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Review

Endocannabinoids and fatty acid amides in cancer, inflammation and related disorders

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Abstract

The long history of the medicinal use of *Cannabis sativa* and, more recently, of its chemical constituents, the cannabinoids, suggests that also the endogenous ligands of cannabinoid receptors, the endocannabinoids, and, particularly, their derivatives may be used as therapeutic agents. Studies aimed at correlating the tissue and body fluid levels of endogenous cannabinoid-like molecules with pathological conditions have been started and may lead to identify those diseases that can be alleviated by drugs that either mimic or antagonize the action of these substances, or modulate their biosynthesis and degradation. Hints for the therapeutic applications of endocannabinoids, however, can be obtained also from our previous knowledge of marijuana medicinal properties. In this article, we discuss the anti-tumor and anti-inflammatory activity of: (1) the endocannabinoids anandamide (arachidonoylethanolamide) and 2-arachidonoyl glycerol; (2) the bioactive fatty acid amides palmitoylethanolamide and oleamide; and (3) some synthetic derivatives of these compounds, such as the *N*-acyl-vanillyl-amines. Furthermore, the possible role of cannabimimetic fatty acid derivatives in the pathological consequences of cancer and inflammation, such as cachexia, wasting syndrome, chronic pain and local vasodilation, will be examined.

Keywords: Anandamides; *Cannabis sativa*; Endocannabinoids; Hybrid; Tetrahydrocannabinol

1. Introduction

The possible therapeutic use of *Cannabis sativa* and its active constituents, the cannabinoids, is currently at the center of a heated political and scientific debate in several countries (Burstein, 1997; Grotenhermen, 1998). Yet, some of the

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social and legal implications of the medical prescription of marijuana, as well as the undesired psychotropic effects of this kind of treatment, may be bypassed if the endogenous counterparts of the psychoactive cannabinoids, the 'endocannabinoids', could serve for the development of new drugs with applications similar to those for which cannabis has been employed in the past (Mechoulam, 1986). The only endocannabinoids known to date were isolated in the early 1990s, and are all derivatives of polyunsaturated fatty acids (Fig. 1): (1) the 'anandamides', i.e. the ethanolamides of the all *cis* $C_{20:3}$, $C_{20:4}$ and $C_{22:4}$ fatty acids, of which the arachidonic acid homologue (simply known as anandamide, AEA) is the best known and most thoroughly studied one (Devane et al., 1992; Hanus et al., 1993); and (2) 2-arachidonoyl-glycerol (2-AG), a well known intermediate in glycerol metabolism whose cannabimimetic properties were discovered only in 1995 (Mechoulam et al., 1995; Sugiura et al., 1995). The anandamides and 2-AG activate to a varied extent the two cannabinoid receptor subtypes identified so far, the CB_1 and CB_2 receptors and, subsequently, exhibit pharmacological properties in vitro and in vivo that are very similar, although not identical, to those exerted by marijuana's psychoactive component, Δ^9-tetrahydrocannabinol (THC) (for recent reviews see Hillard and Campbell, 1997; Di Marzo, 1998; Mechoulam et al., 1998; and the article by R. Mechoulam in this issue). Along with these metabolites, other fatty acid derivatives have been found that exhibit cannabimimetic properties in some assays (Fig. 1). These are: (1) another fatty acid ethanolamide, palmitoylethanolamide (PEA), capable of sharing with the psychoactive cannabinoids an immune cell down-regulatory (Facci et al., 1995; Berdyshev et al., 1997), neuroprotective (Skaper et al., 1996), and analgesic (Calignano et al., 1998; Jag-

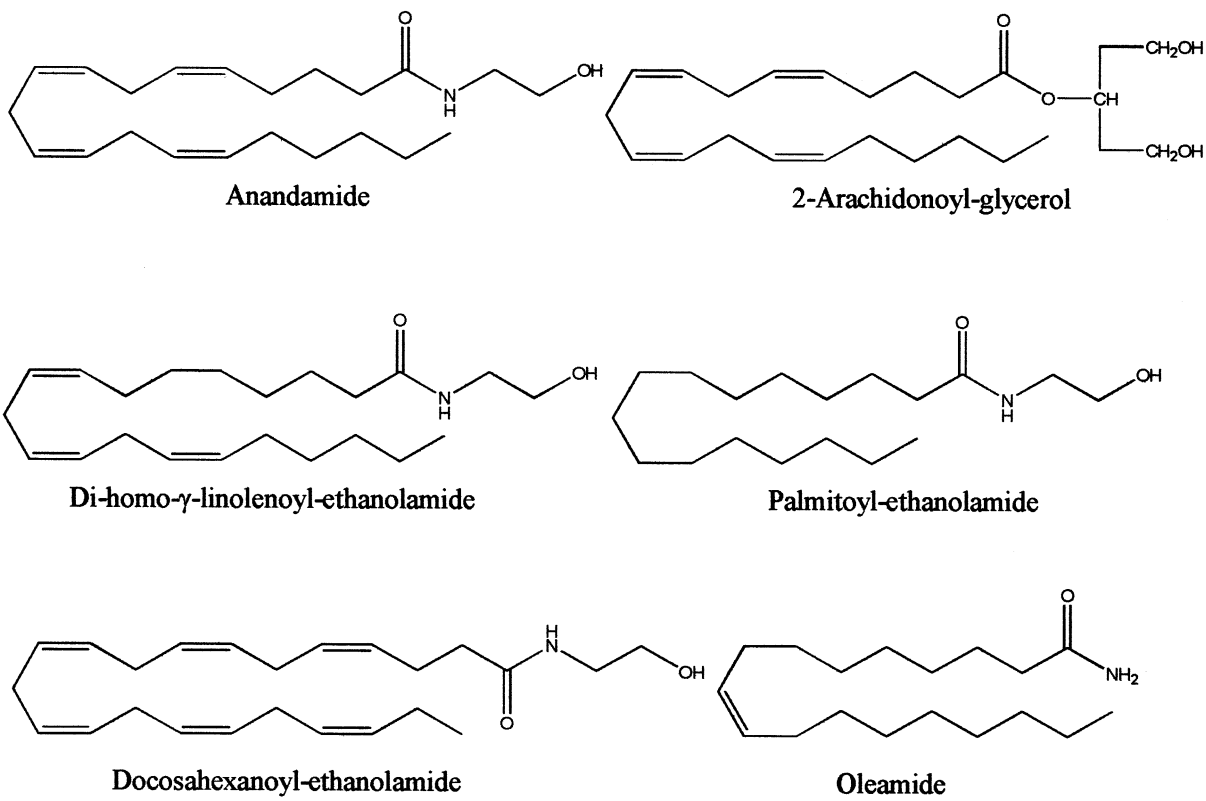

Fig. 1. Chemical structures of endocannabinoids and other natural cannabimimetic fatty acid derivatives.

gar et al., 1998a) activity (for reviews see Schmid et al., 1990; Lambert and Di Marzo, 1999); and (2) oleamide (*cis*-9-octadecenoamide, OA), a recently isolated sleep-inducing factor (Cravatt et al., 1995) which exhibits some cannabimimetic neurobehavioural (Mechoulam et al., 1997) and anti-proliferative (Bisogno et al., 1998) effects (for reviews see Boger et al., 1998; Lambert and Di Marzo, 1999). Unlike the endocannabinoids, however, PEA and OA have very low affinity ($K_i \geq 10$ μM) for CB_1 and CB_2 cannabinoid receptors, and the mechanism of their cannabimimetic activity is still a matter for speculation.

