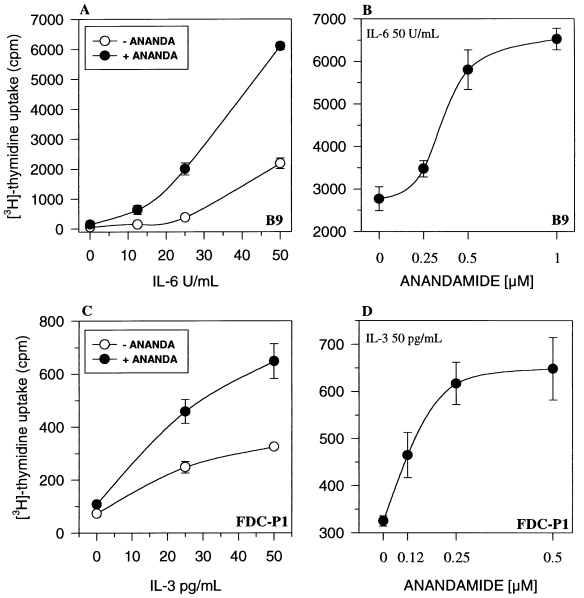

Fig. 2. Effect of anandamide on thymidine uptake in two hematopoietic cell lines. B9 cells were costimulated in serum-free medium with increasing amounts of IL-6 in the presence or in the absence of 1 μM anandamide (A) or with 50 U/ml IL-6 and the indicated concentrations of anandamide (B) for 24 h. FDC-P1 cells were cultured for 48 h in medium supplemented with 1% horse serum with increasing concentrations of IL-3 with and without 0.5 μM anandamide (C) or with a constant concentration (50 pg/ml) of IL-3 and varying concentrations of anandamide (D). [^{3}H]Thymidine uptake (cpm ± S.D.) was measured in triplicate as indicated in Section 2.

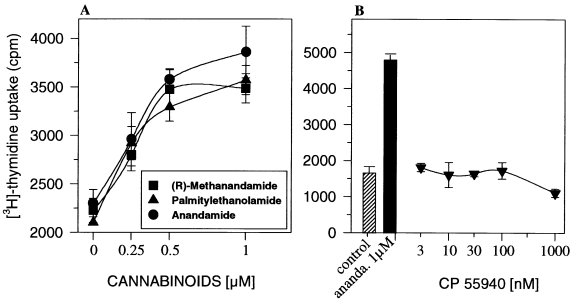

Fig. 3. Comparison of anandamide with other fatty acid analogs and with a synthetic cannabinoid ligand. B9 cells were stimulated in serum-free medium for 24 h with 50 U/ml IL-6 and the indicated concentrations of the fatty acid cannabinoids anandamide, (*R*)-methanandamide, palmitylethanolamide (A) or with the synthetic cannabinoid CP 55940 (B). Thymidine uptake was measured as in Fig. 2.

was concentration-dependent with a maximal activation obtained at 0.5–1 μM for B9 cells and 0.25–0.5 μM for FDC-P1 cells costimulated with either 50 U/ml IL-6 or 50 Ng/ml IL-3 respectively (Fig. 2B,D). In the conditions depicted in Fig. 2B, the addition of 1 μM anandamide increased the cell number by a factor 2.2 (9300 ± 52 B9 cells in treated wells vs. 4200 ± 30 in non-treated control wells), indicating that the enhancement of DNA synthesis was related to a proliferative response.

This synergistic response induced by anandamide was observed in serum-free medium for B9 cells or in very low serum concentration (1%) for FDC-P1 cells and all the experiments reported thereafter were done under the same conditions. Indeed, the effect of anandamide decreased progressively with higher serum concentrations and was totally abolished when

