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Review

Endocannabinoids

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Abstract

The background knowledge leading to the isolation and identification of anandamide and 2-arachidonoyl glycerol, the principal endocannabinoids is described. The structure–activity relationships of these lipid derivatives are summarized. Selected biochemical and pharmacological topics in this field are discussed, the main ones being levels of endocannabinoids in unstimulated tissue and cells, biosynthesis, release and inactivation of endocannabinoids, the effects of 'entourage' compounds on the activities of anandamide and 2-arachidonoyl glycerol, their signaling mechanisms and effects in animals. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Research on *Cannabis sativa* commenced early in the 19th century. The investigations did not lead very far. In contrast to morphine and cocaine, which were isolated from opium and from coca leaves as crystalline salts, cannabis had an active constituent that was quite elusive. It was present in an oily extract which could not be further purified. The first real progress was achieved in the early 1940s by Lord Todd in England and Roger Adams in the US, who independently isolated cannabinol, a very weak psychoactive constituent, and cannabidiol which is inactive. Synthetic studies led to compounds with cannabis-like activity, but the main active component of the plant was still beyond their reach (Mechoulam, 1973).

In the early 1960s we established the structure and stereochemistry of cannabidiol (Mechoulam and Shvo, 1963). The active cannabis constituent, Δ^9-tetrahydrocannabinol (Δ^9-THC), was isolated for the first time in pure form and its structure was elucidated in 1964 (Gaoni and Mechoulam, 1964). These rather tardy identifications reactivated cannabis research. Most of the natural cannabinoids were isolated from the plant soon thereafter and their structures were elucidated (Mechoulam, 1973). Total syntheses were achieved for many of the natural cannabinoids (Mechoulam, 1973). The basic structure–activity relationships regarding cannabimimetic activity (Razdan, 1986) and metabolic routes were established (Agurell et al., 1986). The structure of Δ^9-THC and some synthetic cannabinoids discussed below are presented in Fig. 1.

2. Molecular basis of cannabinoid action

With the clarification of the chemical aspects of cannabis, interest focused on cannabinoid pharmacology and cellular effects. Although we learned much about the overt behavioral effects, the neurochemistry and the neurophysiology of THC action (Paton and Pertwee, 1973; Paton, 1975; Dewey, 1986; Martin, 1986), little was known of its molecular basis until the late 1980s. Two main assumptions had hampered work in this direction. One of these was the presumed lack of stereoselectivity (Dewey et al., 1984). It is assumed that compounds acting through a biomolecule—on an enzyme, on a receptor or directly on a gene—generally show a very high degree of stereoselectivity. This was supposedly not the case with cannabinoids. Synthetic (+)-Δ^9-THC showed cannabimimetic activity of 5–10% compared with that of (−)-Δ^9-THC (Dewey et al., 1984). This observation cast doubt over the existence of a specific cannabinoid receptor and hence of a cannabinoid mediator. However it was established in the mid 1980s that

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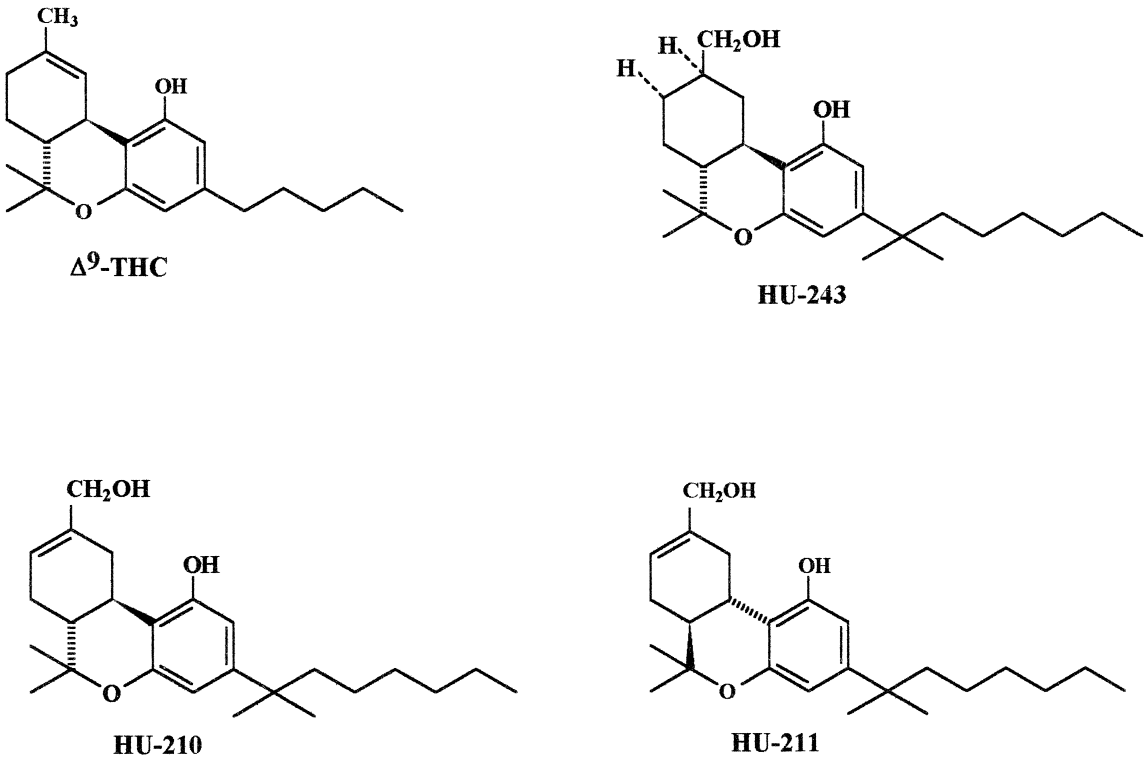


Fig. 1. Structures of some plant and synthetic cannabinoids mentioned in the text.

cannabinoid activity is highly stereoselective and that earlier observations resulted from technical separation problems (Mechoulam et al., 1992). Unfortunately, this presumed low degree of stereoselectivity had delayed research aimed at identification of a receptor–mediator cannabinoid system.

The second assumption was summarized by Paton, who pointed out that ''underlying much of the pharmacology of cannabis is the high lipophilicity of its active principles which is responsible for the slowness of its kinetics, its cumulation, [and] its persistence''. In his view cannabis pharmacology parallels in many ways that of the general anaesthetics, yet it does not produce the expected surgical anaesthesia, as ''there is too great a physicochemical disparity between it and biological membranes into which it is inserted for a volume fraction to be achieved sufficient to produce the phenomenon of full anesthesia'' (Paton, 1975). Δ^9-THC should thus, according to Paton, have belonged to the group of biologically active lipophiles and its effects should be compared with the chronic effects of anesthetics at low dose levels.

This line of thought made it possible to explain the action of cannabinoids without postulating the existence of a specific cannabinoid receptor and of an endogenous mediator of cannabinoid action.

Gill (1976) expanded the above ideas, and found experimental evidence that accounted for the low cannabinoid stereoselectivity without invoking the existence of a classical cannabinoid receptor. First, he compared the activity of cannabinoids and of steroid anesthetics on the order parameter of spin-labelled liposomes. He reported that the steroid anesthetic alfaxolone increased the fluidity of a liposome bilayer in a manner comparable to the effect of the volatile anesthetic, halothane. The same effect was observed with (−)-Δ^9-THC, while cannabinol and cannabidiol decreased the molecular disorder of the lipid bilayer. From these and related experiments Gill and Lawrence (1976) concluded that: ''The molecular pertubation produced by the psychoactive cannabinoids is qualitatively the same as that produced by the general anesthetics, namely, an increased fluidization and disordering of the lipid phase of the cell membrane. The effectiveness of the various cannabinoids in producing this increased fluidization correlates well with their potency as psychoactive agents, which encourages us in the belief that this effect is relevant to the in vivo actions of the cannabinoids''.

Martin (1986) has summarized the early literature on the possible duality of mechanism of action. The data cited show that cannabinoids produce membrane disturbances, but the size of these changes could not be correlated with differences in activity for the various cannabinoids. A typical example is reported by Tamir and Lichtenberg (1983) from our laboratory, who found that while (−)-Δ^9-THC and its dimethylheptyl analog were most effective to fluidize membranes, cannabidiol and the dimethylheptyl analog of (+)-Δ^9-THC had an opposite effect. This relationship is consistent with the known structure–activity relationships of cannabinoids. However the potent cannabi-

mimetic, Nabilone, showed effects similar to those of cannabidiol, rather than those of THC, which was counter to the predictions.