Among the typical therapeutic applications suggested in the past for marijuana and/or THC, alleviation of inflammation, and, subsequently, asthma and some forms of chronic pain, and stimulation of appetite in cancer patients subjected to chemotherapy, have been widely described (Mechoulam, 1986). Furthermore, the possibility that THC-like substances could be used as anti-tumor drugs has been also explored (White et al., 1976). Stemming from this knowledge, it is reasonable to predict that drugs designed from cannabimimetic fatty acid derivatives may be used for the cure or alleviation of these pathological conditions. In this article we shall describe the pharmacological and biochemical evidence in favor and against the possible participation of endocannabinoids in inflammation, tumor cell growth and related disorders, and shall discuss whether there is a case for the use of natural and synthetic cannabimimetic fatty acid amides and esters in these diseases.

2. Endocannabinoids and cancer

The anti-neoplastic activity of THC and its analogues in vitro was first examined in the early 70s. Leukemia cells were, among cancer cells, those whose growth was most potently reduced by THC, an effect probably due to both inhibition of DNA synthesis and, as described later, counteraction of cell differentiation (Carchman et al., 1976; Tucker and Friedman, 1997; Murison et al., 1987). THC was also shown to retard the growth of Lewis lung adenocarcinoma and to inhibit Friend leukemia virus-induced splenomegaly in vivo (Munson et al., 1975; White et al., 1976). Although in these studies non-psychotropic cannabinoids, such as cannabidiol, were found to be less potent than THC, these compounds did exhibit some anti-tumor activity. Furthermore, when several cannabinoid analogues were tested in the same series of experiments, as in the case of the inibition of L1210 murine leukemia DNA synthesis (Carchman et al., 1976), the rank of potency found for these compounds did not correspond to their relative activity as ligands of cannabinoid receptors, as determined in later studies. These observations suggest that the anti-neoplastic effects of THC may not be necessarily mediated by cannabinoid receptors, a hypothesis that finds confirmation also in the recent findings that (1) a non-psychoactive cannabinoid, cannabigerol, inhibits human oral epithelioid cell and NIH 3T3 fibroblast growth in vitro (Baek et al., 1998); and (2) THC induces apoptosis in C6 glioma and PC-3 prostatic carcinoma cells through cannabinoid-receptor independent mechanisms (Sanchez et al., 1998; Ruiz et al., 1999). However, in support of the involvement of stereoselective binding sites at least in a part of the anti-tumoral effects of cannabinoids came the recent observations that the synthetic cannabinoid HU-210, but not its non-psychoactive enantiomer HU-211, disrupt the cytoskeletal organization of differentiated PC-12 cells, an effect due to inhibition of tubulin and actin expression (Tahir et al., 1992; Wilson et al., 1996). Furthermore, recent studies carried out by Galve-Roperh et al. (2000) showed that the apoptotic effect of cannabinoids on glioma cells in vitro can be blocked by a combination of CB_1 and CB_2 cannabinoid receptor antagonists.

A possible way of assessing the participation of cannabinoid receptors and of the endocannabinoid system in the control of cancer cell growth could be to investigate the correlation of CB_1 and CB_2 receptor expression and endocannabinoid biosynthesis with the onset of tumors. This could be done, for example, by analyzing the levels of cannabinoid receptor mRNA and protein, as well as of the endocannabinoids, in tumor cell lines and their corresponding non-tumoral cells. Unfortunately, despite the fact that

sensitive techniques for the rapid measurement of both cannabinoid receptors and endocannabinoids in tissues are now available, no such comparative study has been performed yet, even though it is now well established that these molecules are produced by numerous tumor cells (see Matsuda and Bonner, 1995; Di Marzo, 1998, for reviews). A series of studies carried out in our laboratory, however, established that endocannabinoids can indeed potently inhibit the proliferation of some cancer cells in vitro. AEA was tested for its possible growth inhibitory action on several tumor cell lines, including mouse neuroblastoma and monocytoma, rat heart endothelioma, basophilic leukemia and pheochromocytoma, and human breast cancer cells (HBCCs). A potent and dose-dependent anti-proliferative effect ($IC_{50} = 0.5–6$ μM, depending on the cell line) was only found in the latter cells (De Petrocellis et al., 1998). AEA was shown to owe this effect to the inhibition the G1/S phase of the cell cycle and of the incorporation of [^{3}H]thymidine into DNA, and not to toxicity or induction of apoptosis. Accordingly, the effect was maximal between 2 and 4 days of daily treatment with the compound, i.e. during the exponential phase of HBCC growth. Furthermore, the effect of AEA was not due to arachidonic acid (AA) produced from its hydrolysis, since it was enhanced and not inhibited by inhibitors of fatty acid amide hydrolase (FAAH, the enzyme mostly responsible for AEA metabolism). Accordingly, a metabolically stable AEA analogue, (*R*)-methanandamide (Met-AEA), and AA were more potent and less potent than AEA, respectively. Conversely, several lines of evidence suggest that the anti-mitogenic effect of AEA on HBCCs is mediated by CB_1-like cannabinoid receptors (De Petrocellis et al., 1998; Melck et al., 2000): (1) AEA, 2-AG and HU-210 exhibit the same rank of potency as inhibitors of cell proliferation and in binding assays performed using EFM-19 cell membranes, although this order of potency was different from that reported in CB_1 receptor binding assays; (2) the effect of AEA is attenuated by a low concentration (0.5 μM) of the CB_1 antagonist SR141716A, but not of the selective CB_2 antagonist SR144528; (3) selective ligands of CB_2 receptors do not significantly inhibit HBCC proliferation up to a 10 μM concentration; (4) HBCC membranes contain specific binding sites for [^{3}H]SR141716A which can be displaced by AEA at doses comparable to those necessary to exert inhibition of proliferation; (5) HBCC cells express CB_1 mRNA transcripts as well as a CB_1-immunoreactive protein; (6) MCF-7 cells that had undergone several sub-culturing passages were less sensitive to AEA and exhibited lower levels of [^{3}H]SR141716A binding and of CB_1 mRNA transcripts and immunoreactive protein.

On the basis of the above findings, and in the attempt of suggesting that endocannabinoids play a physiological role as down-regulators of mammary cancer cell growth, another study addressed the issue of whether HBCCs could synthesize and inactivate AEA and its analogues (Bisogno et al., 1998). It was found that a HBCC line, EFM-19 cells, does indeed synthesize AEA from either [^{14}C]ethanolamine and [^{3}H]AA, although it was not possible to demonstrate that a Ca^{2+} ionophore stimulates the formation of the endocannabinoid as in neuronal cells (Di Marzo et al., 1994). Furthermore, it was shown that these cells rapidly uptake [^{14}C]AEA from the culture medium and enzymatically hydrolyze it to [^{14}C]ethanolamine and AA, probably via the catalytic action of FAAH, whose mRNA transcript was present in EFM-19 cell RNA. Interestingly, OA, which is present in high amounts in EFM-19 cells, inhibits [^{14}C]AEA degradation and weakly inhibits cell proliferation ($IC_{50} = 10$ μM) in a fashion sensitive to SR141716A. It was suggested that prolonged treatment of cells with OA may lead to an enhancement of *endogenous* AEA basal levels and subsequent inhibition of proliferation. This hypothesis, if supported by further experiments, would imply that AEA may act as local suppressor of HBCC proliferation.