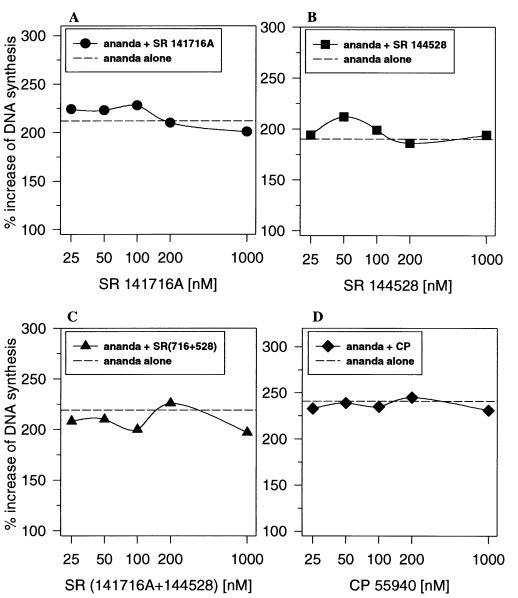

Fig. 4. Absence of antagonistic effect of the cannabinoid receptor antagonists SR 141716 and SR 144528. B9 cells costimulated with 50 U/ml IL-6 and 0.5 μM anandamide were treated for 24 h with the indicated concentrations of the CB1 or CB2 antagonists SR 141716 (A) and SR 144528 (B) respectively or a mixture of both (C). Cells were also identically treated with the 'inactive' CB1-CB2 agonist CP 55940 (D). Data are expressed as percent increase of DNA synthesis as indicated in Section 2.

the optimal 10% concentration required for culture maintenance was used (data not shown).

3.3. Comparison of anandamide with other fatty acid analogs and with a synthetic cannabinoid ligand

Since anandamide binds both CB1 and CB2 receptors, in a first attempt to determine which receptor subtype may be involved, we compared anandamide with two other fatty acid ligands displaying better selectivity. Fig. 3A shows that (*R*)-methanandamide, an analog of anandamide with higher selectivity for CB1 [24], and palmitylethanolamide, an endogenous agonist for CB2 [25], similarly increased the rate of DNA synthesis in the IL-6-costimulated B9 cells within the same range of concentrations as that observed with anandamide ($EC_{50} \approx 0.3$ μM for the three compounds). These data suggested that the two types of receptors, simultaneously present on the cells, were equally efficient in transducing a proliferative signal.

If so, a potent agonist, such as the reference synthetic cannabinoid ligand CP 55940, known to bind and activate CB1 and CB2 with an equal potency and more efficiently than anandamide [3,26], would induce a proliferative signal of at least the same magnitude. As shown in Fig. 3B and in contrast to what was expected, CP 55940 failed to demonstrate any effect on cell activation when tested in the same conditions and over the same range of concentrations as anandamide.

3.4. Cannabinoid receptor specificity studies

The absence of stimulation by the potent synthetic ligand CP 55940 was intriguing enough to raise the question of the receptor specificity of the anandamide-induced effect. In a first attempt to elucidate this question, the antagonists SR 141716 and SR 144528, specific for CB1 and CB2 receptors respectively [11,20], were used alone or in combination with anandamide. Moreover, since the CB1/CB2 ligand CP 55940 was ineffective, this compound was also tested itself as an 'anandamide blocker'. Data presented in Fig. 4 showed that neither the respective specific antagonists alone or in combination nor the inactive bispecific agonist tested over a wide range of concentrations were able to inhibit even partially the DNA synthesis stimulation induced by 0.5 μM anandamide. These results represented the first strong indication that the anandamide-induced effect was not mediated through CB1 or CB2 receptors.

Cannabinoid receptors are coupled to a heterotrimeric G_i-protein which can be selectively inactivated by pretreatment with PTX [8–10]. We therefore compared the growth-enhancing effect of anandamide in the absence or in the presence of PTX. Fig. 5 shows that the anandamide-driven cell growth enhancement was not affected by the presence of the toxin, thus reinforcing the notion of a cannabinoid receptor-independent phenomenon.

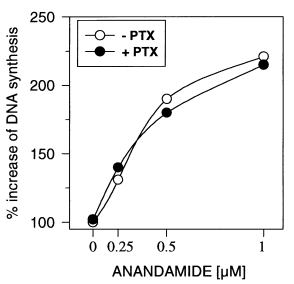

Fig. 5. Absence of effect of pertussis toxin (PTX) on anandamide-induced growth enhancement. B9 cells stimulated in serum-free medium with 50 U/ml IL-6 were preincubated 5 h with 50 ng/ml PTX and then treated with the indicated concentrations of anandamide for 24 h. Data are expressed as percent increase of DNA synthesis as indicated in Section 2.