By the mid 1980s it had become clear that the membrane perturbation theory of cannabis action represents at best only part of the picture. The structure–activity relationships indicated that small changes in the THC molecule could lead to significant changes in activity—a situation incompatible with a non-specific (membrane) mode of action. For example, introduction of a methyl group on the aromatic ring next to the phenolic group (C-2 position) did not alter the activity, while the same modification next to the ether group (C-4 position) eliminated the activity (Glaser et al., 1995). The very high stereospecificity of cannabinoid action also pointed to a more specific mechanism. For example, (−)-HU-210 was found to be several thousand times more active in a variety of tests than was the (+)-HU-211 enantiomer (Fig. 1) (Mechoulam et al., 1992).

Makriyannis (1995) has again reviewed the evidence and has concluded that ''although the cellular membrane may not be the principal target for cannabinoid activity, it nevertheless plays a role in the mechanism of action''.

Results of recent studies show that some effects are indeed non-specific. Chakrabarti et al. (1998) have looked at neurobehavioral effects of anandamide (see below) and at cannabinoid receptor gene expression in mice. They have found that ''The anandamide induced neurobehavioral profile does not seem to correspond to the CB_1 gene expression in the mouse strains, it is therefore unlikely that the CB_1 receptor mediates all the cannabinomimetic effects of anandamide in the brain''. Lerner (1997) has argued that the sleep factor, *cis*-9-octadecenoamide (oleamide), which is chemically related to the endogenous anandamide, may be an endogenous fluidity transmitter that regulates some of the proteins that span the membrane, and has commented that ''it would be this process that general anesthesia is mimicking''. However, oleamide also potentiates anandamide action by inhibiting its rapid degradation, presumably by an action on the specific amidase (Mechoulam et al., 1997) (see below).

In summary, cannabinoids seem to have a dual mechanism of action, (a) through an action on receptors (see below) and, (b) by membrane perturbation. However, while the receptor pathway is well established, it should be kept in mind that the high concentrations of cannabinoid compounds required to produce membrane perturbation in in vitro assay systems would not be likely to be encountered in the cellular environment in animals (Howlett, 1995a).

The first reliable indications that cannabinoids act through receptors were provided by Howlett's group. Howlett and Fleming (1984), using the neuroblastoma N18TG2 cell line as a model system, demonstrated that cannabinoids interact with the adenylate cyclase second messenger pathway in an inhibitory fashion. The level of potency of a variety of cannabinoids to inhibit adenylate cyclase paralleled cannabinoid effects in animal models and in humans (Howlett et al., 1986; Howlett, 1987). Stereospecificity was also demonstrated using the HU-210 and HU-211 enantiomers (Howlett et al., 1990). (−)-HU-210 was several orders of magnitude more potent to inhibit cAMP accumulation and adenylate cyclase activity than was (+)-HU-211. This research approach culminated in the discovery in the brain of specific, high-affinity cannabinoid binding sites, whose distribution is consistent with the pharmacological properties of psychotropic cannabinoids (Devane et al., 1988; Herkenham et al., 1990; for reviews see Pertwee, 1995; Howlett, 1995a,b). Shortly thereafter, Matsuda et al. (1990) and Gerard et al. (1991) cloned the CB1 cannabinoid receptor. A peripheral receptor (CB_2) was identified in the spleen by Munro et al. (1993). Surprisingly the CB_2 receptor has only 44% homology with the CB_1 receptor.

3. Isolation and structure of anandamide

The standard assay for new receptor agonists is displacement of a labeled probe bound to the appropriate receptor. This route was followed in the isolation of anandamide. First, a new probe based on the highly active HU-210 was developed (Devane et al., 1992a). This compound, which has a typical THC-like structure, a K_D in the picomolar range, and high enantioselectivity of both pharmacological activity and binding, was an ideal candidate for labeling, thus producing a novel probe. This was achieved by enantiospecific reduction of the double bond in HU-210 with tritium, leading to [^{3}H]-HU-243 (Fig. 1). Its non-labeled form yields a Δ^9-THC-like response of pigeon drug discrimination ($ED_{50} = 0.002$ mg/kg) at the potency level of HU-210, and binds to the cannabinoid receptor with a K_i of 45 pM, significantly lower than even that of HU-210 ($K_i = 181$ pM).

All plant or synthetic cannabinoids are lipid soluble compounds. Hence, the procedures employed for the isolation of endogenous ligands by our group were based on the assumption that constituents are also lipid soluble, an assumption that ultimately proved to be correct. Porcine brains were extracted with organic solvents, and the extract was chromatographed according to standard protocols for the separation of lipids. The fractions obtained were screened for cannabinoid activity on the basis of their ability to displace radiolabeled HU-243 in a centrifugation-based ligand binding assay. Ultimately a single active constituent, named anandamide, was isolated (Devane et al., 1992b).

A major problem encountered in the isolation of anandamide was its lability: although purity increased on repeated chromatography, the amounts of anandamide diminished rapidly. Improvement of the separation procedure was reported in a later publication (Hanuš et al., 1993). After precipitation of the inactive phospholipids with ace-

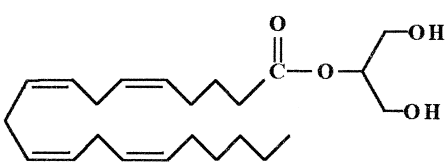
tone, the extract was chromatographed once only with a large number of small fractions collected.

Isolation of minute amounts of a labile natural product from a complicated mixture poses problems for the structural elucidation due to minor impurities of other related constituents, traces of materials originating from the plastic laboratoryware, or even traces of solvents tenaciously bound to the natural product. After laborious elimination of such impurities, the structure of anandamide was deduced from mass spectrometric (MS) and nuclear magnetic resonance (NMR) measurements. Technical details of these measurements and analyses of the results are given by Devane et al. (1992b), Hanuš et al. (1993) and Mechoulam and Fride (1995). Final proof of the structure of anandamide was obtained from a simple synthesis. The original publication reported the identification of one active compound only. A second paper, described two additional constituents shown to be closely related to the first anan-

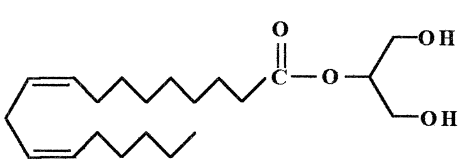
anandamide

7,10,13,16-docosatetraenoylethanolamide

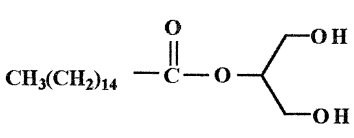
homo-γ-linolenylethanolamide



2-arachidonyl-glycerol



2-linoleoyl-glycerol



2-palmitoyl-glycerol

Fig. 2. Structures of endocannabinoids found in various tissues.

damide as to structure and activity (Hanuš et al., 1993). The structures of the three related active acyl ethanolamides are presented in Fig. 2.

4. 2-Arachidonoyl glycerol (2-Ara-Gl)

The identification of a second cannabinoid receptor (CB_2) in immune cells (Munro et al., 1993) led us to look for additional active endogenous ligands in the gut and later in the spleen, an organ with well established immune functions. Again, this was done by fractionation guided by a binding assay. Canine gut was extracted with methanol and the extract was chromatographed on a silica gel column to yield a fraction that bound to CB_1 in a centrifugation-based ligand binding assay. The active fraction consisted mainly of three compounds which, on the basis of MS measurements, were assumed to be 2-arachidonoyl glycerol, 2-palmitoyl glycerol and 2-linoleoyl glycerol. This assumption was shown to be correct by direct MS and NMR analysis and comparison with synthetic compounds (Mechoulam et al., 1995). 2-Arachidonoyl glycerol was later isolated by Sugiura et al. (1995) and Stella et al. (1997) from brain.

The structures of the three 2-acyl-glycerol esters are presented in Fig. 2.

5. Structure–activity relationships in the anandamide series

Since the identification of anandamide a considerable amount of data has accumulated on structure–activity relationships in the anandamide series (Pinto et al., 1994; Adams et al., 1995; Thomas et al., 1996; Sheskin et al., 1997 and Ref. cited). Tentative SAR regularities as regards binding have already been proposed. As K_i values are very sensitive to the experimental conditions, they vary in the different publications. However, a trend persists. The following tentative relationships for CB_1 binding can be formulated (see Fig. 3).