A series of experiments was also aimed at gaining some understanding of the molecular events that, starting from the activation of CB_1-like receptors by endocannabinoids, lead ultimately to the selective inhibition of HBCC proliferation (De Petrocellis et al., 1998; Melck et al., 1999b, 2000). Mammary cancer cells in culture produce prolactin and use it as an autocrine growth factor. It

was noted that the responsiveness of different mammary cancer cell lines to a monoclonal antibody against prolactin and to AEA were linearly related and this finding, together with the observed selectivity of AEA anti-cancer effect for HBCCs vs. prolactin-insensitive cells, suggested that AEA anti-mitogenic action may be somehow related to prolactin. Four more observations confirmed the hypothesis that AEA may act by blocking endogenous prolactin-induced HBCC proliferation (De Petrocellis et al., 1998; Melck et al., 2000). Firstly, the anti-proliferative effects of sub-maximal doses of prolactin antibody and AEA were not additive, which suggested that the effect of the endocannabinoid was not due to inhibition of prolactin levels. Secondly, AEA inhibited the proliferative action of exogenous prolactin on EFM-19 cells at concentrations lower than those required to inhibit basal proliferation. Thirdly, AEA treatment of the DU-145 prostate cancer cell line-which does not produce endogenous prolactin and yet responds to the *exogenous* hormone by proliferating more rapidly-also led to a strong inhibition of prolactin-induced, but not basal, proliferation. Interestingly, HU-210, 2-AG, but not a selective CB_2 agonist, caused this latter effect, without significantly affecting the basal proliferation of DU-145 cells, whereas, as in HBCCs, the effect of AEA was blocked by a CB_1, but not a CB_2, receptor antagonist. Accordingly, high levels of CB_1 receptor mRNA transcripts and of CB_1-immunoreactive protein, as well as of [^{3}H]SR141716A binding sites, were detected in DU-145 cells. Lastly, and most importantly, AEA potently suppressed the levels of the long form of prolactin receptors in both HBCCs and DU-145 cells in a fashion that could be reverted by co-incubation with SR141716A. These data indicate that AEA anti-mitogenic action in tumor cells is due to CB_1 receptor-mediated inhibition of prolactin action at the level of prolactin receptor expression, and suggest that endocannabinoids may inhibit the proliferation of any tumor cell that at once expresses CB_1 as well as prolactin receptors.

Do endocannabinoids inhibit the mitogenic action of other growth factors on cancer cells? In order to answer to this question, the effect of AEA, 2-AG and HU-210 was also examined on HBCC proliferation induced by the nerve growth factor (NGF), which has been described as a potential tumor-inducing factor for both breast and prostate cancer (Descamps et al., 1998; Geldof et al., 1998). The cannabinoid receptor ligands potently inhibited NGF-induced MCF-7 cell proliferation (IC_{50} 50-600 nM), whereas a selective CB_2 receptor ligand was ineffective. The effect was blocked by selective antagonist of CB_1, but not CB_2, receptors, and was accompanied by a dramatic reduction of the levels of NGF high affinity *trk* receptors (Melck et al., 2000). Thus, it appears that endocannabinoids can act as selective inhibitors of cancer cell proliferation through a growth factor–dependent mechanism that may have little to do with the anti-tumor activities previously described for THC and other cannabinoids. However, the patho-physiological significance of these findings will be appreciated only when a full picture of the role in cancer of prolactin and NGF, as well as of their respective receptors, will be obtained from clinical studies. It will be also of interest to assess whether the effects of AEA and 2-AG on these receptors can be extended to other growth factors and to cells different from mammary or prostate cancer cells. Preliminary data from our laboratories indicate, for example, that AEA exerts a cytostatic action also in rat thyroid cells in culture.

Finally, the intracellular events triggered by CB_1 receptor activation and leading to suppression of the expression of prolactin and NGF *trk* receptors have been partly elucidated. We found that an activator of adenylyl cyclase, forskolin, and an inhibitor of the mitogen-activated protein kinase (MAPK) pathway, PD 098059, block the inhibition of both prolactin and NGF-induced MCF-7 cell proliferation as well as the down-regulation of prolactin and NGF *trk* receptors induced by AEA. Furthermore, an inhibitor of the cAMP-selective protein kinase A — RpcAMPs — mimicked these two effects of the endocannabinoid (Melck et al., 1999a). Finally, AEA was shown to inhibit adenylyl cyclase and to activate MAPK in MCF-7 cells. These data strongly suggest that the suppression of prolactin receptor and *trk* levels is due, at least in part, to CB_1-mediated

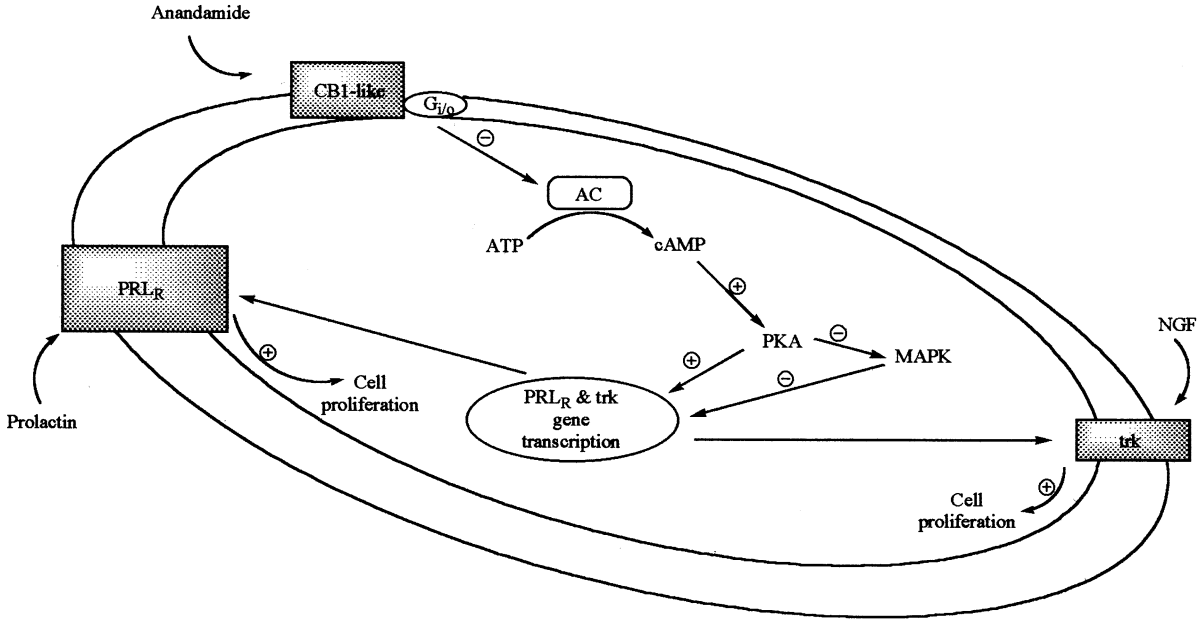

Fig. 2. Schematic representation of the mechanism of endocannabinoid-induced inhibition of human breast and prostate cancer cell proliferation. Human breast and prostate cancer cells in culture respond to prolactin (either endogenous or exogenous) or nerve growth factor (NGF) by proliferating more rapidly. Prolactin and NGF act through their respective receptors, the long form prolactin receptor (PRLr) and the high affinity *trk* NGF receptors, both of which are expressed in these tumor cells. In human breast cancer cells activation of CB_1-like cannabinoid receptors by endocannabinoids or synthetic cannabinoids triggers the inhibition of adenylyl cyclase (AC) and the activation of mitogen-activated protein kinase (MAPK) through G-protein stimulation. MAPK in these cells is also under negative regulation through protein kinase A (PKA)-mediated phosphorylation and, therefore, is activated in part also through CB_1-mediated inhibition of cAMP formation. Both PKA inhibition and MAPK activation by endocannabinoids lead to the suppression of the expression of PRLr and *trk* and, subsequently, to inhibition of prolactin and/or NGF-induced cell proliferation. Further details are discussed in De Petrocellis et al., 1998; Melck et al., 1999a Melck et al. (2000).

