The Biosynthesis, Fate and Pharmacological Properties of Endocannabinoids

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Abstract The finding of endogenous ligands for cannabinoid receptors, the endocannabinoids, opened a new era in cannabinoid research. It meant that the biological role of cannabinoid signalling could be finally studied by investigating not only the pharmacological actions subsequent to stimulation of cannabinoid receptors by their agonists, but also how the activity of these receptors was regulated under physiological and pathological conditions by varying levels of the

endocannabinoids. This in turn meant that the enzymes catalysing endocannabinoid biosynthesis and inactivation had to be identified and characterized, and that selective inhibitors of these enzymes had to be developed to be used as (1) probes to confirm endocannabinoid involvement in health and disease, and (2) templates for the design of new therapeutic drugs. This chapter summarizes the progress achieved in this direction during the 12 years following the discovery of the first endocannabinoid.

Keywords Anandamide · 2-Arachidonoylglycerol · Cannabinoid · Enzyme · Inhibitors

1 Introduction

When the longstanding issue of the mechanism of action of (–)-Δ^9-tetrahydrocannabinol (THC) was solved with the finding of the cannabinoid receptors, studies aimed at finding endogenous ligands for these receptors could be started. These studies culminated in 1992 with the report of the discovery of the first of such ligands, *N*-arachidonoyl-ethanolamine (AEA), which was named anandamide from the Sanskrit word *ananda*, meaning "internal bliss" (Devane et al. 1992). In the following years, the finding of anandamide, which apart from binding to cannabinoid CB_1 (and later also CB_2) receptors could also functionally activate them, led to the revelation that there is a whole endogenous signalling system now known as the *endogenous cannabinoid system*. This comprises, apart from the cannabinoid receptors (Pertwee 1997), other endogenous ligands [named endocannabinoids by our group in 1995 (Di Marzo and Fontana 1995)] and the proteins for their synthesis and inactivation, as well as, possibly, other molecular targets for the endocannabinoids (see Pertwee 2004 for review). First came the finding that a well-known intermediate in phosphoglyceride metabolism, 2-arachidonoyl-glycerol (2-AG), was also able to activate both CB_1 and CB_2 receptors (Mechoulam et al. 1995; Sugiura et al. 1995). The end of the 1990s brought: (1) the finding of the biochemical pathways and the identification of the first enzymes for the formation and inactivation of AEA and 2-AG (Di Marzo et al. 1994; Cravatt et al. 1996; Bisogno et al. 1997b), a breakthrough that was very much facilitated by important similar studies carried out in the 1970s on other lipids belonging to the same families as the two endocannabinoids (Schmid et al. 1990 and Horrocks 1989 for reviews); and (2) the recognition that AEA was a rather promiscuous ligand for several membrane receptors and channels, particularly for vanilloid VR1 receptors (now classified as TRPV1 receptors) (Zygmunt et al. 1999), and as-yet-uncharacterized binding sites in the vascular endothelium (Jarai et al. 1999). Therefore, at the turn of the century it was clear that the endocannabinoid system was going to include new receptors, new ligands and new enzymes. This feeling was confirmed, among other things, by the characterization of: (1) more putative endocannabinoids, all derived from arachidonic acid, i.e. 2-arachidonyl-glyceryl ester (noladin, 2-AGE), *O*-arachidonoyl-ethanolamine (virodhamine, OAE) and *N*-arachidonoyl-dopamine (NADA) (Bisogno et al. 2000;

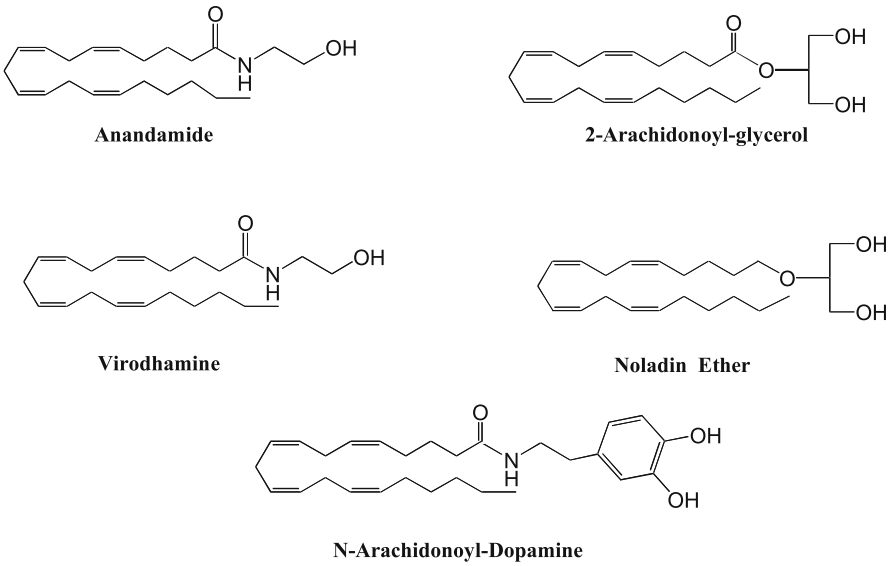

Fig. 1. Established and newly proposed endocannabinoids. Chemical structures of the five endogenous cannabinoid ligands identified so far