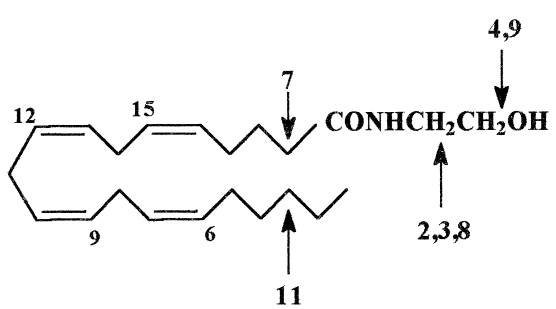


Fig. 3. Anandamide as scaffolding for structure–activity relationships. The numbers on the arrows indicate the portion of the molecule discussed in the text on page 5 in the paragraphs bearing the same numbers.

(1) The number of double bonds on the fatty acid moiety has to be at least 3 or 4; two double bonds only lead to inactivation. This regularity applies to the 20:*x*, n − 6 series only. The effect of a higher number of double bonds has not yet been investigated.

(2) In the 20:4, *n* − 6 series, the unsubstituted amide is inactive; *N*-mono-alkylation (i.e., without a ω-hydroxyl group, as present in anandamide), at least up to a branched pentyl group, leads to significant binding. The following regularities in binding were noted for anandamide-type compounds with the indicated *N*-alkyl moieties: *n*-C_5H_{11} < branched C_5H_{11} < $CHMeCH_2Me$ (either *R* or *S*) < *n*-C_4H_9 < *t*-C_4H_9 < CH_3 < C_2H_5 < $C(CH_3)_2$ < *n*-C_3H_7. The last two compounds were the most active in these homologous series, with K_i values about three times lower than that of anandamide.

(3) *N,N*-dialkylation, with or without hydroxylation on one of the alkyl groups, leads to loss of activity.

(4) Hydroxylation of the *N*-monoalkyl group at the ω-carbon atom (as in anandamide itself) preserves activity, as compared to the parent *N*-alkyl group. However, in most cases, this activity is slightly lower, at least in the relatively potent compounds.

(5) The methyl ether and the phosphate are less active than the parent alcohol. The carboxylic acid derivatives are inactive.

(6) The limited data suggest that, in the n − 3 series, the derived ehtanolamides are either inactive or less active than related compounds in the n − 6 series.

(7) Alkylation or dialkylation of the α-carbon adjacent to the carbonyl group preserves the level of binding in the case of anandamide. α-Monomethylation or α,α-dimethylation of *N*-propyl derivatives potentiates binding and yields led to highly active compounds.

(8) The presence of a chiral center on the *N*-alkyl substituent leads to enantiomers with significantly different levels of binding. One of these compounds, *R*-(+)-arachidonoyl-1′-hydroxy-2′-propylamide [(*R*)-methanandamide], has a 4-fold lower K_i than anandamide and has been shown to be stable to enzyme hydrolysis (Abadji et al., 1994).

(9) The OH group in anandamide can be replaced by a fluorine with about a 10-fold increase in specific binding to CB_1 (Welch et al., 1995; Ryan et al., 1997).

(10) Conjugation of the double bonds leads to reduced activity (Wise et al., 1996).

(11) Branching of the non-carboxylic end of the fatty acid residue enhances binding (Ryan et al., 1997; Seltzman et al., 1997 and Ref. cited).

There are several elementary structure–activity relationships aspects that have yet to be investigated: for example, (a) the minimal and maximal length of the fatty residue; (b) the effect of *trans* in place of *cis* double bonds; (c) the conversion of the fatty acid chain into a leucotriene- or a prostaglandin-type chain. There are preliminary investigations of these aspects (Pinto et al., 1994; Ueda et al., 1995;

Yu et al., 1997; Edgemond et al., 1998), but a clear picture has yet to emerge. Except for 12(*S*)-hydroxy-arachidonoylethanolamide which binds to CB_1, the few compounds so far tested have shown no activity.

No structure–activity relationships studies of the 2-arachidonoyl glycerol series have been reported so far. However unpublished results from our laboratory indicate that, as expected, 1(3)-arachidonoyl glycerol is as potent as 2-arachidonoyl glycerol on binding to both CB_1 and CB_2 and that 2-palmitoyl glycerol and 2-linoleoyl glycerol which accompany 2-arachidonoyl glycerol in the gut, spleen and pancreas do not bind to the receptors.

6. Levels of endocannabinoids in unstimulated tissues and cells

Several analytical methods, mostly based on high pressure liquid chromatography (HPLC), gas chromatography (GC) and HPLC- or GC-MS, have been developed for the quantitation of endocannabinoids, particularly anandamide, in tissues and biological fluids (Fontana et al., 1995; Schmid et al., 1995; Koga et al., 1995, 1997; Sugiura et al., 1996b; Felder et al., 1996; Kempe et al., 1996; Giuffrida and Piomelli, 1998). In rat brain, anandamide was found in concentrations ranging from 'not detectable' (Kempe et al., 1996) to 29 pmol/g tissue (Felder et al., 1996). Comparable amounts were found in porcine and bovine brain (Schmid et al., 1995), whereas higher levels were detected in human brain (up to 148 pmol/g tissue, Felder et al., 1996). On the other hand, in rat brain, the amounts of 2-arachidonoyl glycerol are at least 170 times higher than those of anandamide (up to 4 nmol/g tissue, Sugiura et al., 1995; Stella et al., 1997). Both endocannabinoids have also been detected outside the central nervous system. Anandamide was found in rat kidney (Deutsch et al., 1997), testis (Sugiura et al., 1996b), skin and spleen (Felder et al., 1996), blood plasma (Giuffrida and Piomelli, 1998) and pheochromocytoma PC-12 cells, regardless of whether the latter were undifferentiated or differentiated into sympathetic-like neurons following treatment with nerve growth factor (Bisogno et al., 1998). Detectable amounts of anandamide were also found in human spleen and heart (Felder et al., 1996) and in human breast cancer cells (Bisogno et al., 1998). Much higher levels (up to 20 nmol/g tissue) of the endocannabinoid were reported, for mouse uterus, but only when the organ is least receptive to embryo implantation (Schmid et al., 1997). 2-Arachidonoyl glycerol, but not anandamide, concentrations in the low nanomole per gram tissue range were found in canine spleen, pancreas and gut (Mechoulam et al., 1995; Ben-Shabat et al., 1998). Finally, there is now strong evidence for the presence of endocannabinoids in invertebrate tissues such as sea urchin ovaries and bivalve mussels (Bisogno et al., 1997c; Sepe et al., 1998). Results of the above quantitative studies suggest a few generalisations: (a) in all tissues where both endocannabinoids have been quantitated, anandamide is present in considerably lower amounts than 2-arachidonoyl glycerol; (b) anandamide basal levels are at least 10-fold lower than those of most classical neurotransmitters, but increase significantly in post mortem tissue (Schmid et al., 1995; Felder et al., 1996; Kempe et al., 1996; Sepe et al., 1998); (c) anandamide is often accompanied by higher levels of non-endocannabinoid fatty acid amides such as palmitoyl- and stearoyl-ethanolamide or the sleep-inducing factor, oleamide (Cravatt et al., 1995); (d) non-cannabimimetic monoacylglycerols, such as the mono-palmitoyl, mono-oleoyl and mono-linoleoyl glycerols, accompany 2-arachidonoyl glycerol in all tissues studied, although the polyunsaturated monoglyceride is the major constituent of its family in rat brain (Sugiura et al., 1995).