inhibition of adenylyl cyclase and activation of MAPK (Fig. 2). Indeed, these two intracellular effects are typically produced by substances that activate both CB_1 and CB_2 receptors (see Di Marzo, 1998; and the article by A. Howlett in this issue), and have been described in the past to cause inhibition of MCF-7 cell proliferation under particular culturing conditions (for example see Fenig et al., 1997), and to down-regulate the expression of numerous genes, including *trk* (Ehrhard et al., 1993).

3. Endocannabinoids and the regulation of food intake

The potential importance of cannabinoid-like molecules as appetite stimulants and a therapeutic remedy against nausea and anorexia is well documented in the centuries old history of *Cannabis sativa* (Mechoulam, 1986). More recently, the beneficial effects of THC-based pharmaceuticals (e.g. dronabinol®, marinol®) against cachexia caused by chemotherapy (Bruera, 1992; Gorter, 1999, for reviews) or the wasting syndrome typical of both cancer and AIDS (Grinspoon and Bakalar, 1995, for review) has been described. The discovery of cannabinoid receptors and endocannabinoids, however, prompted a series of studies aimed at understanding whether this endocannabinoid system could be involved in the control of appetite and food-intake, as well as in the feeling of reward originating from the consumption of palatable food. The outcome of these studies can be summarized with the following points: (1) In the invertebrate *Hydra vulgaris*,

possibly the first organism in the evolutionary scale to have developed a neural network, endocannabinoids and cannabinoid receptors seem to be involved in a typical feeding behavior (De Petrocellis et al., 1999); (2) A CB_1 receptor selective antagonist, SR141716A, inhibits palatable food-intake in rodents (Arnone et al., 1997; Colombo et al., 1998; Simiand et al., 1998). Although this effect could be due to the inverse agonist properties of SR141716A (Landsman et al., 1997), the possibility that SR141716A acts by reverting a food-intake stimulating tone by endocannabinoids also exists; (3) AEA induces hyperphagia in rats in a fashion sensitive to SR141716A (Williams and Kirkham, 1999) and, at very low doses, enhances food-intake in mice (Hao et al., 2000); (4) Both cannabinoid receptors and endocannabinoids have been found in the hypothalamus (Gonzalez et al., 1999)-the brain region most likely involved in the regulation of appetite and food-intake-in levels that, in female rats, depend on the phase of the ovarian cycle (Gonzalez et al., 2000). Indeed, craving for palatable food has also been correlated with the various phases of the ovarian cycle (Dye and Blundell, 1997); (5) Both cannabinoid receptors and endocannabinoids have been found in rat limbic forebrain (Bisogno et al., 1999). In this brain region (and more precisely the nucleus accumbens), drugs of abuse, ethanol consumption and rewarding stimuli are thought to induce the release of dopamine. This phenomenon may be the cause of the raise in AEA levels observed recently in the limbic forebrain of THC-tolerant rats (Di Marzo et al., 2000), and may suggest the existence of a correlation also between limbic endocannabinoid/dopamine levels and craving for palatable food (see Gardner and Vorel, 1998, and references cited therein).

In summary, based on the studies published so far, it seems likely that the endocannabinoid system exerts a tonic stimulation of the neural processes regulating food consumption. The molecular mechanisms of this regulatory loop and the neuropeptide(s) involved, however, need to be established. Furthermore, it will be important to find a correlation between dysfunctions of feeding behavior, such as cachexia, anorexia, nausea, etc., and hypothalamic or limbic endocannabinoid levels. This information could be applied to the development of new endocannabinoid-based drugs (such as stable analogues, inhibitors of the biosynthesis or degradation, etc.) for the alleviation of these disorders.

4. Endocannabinoids as anti-inflammatory agents. Role in chronic pain, asthma and local vasodilation

Cannabis preparations have been described by the Indian folklore medicine as a remedy against inflammation, chronic pain and asthma (Mechoulam, 1986), three pathological conditions that derive from cell hyper-reactivity. Today, among the most promising anti-inflammatory compounds from *Cannabis sativa* are the cannabinoid acids (for a recent review see Burstein, 1999), compounds that, unlike THC, are devoid of psychotropic activity and are very weak ligands of either CB_1 or CB_2 receptors. This may suggest that the anti-inflammatory properties of marijuana are not necessarily mediated by cannabinoid receptors. As will be described in this section (see also the review by Berdyshev in this issue), in the case of AEA and its congener PEA, the involvement of cannabinoid receptors in their possible anti-inflammatory properties has also been questioned. Apart from direct in vivo pharmacological data, evidence for the role of the endocannabinoid system in inflammation and, in some cases, inflammatory pain, comes mostly from the finding of: (1) regulatory effects by AEA and PEA on serotonin and pro-inflammatory cytokine production; (2) opposing effects by AEA on the release of the vasoactive peptide calcitonin gene-related peptide (CGRP) from perivascular somatic neurons; (3) AEA, 2-AG and PEA production by stimulated basophils and macrophages; and (4) AEA, 2-AG and PEA inactivation by several blood cell types as well as endothelial cells.

Since the original reports of the anti-inflammatory activity of PEA (see Schmid et al., 1990 for review), the possible therapeutic use of this compound as an anti-inflammatory agent has been