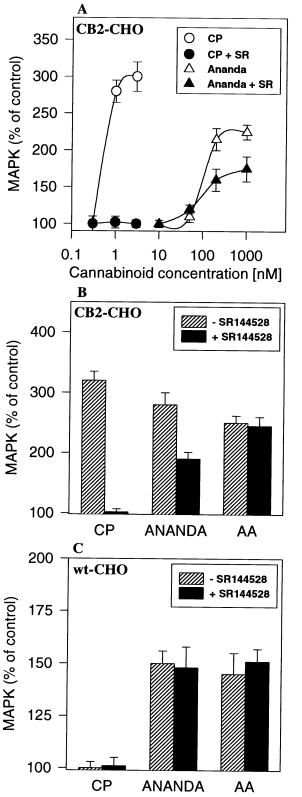

Fig. 6. Anandamide stimulation of MAP kinase. CB2-CHO cells were treated for 10 min with the indicated concentrations of CP 55940 or anandamide in the presence or in the absence of the CB2 antagonist SR 144528 added at 0.5 μM 2 min before (A). The effects of CP 55940 (3 nM), anandamide and arachidonic acid (both 1 μM) were compared in CB2-CHO cells (B) and wt CHO cells (C) with and without 0.5 μM SR 144528. MAP kinase activities were measured in cell lysates as indicated in Section 2.

3.5. Anandamide induces a non-receptor-mediated signal transduction

A cell line transfected with only one cannabinoid receptor subtype which can be specifically blocked by the corresponding antagonist ligand represented a very helpful tool to explore the respective pathways used by anandamide. Synthetic cannabinoid agonists mediate receptor-dependent activation of MAP kinase [8,9]. A similar investigation was carried out with anandamide in comparison with CP 55940. Fig. 6A shows that anandamide stimulated a MAP kinase activity in CB2-transfected CHO similarly to CP 55940. The optimal effect was observed at concentrations of 100–1000 nM which corresponded to those required for cell growth enhancement while 100-fold lower concentrations were needed for CP 55940. Remarkably, whereas the full blockade of cannabinoid receptors by SR 144528 induced a complete inhibition of the CP 55940-driven effect, anandamide in the same conditions was still able to stimulate a strong MAP kinase response which represented about 50% of the total activity. Accord-

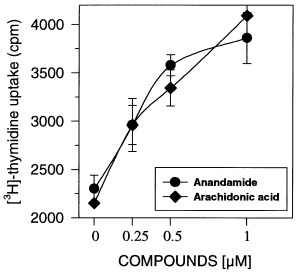

Fig. 7. Comparison of anandamide and arachidonic acid on growth-promoting effect. B9 cells were costimulated with 50 U/ml IL-6 and the indicated concentrations of anandamide or arachidonic acid. After a 24 h incubation, the thymidine incorporation was measured as in Fig. 2.

ingly, in control wt CHO cells, anandamide and CP 55940, at the optimal concentrations of 1 μM and 3 nM respectively, displayed completely different effects: while anandamide stimulated a significant MAP kinase activity which as expected was not affected by the cannabinoid receptor antagonist SR 144528, the specific ligand CP 55940 was totally inactive (Fig. 6C). These data demonstrated that anandamide exhibited the property of transducing a similar transduction signal through both receptor- and non-receptor-mediated mechanisms.

3.6. Comparison of anandamide and arachidonic acid

The effect of anandamide, which is composed of arachidonic acid and ethanolamine coupled through an amide linkage (arachidonic acid *N*-[hydroxyethyl]amide), was directly compared to that of arachidonic acid itself, an important cellular lipid mediator. Data depicted in Fig. 7 show similar concentration-dependent growth-promoting effects of arachidonic acid and anandamide in B9 cells ($EC_{50} \approx 0.3$–0.4 μM for both compounds). This identical effect was probably mediated via a similar pathway since at optimal concentrations both agents were able to activate a receptor-independent MAP kinase cascade in CB2-transfected and control wt CHO cells as well (Fig. 6B,C).