7. Biosynthesis and release of endocannabinoids

There is now considerable evidence for a phospholipid-mediated pathway for the Ca^{2+}-dependent biosynthesis of anandamide, at least in the CNS and the immune system. According to this pathway, anandamide, as previously shown for saturated and monounsaturated acylethanolamides (Schmid et al., 1990), is produced from the hydrolysis of *N*-arachidonoyl-phosphatidylethanolamine catalyzed by a phospholipase D-like enzyme (Di Marzo et al., 1994; Sugiura et al., 1996a, Fig. 4). This pathway accounts for the 'on demand' synthesis of the endocannabinoid in stimulated cells. Indeed, when membrane depolarization of rat central neurons is induced, one can observe de novo formation and release of anandamide and other non-endocannabinoid acylethanolamides (Di Marzo et al., 1994), including those previously shown to be formed through the hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamines (Schmid et al., 1990). In support of the phospholipid-mediated pathway for the synthesis of anandamide, *N*-acyl-phosphatidylethanolamines and *N*-arachidonoyl-phosphatidylethanolamine formation was found to be induced by the same stimuli, leading to acylethanolamide and anandamide biosynthesis (Di Marzo et al., 1994; Cadas et al., 1996). Subsequent studies have focused on the proposed precursor for anandamide, *N*-arachidonoyl-phosphatidylethanolamine leading to: (a) its quantitation in rat brain and testis (Sugiura et al., 1996a,b; Cadas et al., 1997), (b) preliminary characterization of phospholipase D catalyzing its conversion to anandamide (Sugiura et al., 1996a,b; Di Marzo et al., 1996b), and (c) its Ca^{2+}-dependent biosynthesis through the *N*-arachidoylation of phosphatidyl-ethanolamine, using arachidonic acid from the *sn*-1 position of *sn*-1,2-di-arachidonoyl-phosphatidylcholine (Sugiura et al., 1996a,b; Di Marzo et al., 1996b; Cadas et al., 1997) (Fig. 4). This pathway, due to the low levels of ultimate anandamide precursors, the *sn*-1-arachidonate-containing phospholipids, accounts for

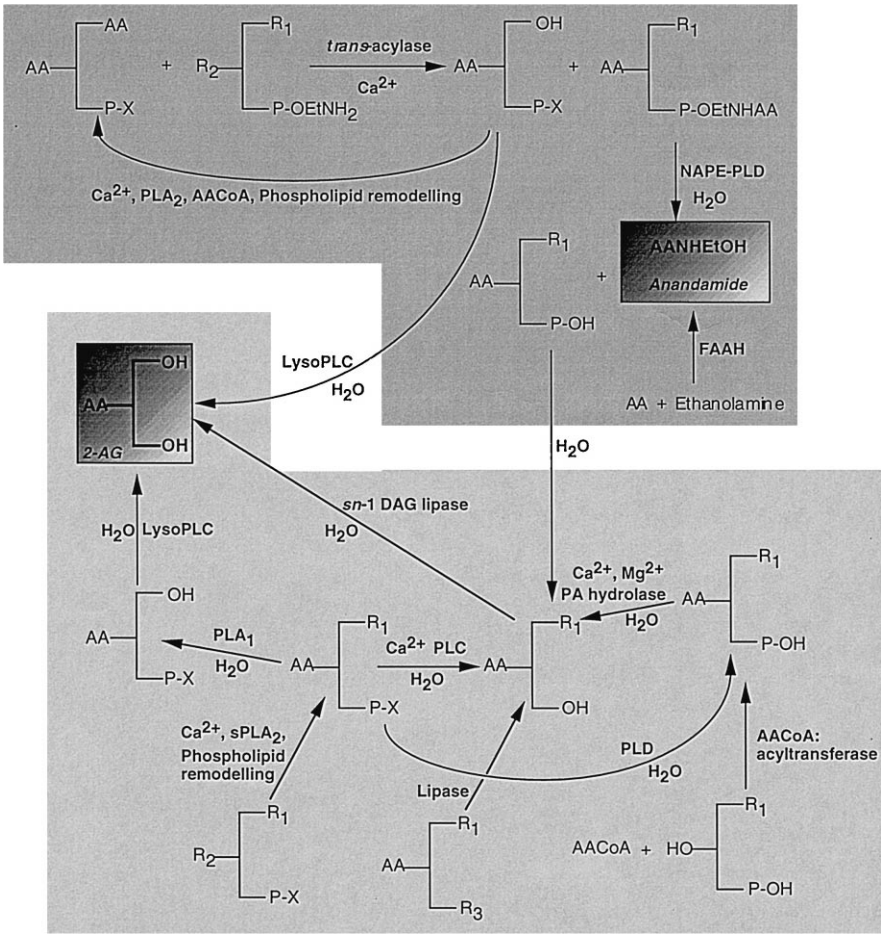


Fig. 4. Biosynthetic pathways for anandamide and 2-arachidonyl glycerol. Dark grey panel: anandamide biosynthesis. Light grey panel: 2-arachidonoyl glycerol biosynthesis. Possible intersections between anandamide and 2-arachidonoyl glycerol synthetic routes are also shown. Calcium-activated phospholipid remodelling may play a role in the formation of precursors for both endocannabinoids. The synthesis of anandamide from ethanolamine and arachidonic acid has been shown only in cell-free systems and with very high concentrations of substrates. AA, arachidonate; cPLA2, SPLA2, cytosolic or secretory phospholipase A2; PLA1, phospholipase A1: NAPE-PLD, phospholipase D selective for *N*-acylphosphatidylethanolamines; FAAH, fatty acid amide hydrolase; P, phosphate group; X, phospholipid base; PLC, phospholipase C; CoA, coenzyme A; PA, phosphatidic acid; MAG, monoacylglycerol; DAG, diacylglycerols; TG, triglycerides.

the low amounts of anandamide compared to those of other acylethanolamides found in most tissues. It has been shown that stimulated cells have an enhanced biosynthesis of *sn*-1,2-di-arachidonoyl-phosphatidylcholine (for example see Kuwae et al., 1997). However, it should be mentioned that another route has been proposed for anandamide biosynthesis, although only in cell-free systems. This route consists of the ATP- and coenzyme A-independent condensation of free arachidonic acid and ethanolamine catalyzed by a 'synthase' (Deutsch and Chin, 1993). This route may lead to selective formation of anandamide preferentially to other acylethanolamides. The 'anandamide synthase' activity, however, showed overall low affinity for ethanolamine and arachidonic acid, and is likely to be due to an 'anandamide amidohydrolase' working 'in reverse' (see below). There may be particular tissues, however, in which this pathway could account for the high levels of anandamide vs. other acylethanolamides detected under particular physiological conditions, such as those typical of the pseudopregnant uterus (Schmid et al., 1997).

The biosynthesis and release of 2-arachidonoyl glycerol were also shown to depend on Ca^{2+} influx into cells. In ionomycin-treated mouse neuroblastoma N18TG2 cells, the monoglyceride was shown to be produced from phospholipase C-independent pathways, and probably through the catalytic action of a *sn*-1-diacylglycerol lipase on *sn*-2-arachidonate-containing diacylglycerols (Bisogno et al., 1997b). N18TG2 cell and rat brain homogenates also proved to have (lyso) phospholipase C activities leading to the formation of 2-arachidonoyl glycerol from (lyso) phosphatidylcholine and lysophosphatidyl-inositol species (Di Marzo et al., 1996a; Ueda et al., 1993). In rat hippocampal slices, 2-arachidonoyl glycerol, but not anandamide, was shown to be produced following electrical stimulation of the Schaffer collaterals, whereas in rat cortical neurons the endocannabinoid was proposed to be biosynthesized through the sequential action of phosphoinositide-selective

phospholipase C and *sn*-1 diacylglycerols lipase (Stella et al., 1997). Finally, 2-arachidonoyl glycerol was shown to be produced by rat platelets challenged with lipopolysaccaride (Varga et al., 1998), and human umbilical vein endothelial cells and aortic smooth muscle cells incubated with either thrombin or the Ca^{2+} ionophore A23187 (Sugiura et al., 1998). There is also chromatographic evidence for the production of a mono- arachidonoylglycerol-like metabolite by fibroblasts treated with platelet-derived growth factor (Hasegawa-Sassaki, 1985) and by dorsal root ganglia stimulated with bradykinin (Allen et al., 1992).

In summary, while a rather specific, albeit non-selective, pathway leads to anandamide biosynthesis, several different mechanisms seem to underlie 2-arachidonoyl glycerol formation in cells. This possibility could explain why the basal levels of the monoglyceride are at least two orders of magnitude higher than those of anandamide. However it should be remembered that diacyglycerol lipase-mediated 2-arachidonoyl glycerol formation also has the purpose of: (a) terminating an agonist-induced diacylglycerol/protein kinase C-mediated intracellular signal (as reviewed by Shears, 1993), and (b) creating an alternative intracellular precursor for arachidonate (and eicosanoid) formation (Hasegawa-Sassaki, 1985; Allen et al., 1992). It is possible that only a minor part of the 2-arachidonoyl glycerol produced by stimulated cells is used to activate cannabinoid receptors.