investigated by Mazzari et al. (1996), who found, after oral administration to rats, a reduction of: (i) substance P-induced mast cell degranulation and extravasation; (ii) passive cutaneous anaphylaxis-induced extravasation; (iii) carrageenan-induced edema and hyperalgesia; (iv) formalin-induced edema; and (v) dextran-induced edema. The involvement of cannabinoid receptors in these effects was not determined, whereas a CB_1-mediated effect against carrageenan-induced thermal hyperalgesia and edema in rats was described later for AEA (Richardson et al., 1998). The finding of the up-modulation by the selective CB_1 antagonist SR141716A of mechanical allodynia produced by injections of complete Freund's adjuvant (CFA) in rat hindpaw (Martin et al., 1999) may have suggested the involvement of endogenous cannabinoids in this model of inflammatory pain. However, in a previous study using the same model, the anti-hyperalgesic effect of AEA was not blocked by SR141716A (Smith et al., 1998). This may indicate that the hyperalgesic effect observed by Martin et al. (1999) with the antagonist could be due to the counteraction of the tonic analgesic effect of an endocannabinoid different from AEA, or to the proposed inverse agonist properties of SR141716A. Furthermore, in the study by Smith and colleagues (1998), the antagonist, when administered alone, failed to alter paw pressure thresholds in either CFA-treated or control rats. Two other studies have shown that administration of either AEA or PEA produces anti-hyperalgesic effects in the rodent formalin paradigm of inflammatory pain. In rats, the two compounds only acted on the second, inflammatory phase of formalin-induced pain response (Jaggar et al., 1998a). In mice, the effects of two compounds were reduced by SR141716A and by the selective CB_2 receptor antagonist, SR144528, respectively, and the two antagonists were also shown to induce hyperalgesia when administered alone (Calignano et al., 1998). More recent work showed no consistent hyperalgesic effect by SR141716A or SR144528 in the formalin test carried out in rats and mice (Hanus et al., 1999; Beaulieu et al., 2000; personal communication by A. Lichtman), nor by either antagonist in the mouse AA-induced ear thickness test (Hanus et al., 1999). Finally, also visceral inflammatory pain can be reduced by AEA and PEA (Jaggar et al., 1998a,b). Unlike AEA, however, PEA did not prevent the viscero-visceral hyperreflexia associated with nerve growth factor (NGF)-induced inflammation of the rat urinary bladder, a finding that, together with the results showing no effects by PEA on the first phase of the formalin anti-nociceptive response (Jaggar et al., 1998a), may suggest that this compound becomes effective as an analgesic only when an inflammatory state is established. These findings underscore the difficulty of assessing both the occurrence of an endocannabinoid inhibitory tone in inflammatory pain, and the participation of cannabinoid receptors in the putative anti-inflammatory actions of exogenous AEA and PEA.

A possible down-regulatory effect by AEA and PEA in airway hyper-reactivity has also been investigated. The former compound did not affect dynamic compliance, total pulmonary resistance, tidal volume or breathing frequency in guinea pigs treated with an aerosol of A23187, but it did reduce airway epithelial injury and pulmonary leukocytosis without preventing peribronchiolar granulocytic accumulation (Stengel et al., 1998). In the study by Berdyshev et al. (1998), both AEA and PEA were reported to weakly inhibit tumor necrosis factor α (TNF-α) levels in bronchoalveolar lavage fluid of LPS-treated mice. AEA was also found to inhibit neutrophil recruitment. These data suggest that the two acylethanolamides have moderate anti-inflammatory activity in the airways, although they may lack the ability to directly relax the airway smooth muscle. In any case, even the weak relaxing effect on tracheal preparations recently described for high μM concentrations of AEA was not mediated by cannabinoid receptors (Yousif and Oriowo, 1999).

In vitro, AEA and PEA act on immune cells to release inflammatory mediators such as serotonin and pro-inflammatory cytokines, but only in one case the involvement of cannabinoid receptors could be suggested. PEA and some potent synthetic cannabinoids were found to inhibit the antigen-induced secretion of serotonin from both rat mast cells and RBL-2H3 cells (Facci et al.,

1995). Surprisingly, AEA inhibited the PEA- and cannabinoid-induced down-regulatory effect. The authors suggested that CB_2, but not CB_1, cannabinoid receptors were responsible for this effect in RBL-2H3 cells. However, subsequent studies (recently reviewed by Lambert and Di Marzo, 1999) showed that PEA is unable to bind with a high affinity to either of the two cannabinoid receptor subtypes. Possible non-CB_1–non-CB_2 receptors may explain the finding by Facci et al., 1995 (recently revisited by Lambert et al., 1999) that PEA competes for the binding of [^{3}H]WIN55,212-2 to membranes prepared from these cells. Berdyshev et al. (1997) reported an inhibitory effect of low nM concentrations of AEA and PEA on LPS-stimulated interleukin-6 and -8 and, in the case of AEA only, TNF-α production. Conversely, the cannabinoid agonist CP-55,940, but not AEA or THC, was shown to *induce* interleukin-8 and β-chemokine monocyte chemotactic protein-1 gene expression in unstimulated HL60 cells (Jbilo et al., 1999). This effect was due to activation of CB_2 receptors, which are not efficiently activated by either THC or AEA (Mechoulam et al., 1998). Incidentally, AEA was also found to enhance the release of the anti-inflammatory interleukin-6 from astrocytes infected with Theiler's murine encephalomyelitis virus (Molina-Holgado et al., 1998). This effect was blocked by the CB_1 antagonist SR141716A, even though controversial data exist on the expression of CB_1 receptors by astrocytes. From these data, a rather confusing scenario emerges where AEA, possibly depending on the concentration used, exerts often opposing effects on pro- and anti-inflammatory cytokines by acting thorough mechanisms that await a full clarification.

Another possible means for endocannabinoids to intervene in inflammation is to modulate the release and action of vasodilatory neuropeptides, much in the same way AEA and 2-AG modulate neurotransmitter activity in the CNS (see Di Marzo et al., 1998a for review). AEA was recently shown to variedly affect the release of CGRP from sensory fibers. At pmol doses, AEA inhibits capsaicin-induced CGRP release from rat hindpaw skin through a CB_1-mediated mechanism. This effect leads to inhibition of plasma extravasation into the hindpaw and to an anti-hyperalgesic action (Richardson et al., 1998). CB_1 receptors, however, are most abundant in neurons intrinsic to the spinal cord and are not expressed in the majority of capsaicin-sensitive CGRPergic sensory neurons (Hohmann and Herkenham, 1999; Farquhar-Smith et al., 2000). Indeed, AEA, but not PEA or 2-AG, was recently found to *induce* CGRP release from perivascular sensory neurons ex vivo through a CB_1 receptor-independent mechanism and via the direct activation of vanilloid receptors (Zygmunt et al., 1999). This effect was exerted in rat mesenteric neurons at concentrations similar to those necessary to AEA to activate CB_1 receptors in other tissues. AEA, at higher concentrations, was also capable of activating either rat (Zygmunt et al., 1999) or human (Smart et al., 2000) VR1 vanilloid receptors in heterologous systems that over-express these receptors. Thus, it is possible that AEA exerts anti-inflammatory or pro-inflammatory responses by either decreasing or increasing CGRP levels. In neurons where CB_1 receptors are localized on capsaicin-sensitive sensory neurons, the CB_1 receptor-mediated inhibitory effect would prevail, whereas in sensory fibers that only express vanilloid receptors, stimulation of CGRP release might occur. Alternatively, if the effect of AEA on VR1 receptors was found to be similar to that of other long chain fatty acid derivatives of capsaicin, such as olvanil (see Szallasi and Blumberg, 1999, for review), it is possible that AEA activation of these receptors is immediately followed by desensitization, which could result in an anti-inflammatory action. Finally, the possibility that AEA may modulate nociceptive responses via spinal VR1 receptors is suggested by the recent finding that the endocannabinoid, although at high μM concentrations, activates inward cation currents in rat dorsal root ganglia in a fashion sensitive to the vanilloid receptor antagonist capsazepine (Smart et al., 2000).