Hanus et al. 2001; Huang et al. 2002; Porter et al. 2002); (2) more possible targets for AEA and some synthetic cannabimimetic compounds (Breivogel et al. 2001); and (3) the biosynthetic enzymes for 2-AG and AEA (Bisogno et al. 2003; Okamoto et al. 2004). Clearly, the history of the endocannabinoid system is far from set, but nevertheless the following sections shall attempt at providing the reader with a picture as updated and as complete as possible of the multi-faceted biochemical and pharmacological aspects of the endocannabinoids (Fig. 1).

2
Biosynthesis and Release of Endocannabinoids

The biosynthetic and metabolic pathways of the two best-studied endocannabinoids, AEA and 2-AG, have several features in common. Both compounds are produced from the enzymatic hydrolysis of precursors derived from the remodelling of membrane phospholipids; both appear to be released and then taken up by cells via diffusion through the plasma membrane, possibly facilitated by a membrane carrier protein; and both are inactivated mostly via intracellular enzymatic hydrolysis. Yet, although overlaps are theoretically possible between the biosynthetic pathways of the two endocannabinoids, fundamentally different enzymes are involved in the formation of AEA and 2-AG. This explains why, as is becoming increasingly clear, the two compounds can be produced independently from each other and why their levels can undergo differential and even opposing changes with different physiological and pathological stimuli. For this reason, the biochemical

pathways underlying the production of the two major endocannabinoids will be discussed here separately. In general, however, the three following commonalities can be observed:

- Both AEA and that portion of 2-AG acting as endocannabinoid (2-AG is in fact also an important intermediate in phosphoglyceride metabolism), are not stored in secretory vesicles but are, instead, synthesized and released "on demand", often following Ca^{2+} influx, which causes activation of Ca^{2+}-dependent biosynthetic enzymes (Di Marzo et al. 1998b).
- Pharmacological and electrophysiological data have shown that activation of metabotropic (glutamate or muscarinic) receptors, either cooperatively with or independently from Ca^{2+}-influx, can also induce the formation of non-chemically identified endocannabinoids acting as retrograde synaptic signals (Kim et al. 2002; Brenowitz and Regehr 2003; Ohno-Shosaku et al. 2003).
- The formation of both compounds is accompanied by the biosynthesis of cannabinoid-inactive or weakly active congeners, which have been suggested to exert an enhancement of AEA and 2-AG actions via various mechanisms collectively referred to as "entourage" effects (Ben-Shabat et al. 1998; Mechoulam et al. 1998b for review).

2.1 Biosynthesis of AEA and Other *N*-Acylethanolamines

AEA belongs to the family of the *N*-acylethanolamines (NAEs), which have been investigated since the 1960s. Work performed by H. Schmid and co-workers long before the discovery of AEA had shown that these compounds are biosynthesized via a phospholipid-dependent pathway (Fig. 2), i.e. the enzymatic hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamines (NAPEs) (Schmid et al. 1990, 1996, 2002a; Hansen et al. 1998, for reviews). The enzyme catalysing this reaction is a phospholipase D selective for NAPEs (NAPE-PLD), which, in turn, are produced from the transfer to the *N*-position of phosphatidylethanolamine of an acyl group from the *sn*-1 position of phospholipids (PE), catalysed by a Ca^{2+}-dependent *trans*-acylase. Already in these early studies it appeared clear that NAPE-PLD was quite different from other PLD enzymes, and that this enzyme as well as the *trans*-acylase exhibited no selectivity for a particular fatty acid moiety. After the discover of AEA, this route was shown to underlie also the biosynthesis of this endocannabinoid in central neurons after depolarization (Di Marzo et al. 1994). Subsequent studies confirmed the occurrence of *N*-arachidonoyl-phosphatidylethanolamine (NArPE), the NAPE precursor of AEA, in murine brain, testes and leukocytes (Sugiura et al. 1996a,b; Di Marzo et al. 1996a,b; Cadas et al. 1997), and showed that NAPE-PLD lacks the transphosphatidylation activity typical of other PLD enzymes (Petersen and Hansen 1999), is dependent on Ca^{2+} for optimal activity (Ueda et al. 2001a) and is stimulated by polyamines (Liu et al. 2002). In fact, all the previous information gained on roughly purified fractions of NAPE-PLD have been recently confirmed by its cloning, which in addition showed that the enzyme belongs to the zinc