So far, there have been only a few studies aimed at correlating endocannabinoid biosynthesis with particular physiopathological conditions. Anandamide, or other acylethanolamides and *N*-acyl-phosphatidylethanolamines, were shown to be formed following neuronal damage induced by either glutamate or sodium azide (Hansen et al., 1997), myocardial infarction and brain ischemia (reviewed in Schmid et al., 1990). Anandamide and palmitoylethanolamide biosynthesis was stimulated by immunological challenge of a widely used model of mast cells, the RBL-2H3 cell line (Bisogno et al., 1997a). These cells respond to the two acylethanolamides in opposite ways, palmitoylethanolamide being a potent inhibitor of degranulation, and anandamide antagonizing the palmitoylethanolamide effect (Facci et al., 1995). More recently, the production of anandamide from macrophages and of 2-arachidonoyl glycerol from platelets has been correlated with the hypotensive state subsequent to haemorrhagic or septic shock in rats (Wagner et al., 1997; Varga et al., 1998). This finding, together with the observation that ionomycin treatment of sympathetic-like neurons—obtained by treating PC and 12 cells with nerve growth factor—does not result in any significant stimulation of anandamide/acylethanolamide/*N*-acyl phosphatidylethanolamine biosynthesis (Bisogno et al., 1998), could suggest that hypotensive endocannabinoids are not derived from sympatholytic activity. Finally, in mouse uterus, the formation of anandamide, with inhibitory activity on blastocyst hatching, was found to peak simultaneously with uterine refractoriness to embryo implantation (Schmid et al., 1997).

8. Inactivation of endocannabinoids

Classical neurotransmitters are usually inactivated by facilitated re-uptake from neurons and/or astrocytes and subsequent enzymatic degradation. This principle, when applied to anandamide and 2-arachidonoyl glycerol poses two major questions: (1) What is the need of a 'carrier' mechanism for the re-uptake of molecules that, being lipophilic, can easily diffuse through the cell membrane? (2) If, as their chemical structures would suggest, anandamide and 2-arachidonoyl glycerol are degraded to arachidonic acid, would this reaction not represent a potential mechanism for the formation of other bioactive, arachidonate-derived molecules? Both these questions were addressed during studies on endocannabinoid inactivation.

Anandamide was found to be inactivated by both rat central neurons and astrocytes, as well as by tumoral cell lines, through sequential re-uptake and enzymatic hydrolysis (Deutsch and Chin, 1993; Di Marzo et al., 1994). Uptake was rapid, temperature-dependent, selective for anandamide over other acylethanolamides and saturable, thus strongly suggesting the presence of a facilitated transport mechanism (Di Marzo et al., 1994). More recent studies have confirmed the presence of 'carrier' proteins for the facilitated diffusion of anandamide through the plasma membranes of RBL-2H3 cells (Bisogno et al., 1997a), cerebellar granule cells (Hillard et al., 1997) and rat cortical neurons and astrocytes (Beltramo et al., 1997). The actual physiological relevance of such 'carriers' for anandamide inactivation in vivo, at least in the CNS and cardiovascular system, was suggested by results of studies showing that the analgesic and hypotensive action of the endocannabinoid could be enhanced by co-administration of AM-404, a selective inhibitor of anandamide recapture by rat neurons and astrocytes (Beltramo et al., 1997; Calignano et al., 1997a).

Despite the proven existence and functional importance of anandamide-facilitated transport mechanisms, a measurable part of the acylethanolamide is recaptured by cells, also via passive diffusion through the cell membrane. This was shown by the observation that some of the exogenous anandamide can be taken up by intact cells, also at 0–4°C (Di Marzo et al., 1994; Bisogno et al., 1997a; Beltramo et al., 1997). Passive diffusion is probably entirely responsible for 2-arachidonoyl glycerol recapture by RBL-$2H_3$ cells. Recapture, apart from not being sensitive to low temperatures, was inhibited by di-/polyunsaturated monoacylglycerols, and was accompanied by rapid esterification of part of 2-arachidonoyl glycerol into membrane phospholipids (Ben-Shabat et al., 1998; Di Marzo et al., 1998). Once inside the cells, anandamide and 2-

arachidonoyl glycerol are immediately degraded to arachidonic acid and ethanolamine or glycerol, respectively, through the enzymatic hydrolysis of their amide/ester bonds (Deutsch and Chin, 1993; Di Marzo et al., 1994, 1998) (Fig. 5). Interestingly, very little of the arachidonate produced from the hydrolysis of micromolar concentrations of the two endocannabinoids was found in the culture medium or associated with cells, most of it having been immediately re-incorporated into membrane phospholipids (Di Marzo et al., 1994, 1998). This finding suggests that, under physiological conditions, where nanomolar quantities of endocannabinoids are produced and quickly recaptured and hydrolyzed by cells, arachidonic acid produced from anandamide and 2-arachidonoyl glycerol breakdown is not likely to be converted into eicosanoids. In pharmacological studies carried out with greater amounts of the two compounds, however, the possibility that some of the effects observed for the endocannabinoids may be due to their conversion into arachidonate and its derivatives should always be taken into account (for example, see Ellis et al., 1995; Jarrahian and Hillard, 1997).

The enzyme responsible for anandamide hydrolysis, originally named 'anandamide amidohydrolase' or 'amidase', has been thoroughly studied (for a recent review on the kinetics, inhibition and subcellular/tissue distribution of this enzyme see Di Marzo and Deutsch, 1998). Starting from the knowledge that 'anandamide amidohydrolase' could also efficiently recognize the sleep-inducing factor, oleamide, as a substrate (Maurelli et al., 1995), Cravatt et al., who had purified, cloned and characterized the enzyme catalyzing oleamide hydrolysis, expressed the latter protein in COS-7 cells and showed that it could recognize anandamide as the preferential substrate (Cravatt et al., 1996). The enzyme was therefore named 'fatty acid amide hydrolase' (FAAH) in view of its ability to catalyze the hydrolysis of at least two distinct classes of bioactive fatty acid amides.

Later it was shown that the recombinant enzyme could be forced to work 'in reverse' and catalyze the synthesis of anandamide and oleamide from high concentrations of fatty acids and ethanolamine or ammonia (Kurahashi et al., 1997; Arreaza et al., 1997). Furthermore, recombinant FAAH was found also to catalyze the hydrolysis of 2-arachidonoyl glycerol with higher efficiency than anandamide (Goparaju et al., 1998). Accordingly, partially purified FAAH from N18TG2 and RBL-2H3 cells also catalyzed the hydrolysis of 2-arachidonoyl glycerol (Di Marzo et al., 1998). These findings explain why inhibitors of FAAH, such as the arachidonoylfluoromethylketone and methylarachidonylfluorophosphonate (reviewed by Di Marzo and Deutsch, 1998), elevate the levels of 2-arachidonoyl glycerol in intact N18TG2 and RBL-2H3 cells (Di Marzo et al., 1998). However, 2-arachidonoyl glycerol enzymatic hydrolysis also occurs in cells expressing very little, if any, FAAH, suggesting that several lipases can contribute to the degradation of this endocannabinoid by tissues. Another mechanism has been proposed for the catabolism of anandamide also, consisting of its enzymatic oxidation by enzymes of the arachidonate cascade, e.g., lipoxygenases, cytochrome *P*450 oxigenases and cycloxygenase-2 (Bornheim et al., 1993; Ueda et al., 1995; Hampson et al., 1995; Yu et al., 1997) (Fig. 4).