Unlike the findings discussed above, the general picture of the formation and inactivation of endocannabinoids and PEA in inflammatory and endothelial cells and, possibly, in sensory neurons, seems to be much clearer. Rat basophilic leukemia (RBL-2H3) cells, a widely employed model for the

study of mast cells, were shown to respond to ionomycin stimulation and immunological challenge-two stimuli leading to cell degranulation and serotonin/histamine release — by producing AEA, PEA and other fatty acid ethanolamides (Bisogno et al., 1997). The same cells inactivate AEA and PEA through uptake processes followed by enzymatic hydrolysis of the amide bond, catalyzed by FAAH (Bisogno et al., 1997; see the reviews by Hillard and Ueda et al. in this issue on the AEA transporter and FAAH, respectively). A facilitated diffusion mechanism for AEA was described in RBL-2H3 cells and shown later to be inhibited by phloretin and, particularly, fatty acid vanillyl-amides such as olvanil, linvanil and arvanil (see below) (Di Marzo et al., 1998b; Melck et al., 1999b). Intact RBL-2H3 cells were also shown to inactivate 2-AG through enzymatic hydrolysis as well as direct esterification into membrane phospholipids (Di Marzo et al., 1998c). Human mast cells also uptake and hydrolyze AEA (Maccarrone et al., 2000b). Macrophages also produce AEA and PEA together with 2-AG on stimulation with ionomycin (Di Marzo et al., 1996, 1999a). Two inflammatory stimuli, lipopolysaccharide (LPS) and platelet activating factor (PAF), induce 2-AG and/or AEA formation in mouse RAW 264.7 macrophages (Pestonjamasp and Burstein, 1998) and rat circulating macrophages (Varga et al., 1998; Di Marzo et al., 1999a). Macrophages contribute to the homeostasis of endocannabinoids also by inactivating these compounds through several pathways (Bisogno et al., 1997; Di Marzo et al., 1999a). Platelets also contribute to endocannabinoid inactivation (Maccarrone et al., 1999), and to the production of 2-AG (but not AEA) on stimulation with LPS (Varga et al., 1998). Interestingly, the enzymatic hydrolysis of 2-AG by platelets (and macrophages) was down-regulated by LPS (Di Marzo et al., 1999a), whereas nitric oxide (NO) seems to play a role in the up-regulation of the activity of the AEA membrane transporter in monocytes, platelets and endothelial cells (Maccarrone et al., 1998, 1999, 2000a). These findings indicate that inflammatory stimuli and mediators may determine the levels of endocannabinoids at the site of inflammation by regulating not only their biosynthesis but also their inactivation. Finally, a recent study suggested that AEA and 2-AG formation can be stimulated also in other cell types involved in the control of vascular permeability, i.e. endothelial cells and sensory neurons (see also the review by G. Kunos in this same issue). A Ca^{2+} ionophore, thrombin and acetylcholine were shown to induce 2-AG formation by rat aorta and human umbilical vein endothelial cells (Mechoulam et al., 1998; Sugiura et al., 1998), whereas the formation of AEA by perivascular sensory neurons stimulated with high Ca^{2+} concentrations was suggested on the bases of pharmacological data (Ishioka and Bukoski, 1999). If supported by further and more physiologically relevant data, these findings would indicate that several cell types may concur to regulating the homeostasis of endocannabinoids at the site of inflammation.

Does the finding of AEA, PEA and 2-AG formation in inflammatory and endothelial cells in vitro have a correspondent in animal models of inflammation? Only a few studies have addressed so far this question. In one case, Kondo et al. (1998) examined the levels of several acylethanolamides, including PEA and AEA, as well as of their phospholipid biosynthetic precursors, the *N*-acyl-phosphatidylethanolamines (see the review by Schmid in this issue), in rat testes treated with cadmium chloride, a chemical known to induce inflammation and degeneration of this tissue. The authors obtained a striking increase of the amounts of all acylethanolamides and, correspondingly, of their precursors, in cadmium-treated vs. vehicle-treated testes. PEA formation appeared to be stimulated to an extent much higher than AEA biosynthesis. In another set of studies, we measured the levels of AEA, PEA and 2-AG in rat hindpaw skin after injection of 1, 2.5 or 5% formalin, as well as in the NGF- or turpentine-treated rat urinary bladder. We found no statistically significant variation in the levels of these compounds, nor in FAAH-like activity (Table 1), thus suggesting that endogenous cannabimimetic fatty acid derivatives may not be locally released in these animal models of inflammation (Beaulieu et al., 2000; Di Marzo and Rice, unpublished observations).

5. Natural and synthetic endocannabinoid analogues in cancer, inflammation and related disorders

From the experimental evidence described in the previous sections it can be concluded that there is a potential for the use of endocannabinoids as therapeutic agents against the growth of some cancer cells, possibly for the cure of cachexia and wasting syndromes, and for the treatment of inflammatory diseases. Both AEA and, particularly, 2-AG, however, are rapidly degraded by cells both in vitro (Deutsch and Chin, 1993; Di Marzo et al., 1998c, 1999a) and in vivo (Willoughby et al., 1997; Jàrai et al., 2000). Furthermore, the major product of their degradation, AA, is the precursor of a plethora of bioactive molecules, the eicosanoids. Therefore, it is hard to predict that endocannabinoids per se could be used as therapeutic agents, and it will be important to design new metabolically stable analogues for these compounds. If these synthetic substances are also capable of interacting with other molecular targets involved in the inhibition of tumor cell proliferation, the induction of appetite and the control of inflammation and pain, it would be possible, in principle, to obtain ultra-potent anti-tumor, anti-inflammatory and analgesic drugs. This possibility is all the more attractive if one takes into consideration that cancer patients often suffer not only from the loss of body weight but also from chronic pain and inflammatory pathologies.

Bearing in mind the analgesic, anti-inflammatory (Szallasi and Blumberg, 1999) and anti-tumor (Morre et al., 1995) properties of capsaicin and/or its long chain derivatives, we synthesized a series of *N*-acyl-vanillyl-amines (Fig. 3). Some of these compounds were shown to be potent inhibitors of AEA re-uptake, as well as partial agonists for CB_1, but not CB_2, receptors, and to be capable of activating efficaciously also VR1 vanilloid receptors (Melck et al., 1999b). We found that one of these compounds, arvanil, was among the most potent inhibitors of basal HBCC and prolactin-induced DU-145 cell proliferation developed to date. Arvanil also inhibited NGF-induced MCF-7 proliferation by suppressing the expression of *trk* receptors. These effects were partially counteracted by either SR141716A or the vanilloid receptor antagonist capsazepine (Melck et al., 1999b, 2000), suggesting that arvanil behaved under these conditions as a 'hybrid' activator of CB_1 and VR1 receptors. Arvanil was also found to exert very strong analgesic effects in animal models of spinal, supra-spinal and inflammatory pain (V. Di Marzo, C.S. Breivogel, T. Bisogno, D. Melck, G. Patrick, Q. Tao, A. Szallasi, R.K. Razdan, B.R. Martin, submitted.; V. Di Marzo and A.C. Rice, unpublished observations) and to induce rapidly desensitizable vasodilation in rodents both in vivo and in vitro (V. Di Marzo, Z. Jàrai, T. Bisogno and G. Kunos, unpublished observations). Although the fact that arvanil is

Table 1
Levels (pmols/mg extracted lipids, means ± SEM) of anandamide (AEA), palmitoylethanolamide (PEA) and 2-arachidonoyl-glycerol (2-AG) in control and inflamed rat hindpaw or bladder[a]