4. Discussion

In this study, we showed that anandamide, an endogenous ligand for central (CB1) and peripheral (CB2) cannabinoid receptors [12], enhanced the growth of two cytokine-dependent murine hematopoietic cell lines, the IL-3-dependent myeloblastic cells FDC-P1 and the IL-6-dependent lymphoid cells B9, in condition of low serum level. Our results are in line with a previous study [15] which demonstrated in similar conditions the same growth-inducing effect for anandamide in different cytokine-dependent myeloid and erythroid cell lines. This suggested a general synergistic effect of anandamide whatever the hematopoietic lineages and the cytokines required for their growth. Besides, this effect was not limited to anandamide but was extended to other fatty acid cannabinoid ligands such as the CB1-selective agonist (*R*)-methanandamide [24] and the CB2 agonist palmitylethanolamide [25]. However, the equipotent (CB1 and CB2) cannabinoid ligand CP 55940, which binds cannabinoid receptors with a 100-fold higher potency than anandamide [26], was inactive in these models (this study, [15]). The lack of effect of an agonist which in other models activates biological functions of cannabinoid receptors with higher efficiency than anandamide [7,8,10,11,27] was a surprising datum which led us to determine whether the anandamide-induced effect described in this model was a specific cannabinoid receptor-mediated process.

It came out from receptor specificity studies that neither the CB1 antagonist SR 141716A nor the CB2 antagonist SR 144528, which bind and saturate their respective receptors at nanomolar concentrations [11,20], was able alone or in association to inhibit the effect of anandamide. Moreover, the 'non-inducer' CB1-CB2 agonist CP 55940, which was thus used as an 'antagonist', also failed to inhibit the effect of anandamide. These results represented the first evidence for a non-CB1- or -CB2-mediated process.

However, though two G_i-protein-coupled cannabinoid receptors have been described so far, the presence of other G_i-protein-coupled receptors which could account for the effect of anandamide cannot be totally excluded. Nonetheless, the absence of effect of PTX, a G_o-G_i-protein inhibitor, on the enhancing property of anandamide allowed us to rule out such an hypothesis. Therefore, considering these results, we could assume not only that the growth-promoting effect induced by anandamide was CB1 or CB2 receptor-independent, but also that other putative G_o-G_i-linked receptors were not involved any more. However, the involvement of an as yet unidentified PTX-independent cannabinoid receptor cannot be totally excluded.

Our findings are in contrast to the study cited above [15] where the authors, in a similar model of cytokine-dependent cell lines, described the anandamide effect as a specific CB2 receptor-mediated process. Their assertion was based on the fact that one CB2-positive hematopoietic cell line which was non-responsive to anandamide became sensitive to its stimulating effect after overexpression of CB2 receptors by transfection. However, it cannot be excluded that the transfection induced changes or perturbations making the cells more sensitive to a 'non-specific' effect of anandamide. Alternatively, overexpression of a high number of CB2 receptors may artificially guarantee specificity to some extent in these particular transfected cells but this does not preclude the possibility of a 'non-specific' effect in normal non-transfected cells. Several lacking controls should have been done to ascertain the role of CB2 receptors in their models: a study of CB1-transfected counterparts, use of PTX to provide demonstration of a role of the G_i-linked cannabinoid receptors, receptor blockade by synthetic agonists which were also reported to be inactive, comparison with hematopoietic cell lines which were reported to be double negative $CB2^-/CB1^-$. In the absence of relevant controls, the CB2 specificity of anandamide in this model remains speculative.