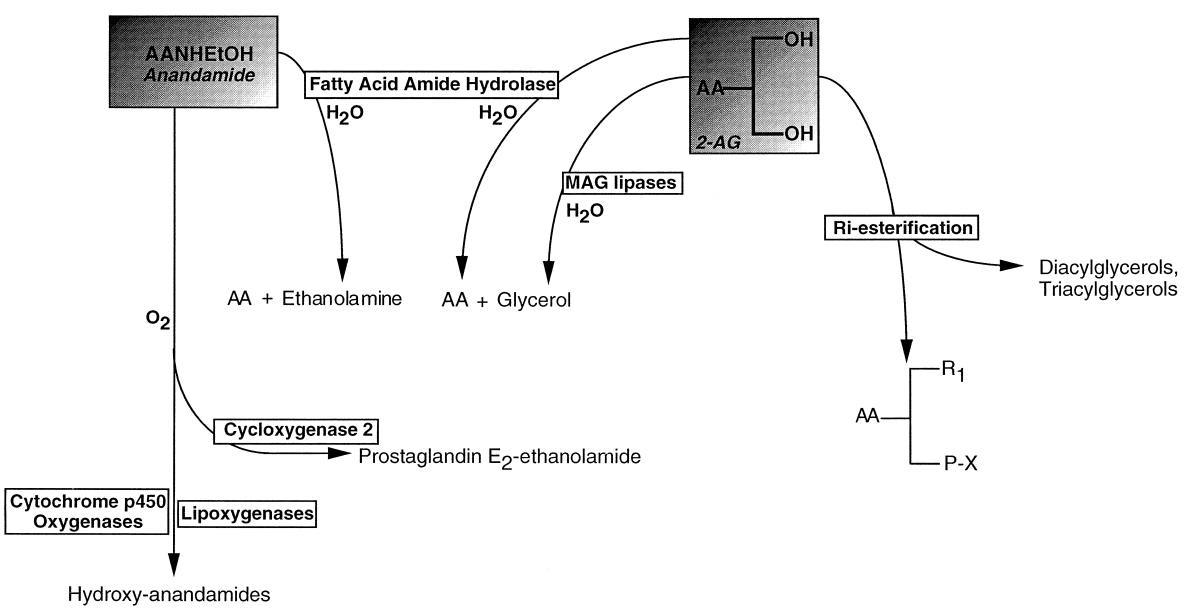


Fig. 5. Catabolism of anandamide and 2-arachidonoylglycerol. Several pathways may contribute to the inactivation of the two endocannabinoids once they have diffused through the cell membrane. Both hydrolysis of the amide bond and oxidation catalyzed by enzymes of the arachidonate (AA) cascade have been proposed for anandamide. As to 2-arachidonoylglycerol (2-AG), hydrolysis of the ester bond and esterification into phosphoglycerides or neutral glycerolipids have been found to occur in intact cells. Fatty acid amide hydrolase plays a key role in the inactivation of both metabolites. AA produced from the hydrolysis of endocannabinoids is immediately reincorporated into membrane phospholipids.

However, these reactions have were observed only in cell-free or cell-expression systems. Lipoxygenase-mediated oxidation of anandamide has recently been described to occur also in living cells, i.e., human platelets and polymorphonuclear leukocytes (Edgemond et al., 1998). 12 (*S*)-hydroxy-arachidonoylethanolamide, a compound with the same affinity as anandamide for CB_1 receptors, but more resistant to inactivation, was formed in both cases.

9. Regulation of FAAH and 'entourage' effects

Three recent observations suggest strongly that FAAH-catalyzed enzymatic hydrolysis plays an important role in the physiological inactivation of endocannabinoids, particularly that of anandamide. First, this compound is hydrolyzed to arachidonic acid in vivo within a few minutes of its injection in mice (Willoughby et al., 1997). Second, co-administration of anandamide with an inhibitor of FAAH, phenylmethylsulphonyl-fluoride, significantly potentiates anandamide effects in the 'tetrad' of behavioural tests in mice (Compton and Martin, 1997). Finally, FAAH distribution in the brain is highest in areas rich in CB_1 receptors, e.g., the neocortex, hippocampus, thalamus and some regions of the cerebellum (Thomas et al., 1997b), where the physiological action of endocannabinoids is more likely to occur and needs to be rapidly terminated. One can predict, based on these observations, that several strategies for the regulation of endocannabinoid breakdown exist in cells.

The fact that anandamide and 2-arachidonoyl glycerol are always accompanied by an 'entourage' of congeners with little or no activity at cannabinoid receptors and no clear action on other molecular targets, suggests that these compounds may behave as endogenous modifiers of endocannabinoid activity. But how would these 'entourage' compounds affect endocannabinoid activity? A possible answer to this question came from the observation by Katayama et al. (1997) that the activity of FAAH in rat intestine homogenates could be masked by the presence of intestinal lipids, including free fatty acids and monoglycerides. Notably, FAAH can recognize not only anandamide and 2-arachidonoyl glycerol, as substrates, but also non-endocannabinoid fatty acid derivatives such as oleamide, oleoyl-, linoleoyl- and palmitoyl-ethanolamide, and monooleoyl-, monolinoleoyl- and monolinolenoyl-glycerol (Maurelli et al., 1995; Cravatt et al., 1996; Schmid et al., 1996; Bisogno et al., 1997a; Goparaju et al., 1998; Di Marzo et al., 1998). As a consequence of this property, these compounds inhibit anandamide hydrolysis in both cell-free systems and intact cells (Maurelli et al., 1995; Di Tomaso et al., 1997; Mechoulam et al., 1997; Di Marzo et al., 1998). Moreover, oleoylethanolamide also counteracts the 'carrier'-mediated uptake of anandamide by cerebellar granule cells (Hillard et al., 1997), and both 1(3)- and 2-linoleoylglycerol significantly reduce 2-arachidonoyl glycerol diffusion into cells (Ben-Shabat et al., 1998). Therefore, it was possible to hypothesize that fatty acid derivatives, released during—or present before—anandamide and 2-arachidonoyl glycerol production by stimulated cells, might potentiate the actions of the two endocannabinoids by minimizing their inactivation by cells, thereby increasing their availability for cannabinoid receptor activation (Fig. 6). It was consistent with this hypothesis that oleamide was found to greatly increase the efficiency of anandamide binding to CB_1 receptors (Mechoulam et al., 1997), and 2-palmitoyl- and 2-linoleoylglycerol had an analogous facilitatory action on 2-arachidonoyl glycerol binding to both CB_1 and CB_2 receptors as well as on the 2-arachidonoyl glycerol inhibitory effect on forskolin-induced adenylate cyclase (Ben-Shabat et al., 1998). These 'entourage' effects were less pronounced in the presence of phenylmethylsulphonyl-fluoride—which inhibits FAAH-catalyzed hydrolysis of both anandamide and 2-arachidonoyl glycerol (Goparaju et al., 1998)—thus suggesting that these effects are indeed due, at least in part, to inhibition of endocannabinoid hydrolysis by the 'entourage' compounds. Moreover, both oleamide and a mixture of 2-palmitoyl- and 2-linoleoyl glycerol significantly potentiated the activity of subthreshold or submaximal doses of either anandamide or 2-arachidonoyl glycerol, in the 'tetrad' of in vivo behavioural assays in mice (Mechoulam et al., 1997, Ben-Shabat et al., 1998). Oleamide also synergized with anandamide to inhibit lymphocyte (Langstein et al., 1996) and human breast cancer cell proliferation (Bisogno et al., 1998). Since the endocannabinoid inactivation mechanisms described above play an important role in endocannabinoid homeostasis in vivo, and the 'entourage' compounds inhibit these mechanisms, it was reasonable to predict that pharmacological doses of these substances can raise endogenous anandamide and/or 2-arachidonoyl glycerol levels, thus exhibiting 'indirect' cannabimimetic properties. This was found to be the case for oleamide, which shares with anandamide a significant, albeit weak, activity in the 'tetrad' of tests (Mechoulam et al., 1997), as well as a weak anti-proliferative action on lymphocytes and breast cancer cells (Langstein et al., 1996; Bisogno et al., 1998). However, it was only shown in the latter cells that the oleamide cannabimimetic action is mediated by endogenous cannabinoids. Recent findings suggest that some of the oleamide effects in the CNS may possibly be due to allosteric modulation of 5-HT receptors (Thomas et al., 1997a) or inhibition of astrocyte gap junctions (Boger et al., 1998), as has already been shown for anandamide (Venance et al., 1995). Surprisingly THC does not inhibit gap junction communication. Since recent evidence suggests that also anandamide may exert part of its effects via 5-HT receptors (see below, Fride et al., 1998; Kimura et al., 1998), it is possible that the effects of oleamide may in part be mediated by increasing the availability of anandamide due to oleamide inhibition of FAAH (Mechoulam et al., 1997). Also, oleoyl- linoleoyl- and palmitoylethanolamide have been found also to exert slight