	Hindpaw *Control* ($n=9$)	Hindpaw *Formalin* 2.5% ($n=5$)	Bladder *Control* ($n=3$)	Blader *Turp* ($n=3$)	Bladder NGF ($n=3$)
AEA	0.69 ± 0.14	0.53 ± 0.19	0.21 ± 0.11	0.19 ± 0.11	0.16 ± 0.05
PEA	5.6 ± 1.0	3.64 ± 0.52	3.35 ± 1.7	2.56 ± 1.5	2.63 ± 0.66
2-AG	51.1 ± 9.0	79.8 ± 12.3	36.7 ± 13.6	43.6 ± 9.6	43.6 ± 6.5

[a] The metabolites were extracted and purified as described previously (Beaulieu et al., 2000) and then derivatized and quantified by isotope dilution gas chromatography mass spectrometry in the presence of the appropriate deuterated standards (Bisogno et al., 1999). NGF, nerve growth factor, 10 μg for 2 h; Turp, turpentine, 50% in olive oil for 2 h. Formalin was injected into the paw in a 0.9% saline solution, whereas NGF and turpentine were delivered intravesically into bladder lumen via transurethal catheter. Tissue was collected and extracted 1 h (in the case of hindpaws) or 2 h (in the case of bladder) after the treatment, when the inflammatory response peaks. Analogous results were obtained with 1% and 5% formalin. See (Beaulieu et al., 2000) for further details.

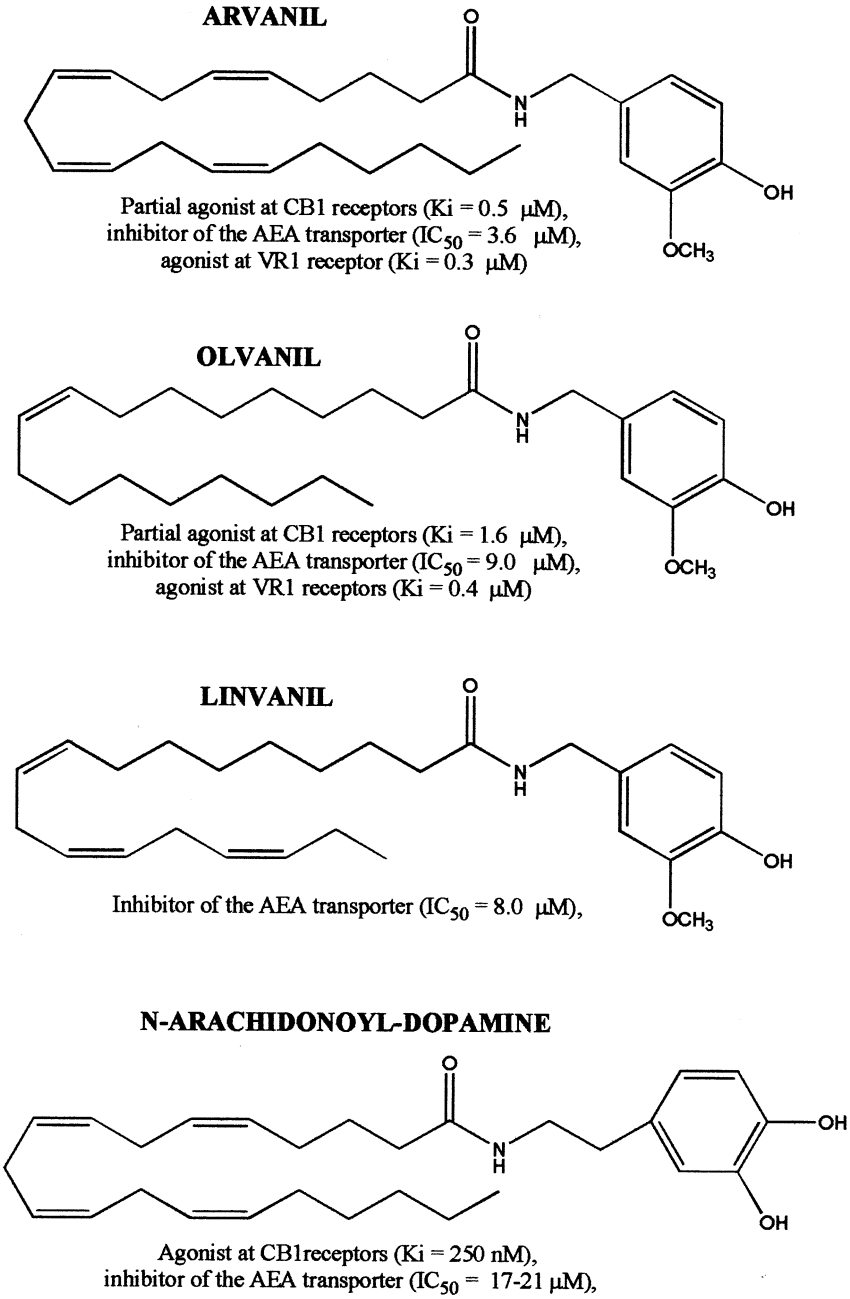

Fig. 3. Chemical structures of possible multi-targeted and metabolically stable synthetic cannabimimetic compounds. The all-*cis* unsaturated *N*-acyl-vanillyl-amides are inhibitors of anandamide facilitated transport as well as activators of cannabinoid and vanilloid receptors. *N*-arachidonoyl-dopamine also inhibits anandamide facilitated transport and activates CB_1 receptors, while its capability of binding to dopamine receptors is being evaluated.

not a good substrate for FAAH certainly contributes to its potent pharmacological activity in vivo, these data indicate that the development of functional 'chimeric' ligands of cannabinoid re-

ceptors and other sites of action can be a feasible and successful strategy to obtain potent anti-tumor, analgesic and anti-inflammatory agents. Encouraged by these results we are now studying a series of *N*-acyl-dopamine analogues synthesized in V. Bezuglov's laboratory in Moscow. Our preliminary data suggest that, like arvanil, one of these compounds, *N*-arachidonyl-dopamine (Fig. 3), is a good ligand of CB_1, but not CB_2, receptors ($K_i = 250$ nM and ≥ 8 μM, respectively), with very potent activity against HBCC proliferation ($IC_{50} = 0.25$ μM) (T. Bisogno, D. Melck, M.Y. Bobrov, L. De Petrocellis, N.N. Gretskaya, V. Bezuglov and V. Di Marzo, submitted). The respective contribution of dopamine D2 receptors (which are present in HBCCs and retard their proliferation by inhibiting endogenous prolactin release [Johnson et al., 1995]) and CB_1 receptors to this latter effect is being currently evaluated.