It has been shown that stimulation of cannabinoid receptors by synthetic ligands induces a MAP kinase signaling pathway [8,9]. In this study, using the model of CB2-transfected CHO cells, we reported a similar stimulation of MAP kinase with the natural endogenous cannabinoid anandamide in the range of concentrations required for cell growth enhancement. Transfected CHO cells therefore represent a very useful tool and an additional model to go deeper into the question of the cannabinoid receptor specificity of anandamide. This model allowed us to demonstrate that when cells were exposed to saturating concentrations of the specific antagonist SR 144528 and thus in absence of available receptors, anandamide was still able to exhibit a MAP kinase activity

which represented 50% of the total activity. This ability of anandamide to elicit a non-receptor-mediated MAP kinase activation was further confirmed in control wt CHO cells. These data evidenced the dual function of anandamide able to induce signal transduction through both receptor- and non-receptor-mediated pathways. In contrast, the effect of the reference compound CP 55940 was totally abolished by SR 144528 in CB2-CHO cells, indicating for this ligand a strictly specific receptor-related process also attested by an absence of effect in wt CHO cells. In addition, these data could imply that the growth-enhancing effect observed with anandamide on hematopoietic cells could be mediated through a receptor-independent activation of a MAP kinase cascade. One hypothesis to explain the synergistic effect of anandamide with cytokine growth factors could be that activation in parallel of two different pathways (possibly MAP kinase and JAK/STAT kinase) leads to enhanced cell cycling. Conversely, the absence of a growth-promoting effect of the specific cannabinoid receptor ligand CP 55940 in hematopoietic cells would likely mean that, in these cells, CB2 receptors are not functionally linked to a MAP kinase pathway or that CB2 receptors modulate intracellular events downstream of the MAP kinase activation that are different from those triggered directly by anandamide in a receptor-independent way. Further investigations are needed to elucidate this point.

Nevertheless, the hypothesis that anandamide stimulates cell growth via a receptor-independent MAP kinase activation is relevant with the observation that anandamide, at similar concentrations as those used in this study, induces a non-receptor-mediated release of arachidonic acid via stimulation of phospholipase A_2 (PLA_2) [19]. Arachidonic acid released after activation of membrane-associated PLA_2 represents an important lipid messenger activating different subgroups of MAP kinase such as $p42^{MAPK}$ and $p44^{MAPK}$ (ERK) via the eicosanoid cascade or C-jun N-terminal kinase (JNK) via the activation of NADPH oxidase and independently of a downstream eicosanoid biosynthesis [28]. These data added to the fact that arachidonic acid acts as an important mediator involved in cellular proliferation and mitogenesis [28–30] led us to compare the arachidonic acid derivative, anandamide, with arachidonic acid itself. We found an identical stimulatory effect of both compounds on cell growth, within the same range of concentrations and to the same extent, reinforcing the notion of a direct activation without any receptor requirement. Accordingly, we also observed a very similar activation of MAP kinase by both agents in control and CB2-transfected CHO cells, strengthening the assumption that a receptor-independent activation of a MAP kinase pathway was involved. Two hypotheses can be put forward to explain the similarity between anandamide and arachidonic acid. First, the lipophilic anandamide may directly stimulate membrane-bound PLA_2 and arachidonic acid production as already reported [19] which in turn activates cell growth. Second, hydrolytic degradation of the unstable anandamide into arachidonic acid may represent another possibility. In any case, fatty acid cannabinoids, in addition to their receptor-mediated actions largely documented in the literature, are directly able to activate an arachidonic acid-like signaling cascade leading to cell activation. Interestingly, it has been recently reported that arachidonic acid stimulates downstream a c-*fos* serum response element in fibroblasts [31]. If this result can be extrapolated to hematopoietic cells, it would explain why cell activation by anandamide (this study, [15]) or by arachidonic acid (this study) was only revealed in serum-starved cells.

Whatever the precise mechanism underlying the non-receptor-mediated effects of endogenous fatty acid cannabinoids, we clearly demonstrate for the first time in this study that these messengers were able to directly activate a fundamental biological process such as cell growth by bypassing their receptors. Moreover, evaluation of cannabinoid-induced biological effects imposes that steps must be taken to discriminate between receptor- and non-receptor-mediated actions. With regard to the totally different cellular events that can be elicited by either method, this represents a fundamental aspect for the understanding and the deciphering of the physiological role of endogenous cannabinoids.

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