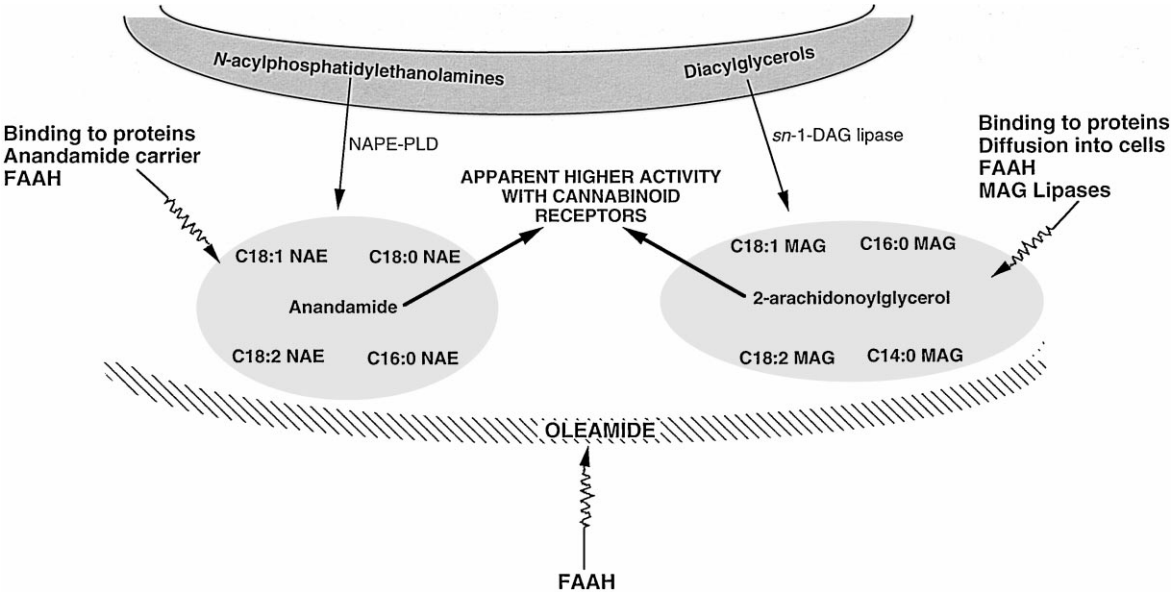


Fig. 6. 'Entourage' effects for anandamide and 2-arachidonoylglycerol action at cannabinoid receptors. Both anandamide and 2-arachidonoylglycerol are present in cells together (and in some cases are co-biosynthesized) with higher amounts of non-endocannabinoid congeners. These seemingly inactive compounds may exert a 'protective' action against endocannabinoid binding to proteins, recapture by cells and enzymatic hydrolysis, possibly by competing for the same binding sites. This would lead to apparent potentiation of endocannabinoid activity. Oleamide is more abundant than anandamide in all cell types so far studied and can exert an analogous protective action through competitive inhibition of fatty acid amide hydrolase (FAAH)-catalyzed hydrolysis of endocannabinoids. NAE, *N*-acylethanolamine; MAG, monoacylglycerol; NAPE-PLD, phospholipase D selective for *N*-acylphosphatidylethanolamines; DAG, diacylglycerol.

anandamide-like pharmacological effects, both in vivo and in vitro (Fride et al., 1997; Berdyshev et al., 1997), but no data are available on the possible mechanism of action. Non-endocannabinoid fatty acid derivatives may also act by interfering with the functionality of membrane proteins (including those of cannabinoid receptors) as suggested previously for unsaturated fatty acids (reviewed by Khan et al., 1995; Di Marzo, 1995) and more recently for oleamide (Lerner, 1997), or by inhibiting the binding of endocannabinoids to plasma proteins (or even to glassware and plastic tubes), thus enhancing their apparent activity.

10. Endocannabinoids and intracellular signalling mechanisms

The intracellular signalling events triggered by the activation of cannabinoid receptors have been extensively reviewed (Howlett, 1995a,b; Felder and Glass, 1998). The ability of anandamide and 2-arachidonoyl glycerol to induce several of these events, as well as some less conventional effects of anandamide on other intracellular signalling pathways, have been critically discussed (Di Marzo, 1998). Detailed discussion of this important aspect of endocannabinoid biochemistry is, therefore, beyond the scope of the present article. It may, however, still be worth categorizing the intracellular actions of anandamide and 2-arachidonoyl glycerol as: (a) CB_1- and/or CB_2-mediated actions—e.g., modulation of forskolin-induced adenylate cyclase, inhibition of N- or P/Q-type Ca^{2+} channels, activation of inwardly-rectifying K^+ channels, activation of mitogen-activated protein kinase—studied by means of selective antagonists for these two receptors; (b) actions which are not mediated by either of the two known cannabinoid receptor subtypes, but are nonetheless due to interactions with G-proteins—e.g., activation of arachidonate release, inhibition of gap–junction-mediated Ca^{2+} signalling in astrocytes—as determined by studying the effect of pertussis toxin; (c) non-cannabinoid receptor-mediated actions—e.g., inhibition of L-type Ca^{2+} channels, modulation of 5-HT or *N*-methyl-D-aspartate (NMDA) transmembrane signalling, etc.—which cannot be blocked by cannabinoid receptor antagonists or by pertussis toxin.

Further insights into the latter two types of endocannabinoid intracellular actions may lead to surprising discoveries (such as the finding of novel cannabinoid receptor subtypes) and widen the range of potential physiological and pharmacological effects induced by anandamide and 2-arachidonoyl glycerol.

11. Pharmacology of endocannabinoids

The widespread distribution of endocannabinoids in animal tissues (see above) suggests a wide range of effects of this family of modulators. Substantial evidence is available to support this prediction.

The first report that anandamide produced in vivo effects similar to those of plant-derived or synthetic cannabinoid receptor ligands appeared in 1993 (Fride and Me-

choulam, 1993). The response of mice to i.p. injections of anandamide was measured in the 'tetrad', which is a series of four non-specific measurements, but which together are assumed to be predictive of psychoactive cannabimimetic drug effects. The effects of anandamide included inhibition of motor activity, catalepsy, hypothermia and hypoalgesia. These results were consistent with in vitro results for anandamide intracellular actions (Vogel et al., 1993; Felder et al., 1993 and data above).

As discussed above anandamide is only one member of a putative family of endogenous fatty acid ethanolamides which activate cannabinoid receptors. Thus ethanol amides of docosatetraenylic, homo-γ-linolenyl, and mead acid have also been shown to display cannabinoid activities in vivo and in vitro (Hanuš et al., 1993; Barg et al., 1995; Priller et al., 1995). Palmitoylethanolamide, which has been reported to bind to CB_2 only, down modulates mast cell activation in vitro (Facci et al., 1995). The ester endocannabinoid, 2-arachidonoyl glycerol, also displays cannabinoid-like activities in vivo and in vitro (Mechoulam et al., 1995).

Cannabimimetic effects of endocannabinoids have now been reported for a variety of experimental settings, including inhibition of the dopaminergic nigrostriatal system (Gueudet et al., 1995; Souilhac et al., 1995; Cadogan et al., 1997), interference with learning and memory (Terranova et al., 1995; Mallet and Beninger, 1996; Stella et al., 1997), drug discrimination (Wiley et al., 1995, 1997), activation of the hypothalamo-pituitary–adrenal axis (Weidenfeld et al., 1994; Wenger et al., 1997a,b), decrease in prolactin release by the hypothalamus (Fernandez-Ruiz et al., 1997), decrease in intraocular pressure (Pate et al., 1995), hypotension and bradycardia (Varga et al., 1995), immune modulation (Schwarz et al., 1994; Lee et al., 1995; Ouyang et al., 1998) and inhibition of the electrically evoked twitch response in the isolated mouse vas deferens (Mechoulam et al., 1992; Pertwee et al., 1994). Moreover, the response to anandamide, similarly to that to Δ^9-THC, is only seen in the mature animal, since it could not be detected in very young, developing mice (Fride and Mechoulam, 1996b). This age difference in the response to cannabinoids led us to undertake a clinical trial with Δ^8-THC in young children, against vomiting due to cancer chemotherapy. We assumed that while THC-type side-effects may be low or absent, the antivomiting effect may still be present as it does not seem to be CB_1-induced. Indeed no psychotropic effects were noted even with high doses, while all vomiting was blocked (Abrahamov et al., 1995). Hence cannabinoids may be developed in the future as pediatric drugs.

As further data on the pharmacological effects of anandamides appeared, there emerged a number of differences between anandamide and the prototype cannabinoid, Δ^9-THC. For example, the anandamides show partial agonist activities in some of the assays used in vivo and in vitro (Mackie et al., 1993; Vogel et al., 1993; Fride et al., 1994; Barg et al., 1995; Mechoulam and Fride, 1995). Very low doses of anandamide (about 1000 times lower than those required to induce the well known inhibitory effects), but not of Δ^9-THC, were shown to induce stimulatory effects in the 'tetrad' of tests and on phagocytosis (Sulcova et al., 1998). Further, up to 5×10^{-5}-fold lower doses than those required for inhibitory effects were found to inhibit Δ^9-THC-induced cannabimimetic effects in vitro and in vivo (Fride et al., 1995). Very low concentrations of Δ^9-THC and synthetic cannabinoids were found to enhance CB_2 receptor-mediated B-cell growth (Derocq et al., 1995). No effects of very low doses of anandamide were reported upon in that study. It thus remains to be seen whether the 'very-low-dose' effects of anandamide are confined to CB_1 or also to CB_2 receptor-mediated mechanisms.