In the previous sections, we have discussed the possible role of PEA in inflammation and briefly mentioned the effect of OA on HBCC proliferation. PEA and, particularly, OA are substrates for FAAH (see the article by Ueda et al. in this issue) and are usually found in cells in higher amounts than AEA (Lambert and Di Marzo, 1999, for review). Therefore, it has been suggested that some of their pharmacological properties are due to inhibition of AEA inactivation and subsequent increase of endogenous AEA levels (Mechoulam et al., 1997; Lambert and Di Marzo, 1999). In agreement with this hypothesis, OA has been found to synergize with AEA as an anti-proliferative agent for human lymphocytes and HBCCs (Langstein et al., 1996; Bisogno et al., 1998). A reciprocal synergic effect by PEA and AEA in the inhibition of the formalin-induced anti-nociceptive response has also been reported (Calignano et al., 1998). This latter effect was counteracted by two antagonists of either CB_1 or CB_2 receptors, i.e. SR141617A and SR 144528, respectively, and was, therefore, interpreted as the capability of CB_2-like receptors to synergize with CB_1-induced anti-nociception, and vice versa. We found that PEA did not inhibit HBCC or human prostate cancer cell proliferation per se, but significantly enhanced the anti-proliferative effects of AEA by

Table 2
Effect of palmitoylethanolamide and other substances on anandamide inhibition of human breast and prostate cancer cell proliferation (expressed as% control cell proliferation, 100 = no effect, 0 = maximal effect)[a]

	MCF-7 (basal)	EFM-19 (basal)	MCF-7 (NGF-induced)	DU-145 (PRL-induced)
Anandamide (1 μM)	73.7 ± 3.2	64.3 ± 9.3	38.1 ± 0.2 ($IC_{50} = 0.55$ μM)	57.4 ± 1.7
Palmitoylethanolamide	96.5 ± 3.5	96.0 ± 4.2	98.3 ± 2.5	92.3 ± 4.8
Anandamide + palmitoylethanolamide	40.4 ± 6.2[b]	33.4 ± 5.3[b]	5.3 ± 4.2[c] ($IC_{50} = 0.1$ μM[c])	0[d]
BML-190 (5 μM)	98.5 ± 2.1	98.7 ± 2.6	98.9 ± 2.0	N.D.
Anandamide + BML-190	67.7 ± 2.5	66.1 ± 0.7	40.2 ± 9.0	N.D.
SR144528 (0.5 μM)	N.D.	70.4 ± 9.8	N.D.	N.D.
Anandamide + palmitoylethanolamide + SR144528	N.D.	34.3 ± 6.6	N.D.	N.D.

[a] The concentration of palimtoylethanolamide used was 5 μM for studies on the effect on basal proliferation and 2.5 μM for studies on NGF- and PRL-induced proliferation. For NGF-induced MCF-7 cell proliferation, the IC_{50} values for the effect of anandamide with or without 2.5 μM palmitoylethanolamide are also shown. NGF, nerve growth factor; PRL, prolactin; BML-190, a selective agonist of CB_2 receptors; SR144528, a selective antagonist of CB_2 receptors; N.D., not determined.

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] $P < 0.005$, as compared by ANOVA to anandamide only. For further details on cell proliferation assays (see De Petrocellis et al., 1998; Melck et al., 1999a, 2000).

about 5-fold (Table 2, D. Melck, L. De Petrocellis, T. Bisogno and V. Di Marzo, manuscript in preparation). This 'entourage' synergic effect was not mimicked by a selective CB_2 agonist, and was not counteracted by the CB_2 antagonist, SR144528. This finding suggests that CB_2 receptors are not necessarily involved in the synergic effects of PEA on AEA actions, and that other molecular explanations must be looked for in order to explain this phenomenon. In any case, the synergic actions by PEA and OA on AEA cytostatic and analgesic effects could be exploited by devising appropriate anti-cancer and anti-inflammatory 'cocktails', much in the same way a mixture of 2-AG with non-cannabimimetic 2-acylglycerols was recently shown to exhibit higher pharmacological activity in vivo than 2-AG alone (Ben-Shabat et al., 1998).

6. Concluding remarks

The data described in this article point to endocannabinoids as possible new templates for the development of potent anti-inflammatory and anti-tumor drugs. However, whether these compounds behave as endogenous modulators of hyper-reactivity and cancer cell proliferation under patho-physiological conditions still remains to be fully established. This is not a trivial issue since, if these disorders can be associated with a defective regulation of endocannabinoid levels, it will be possible to use as possible remedies not only endocannabinoid-based agonists, but also inhibitors of endocannabinoid biosynthesis, action and inactivation. In fact, several inhibitors of AEA facilitated transport and enzymatic hydrolysis, devoid of CB_1-binding and psychotropic activities, have been developed to date (see Khanolkar and Makriyannis, 1999; Di Marzo et al., 1999b, for reviews) and used to enhance the pharmacological effects of the endocannabinoid in vivo.

Another point that deserves further investigation is the actual participation of cannabinoid receptors to the anti-inflammatory and anti-proliferative effects of endocannabinoids. As mentioned above, although there is strong evidence suggesting that AEA cytostatic action on HBCCs is mediated by CB_1-like receptors, other mechanisms have been described for the anti-neoplastic activity of plant cannabinoids. However, a very recent investigation suggested that also the apoptotic effects of cannabinoids on C6 glioma cells might be due to activation of cannabinoid receptors and subsequent ceramide accumulation, and would result in blockade of malignant gliomas in vivo (Galve-Roperh et al., 2000). The anti-inflammatory effect and the inhibition of chronic-pain induced by AEA also do not seem to be uniquely mediated by cannabinoid receptors, and the participation of other sites of action, such as vanilloid (see Zygmunt et al., 2000) and opioid receptors (Manzanares et al., 1999), and non-CB_1–non-CB_2 cannabinoid receptors (see, for example, Jàrai et al., 1999), needs to be thoroughly investigated. These studies will tell us whether we can use potent non-endocannabinoid agonists of cannabinoid receptors, alone or in combination with other drugs, for the treatment of cancer and inflammation.

7. Note added to the proof

In a paper which appeared in press during the revision of this review, AEA was shown to induce apoptosis in several tumor cell lines by binding to sites of action different from CB_1/CB_2 receptors. Both the binding of AEA to these sites and AEA apoptotic action were reversed by capsazepine, a vanilloid receptor antagonist (Maccarrone, M., Lorenzon, T., Bari, M., Melino, G., Finazzi-Agro, A., J. Biol. Chem., epub ahead of time, in press). This finding confirms that AEA can also produce functional effects via vanilloid receptors and underlines the importance of 'hybrid' CB_1/VR1 receptor agonists as possible anti-cancer agents.

Recently, Ross R.A., Brockie H.C. and Pertwee R.G. (Eur. J. Pharmacol., 401, 121–130, 2000) reported that both the synthetic CB_1/CB_2 receptor agonist CP55,940 and PEA inhibit the lipopolysaccharide-induced release of nitric oxide in macrophages (which do biosynthesize PEA). The effect of CP55,940, but not PEA, was blocked by the CB_1 anatagonist SR144528. Furthermore, PEA, unlike CP55,940, decreased the E_{max} value,

but did not enhance the EC_{50}, for lipopolysaccharide-induced release of nitric oxide. These data suggest that this effect of PEA is not mediated by CB_2-like receptors. In another study, cannabidiol, which has very little affinity for CB_1/CB_2 receptors, was shown to cause: (i) a dose-dependent suppression of lymphocyte proliferation, both mitogen-stimulated and antigen-specific, (ii) blockade of the Zymosan-triggered reactive oxygen burst by peritoneal granulocytes, and (iii) suppression of the lipopolysaccharide-induced rise in serum tumor necrosis factor in CB57/BL mice (Malfait, A.M., Gallily, R., Sumariwalla, P.F., Mailk, A.S., Andreakos, E., Mechoulam, R. and Feldman, M., Proc. Natl. ACad. Sci. USA, 97, 9561–9566, 2000). These effects were proposed to be at the basis of the inhibition by CBD of murine collagen-induced arthritis in mice, and emphasize how the anti-inflammatory actions of cannabimimetic compounds are not necessarily mediated by CB_1/CB_2 receptors.

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