In a model of the analgesic response to intrathecal injections of Δ^9-THC or anandamide, Welch et al. (1995) noticed several differences between the analgesia induced by the two compounds and, interestingly, also saw an inhibitory effect of anandamide on Δ^9-THC-induced analgesia. We speculated at the time that very low doses of anandamide may exert their effects through activation of a CB_1 receptor Gs protein-coupled transduction pathway, as opposed to the well established Gi protein pathway (Fride et al., 1995). Recently, Felder et al. (Felder et al., 1998; Glass and Felder, 1997) have indeed presented evidence for the existence of a Gs protein linkage to the CB_1 receptor (see also Axelrod and Felder, 1998). Since levels of anandamide in the brain are low (see above), it is possible that the effects induced by very low, exogenously administered doses of anandamide reflect in part the functional activity of the endocannabinoid system under normal conditions.

Low doses of anandamide or Δ^9-THC (0.02 mg/kg), administered daily during the last trimester of pregnancy in rats, result in decreased levels of prostaglandins in the dams, prolonged pregnancy and increased frequency of stillbirths (Wenger et al., 1997a,b). Interestingly, much higher daily doses of anandamide or Δ^9-THC (both 20 mg/kg) during the last trimester of pregnancy in mice did not result in abnormality of litters or of gestational parameters. Instead, these doses induced behavioral alterations throughout the life of the offspring, reminiscent of a permanent 'high' (Fride and Mechoulam, 1996a,b and unpublished data).

Dey et al. presented elegant data in a series of publications showing that anandamide and CB_1 receptors are abundantly present in the uterus, and can prevent implantation of the embryo (Das et al., 1995; Schmid et al., 1997). Previously, anandamide was found to inhibit the sperm-fertilizing capacity in sea urchins (Schuel et al., 1994). Together, these observations suggest a role for anandamide in fertility, inhibiting reproduction, possibly when environmental circumstances are unfavorable. Needless to say, much more work needs to be done to further test these hypotheses.

Anandamide and 2-arachidonoyl glycerol were also recently found to selectively and potently inhibit the proliferation of human breast cancer cells in vitro (De Petrocellis et al., 1998). The effect of anandamide was shown to be mediated by a CB_1-like receptor and to be due to inhibition of the synthesis of the long form of prolactin receptor.

Anandamide has been shown to reduce blood pressure and induce bradycardia via CB_1 receptor activation (Varga et al., 1995; Lake et al., 1997). This depressor effects is, at least in part, terminated by a reuptake mechanism (Calignano et al., 1997a). Vasorelaxation has also been observed upon administration of anandamide (Zygmunt et al., 1997; Pratt et al., 1998), while there are reports of both anandamide (Randall et al., 1996) and 2-arachidonoyl glycerol (Sugiura et al., 1998) release from stimulated vascular tissue. In this case anandamide was only identified by chromatography. Hence it has been suggested that the endocannabinoids exert their circulatory effects through CB_1 receptor activation after their release into the vasculature (Randall et al., 1996; Randall and Kendall, 1997, 1998a,b). Whether the endocannabinoids represent an endothelial-derived hyperpolarizing factor is currently under debate (Plane et al., 1997; Wagner et al., 1997; Zygmunt et al., 1997; Pratt et al., 1998; Randall and Kendall, 1998a,b). Endocannabinoids may represent the endothelial derived hyperpolarizing factor in some vascular tissues but not in others. Thus, it still has to be clarified whether endocannabinoid-induced cardiovascular effects are mediated by endothelial derived hyperplarizing factor-induced vasorelaxation or by other mechanisms such as an increase in arachidonic acid (Pratt et al., 1998).

It is surprising that, although the possibility of anandamide being a putative endothelial derived hyperpolarizing factor is under study by numerous groups, no work on the second endocannabinoid, 2-arachidonoyl glycerol, has yet been reported. We have found that carbachol, which is known to liberate a hyperpolarizing substance from the vascular endothelium via activation of the muscarinic receptor, causes a potent increase of 2-arachidonoyl glycerol levels in rat aorta (from about 0.7 nmol/g wet weight to about 3.778 nmol/g wet weight). 2-Arachidonoyl glycerol (12 mg/kg) causes a short lived decrease in blood pressure in the mouse on i.v. administration, from 122 to 82 mm Hg in mean arterial pressure which wanes after 18 min. As 2-arachidonoyl glycerol is rapidly hydrolysed in vivo, we tested a stable 2-arachidonoyl glycerol analog, namely 2-arachidonyl glyceryl ether, in which the labile ester moiety of 2-arachidonoyl glycerol is replaced by a stable ether one. This synthetic ether reduces blood pressure more strongly, from 135 mm Hg to 72 mm Hg, an effect which lasted for at least 30 min (Mechoulam, Fride, Ben-Shabat, Meiri, Horovitz, unpublished observations).

Tolerance to repeated injections of anandamide has been shown in the 'tetrad' (Fride, 1995) and also for antinociception following intrathecal administration (Welch, 1995). However, unlike Δ^9-THC-induced tolerance, no cross-tolerance to dynorphic systems resulted in the latter case (Welch, 1997). In addition, tolerance did not appear for inhibition of intestinal motility, leading to the speculation that intestinal effects are mediated by CB_2 receptors (Fride, 1995). This would contrast with a recent report (Calignano et al., 1997b), providing evidence for CB_1 receptor-mediated inhibition of intestinal motility after anandamide administration.

There are also reports of direct interactions with receptors other than CB receptors. Thus Hampson et al. (1998) have shown a direct inhibitory as well as enhancing effect of anandamide on NMDA receptor-dependent neurotransmission, which was not seen with Δ^9-THC. There is evidence for an indirect NMDA receptor involvement in CB_1 receptor-mediated hyperalgesia inhibition based on an in vivo model of spinal nociception (Richardson et al., 1998).

The curious lack of antagonism by the widely used specific CB_1 receptor antagonist, SR141716A (Rinaldi-Carmona et al., 1994), against anandamide in the 'tetrad' (Adams et al., 1998; Fride et al., 1998) suggests that anandamide may also interact with other neurotransmitter systems. A previous observation by Fan (1995) that cannabinoids may directly inhibit 5-HT_3 receptor activity, as well as the similarities in therapeutic potential between 5-HT_3 receptor antagonists and cannabinoids, in conditions such as nausea in cancer patients undergoing chemotherapy (Greenshaw, 1993; Abrahamov et al., 1995; Fride and Mechoulam, 1996a,b), analgesia, inflammations, neuroprotection and sleep/wakefulness (see for example Mechoulam, 1986; Greenshaw and Silverstone, 1997) prompted the investigation of possible interactions between 5-HT_3 receptor ligands and anandamide. We now have substantial evidence from behavioral experiments that, indeed, anandamide may, at least partly, exert its CNS effects via 5-HT_3 receptors and perhaps also via 5-HT_2 receptors (Fride et al., 1998). Kimura et al. (1998) recently reported that, at micromolar concentrations, anandamide can bind to 5-HT_1 and 5-HT_2 receptors. These various findings may have important implications for the design of new drugs for conditions influenced by both 5-HT and cannabinoid receptor agents.

12. Quo vadimus?

Over the last 35 years, particularly in the last decade, the cannabinoid field, that initially appealed to a very small group of researchers, has become of wide interest. Predictions as to the paths a branch of science will follow have some of the nature of crystal-gazing. Still we believe that we shall soon see advances of major general importance, such as (a) understanding of the interactions between the endocannabinoids and other mediators, specially serotonin, gamma amino butyric acid (GABA), glutamate

and dopamine; (b) clear definition of the physiological roles of the endocannabinoids, possibly as protectors against neurodegeneration and neural damage, as participants in blood pressure regulation, as immune system regulators, in addition to their somewhat better established roles in memory and cognition; (c) introduction of cannabinoids as novel drugs, possibly against trauma, stroke, neurodegeneration, cardiac ischemia and inflammatory diseases, and perhaps work will start on the presumed importance of the cannabinoid system in the chemical nature of emotions.

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