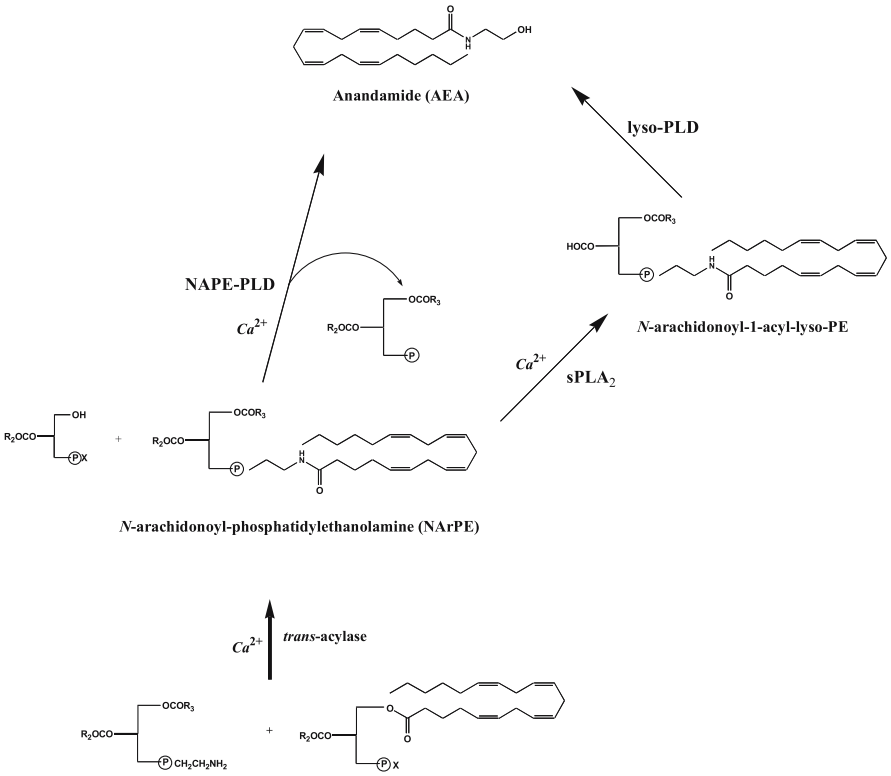

Fig. 2. Major biosynthetic pathways and enzymes for the endocannabinoid anandamide (AEA) and other *N*-acylethanolamines. The *circled P* indicates a phosphate group. *N-ArPE*, *N*-arachidonoyl-phosphatidylethanolamine; *PE*, phosphatidylethanolamine; *PLD*, phospholipase D; *sPLA*$_2$, secretory phospholipase A$_2$ of group IB

metalloproteinase family of hydrolases of the β-lactamase fold (Okamoto et al. 2004). Several independent lines of evidence strongly suggest that this pathway is the one mostly responsible for AEA biosynthesis in intact cells, and in particular:

- The finding of a similar distribution of NArPE and AEA in nine different brain areas (Bisogno et al. 1999a), and of increasing levels of both NArPE and AEA in rat brain at different stages of development (Berrendero et al. 1999), confirms a precursor/product relationship for the two compounds.
- The Ca^{2+} sensitivity of both the *trans*-acylase and NAPE-PLD is in agreement with the fact that AEA biosynthesis is triggered by neuronal depolarization and other Ca^{2+} mobilizing stimuli.
- The fact that this biosynthetic pathway is common to other NAEs, and that the percentage fatty acyl chain composition of these compounds in tissues is ultimately dependent on that of the *sn*-1 position of phospholipids (Fig. 2), explains why AEA is the minor of its congeners.

However, a recent study (Sun et al. 2004) also highlights another possible way for NAPEs to be transformed into NAEs, at least in cell-free homogenates, i.e. via the sequential action of a group IB secretory phospholipase A_2 (PLA_2), with the formation of *N*-acyl-1-acyl-lyso-PE, followed by the action of a lyso-PLD enzyme distinct from the known NAPE-PLD (Fig. 2).

2.2 Biosynthesis of 2-Arachidonoylglycerol

Although probably over-estimated due to artefactual production, for example following rat decapitation (Sugiura et al. 2001), the levels of 2-AG in unstimulated tissues and cells, but not in the blood or cerebrospinal fluid (CSF), are usually much higher than those of AEA, and sufficient in principle to permanently activate both cannabinoid receptor subtypes (Sugiura et al. 1995; Stella et al. 1997). This simple observation, and the fact that this compound is at the crossroads of several metabolic pathways and is an important precursor and/or degradation product of phospho-, di- and triglycerides, as well as of arachidonic acid, indicates that the 2-AG found in tissues is not uniquely used to stimulate cannabinoid receptors, although the one measured in extracellular fluids, such as serum and CSF, probably is. While an enhancement of intracellular Ca^{2+} is necessary and sufficient for AEA biosynthesis, 2-AG formation is triggered also, but not only, by Ca^{2+}-mobilizing stimuli (and, hence, also, but not only, following neuronal depolarization). In fact, the most important biosynthetic precursors of 2-AG are the *sn*-1-acyl-2-arachidonoylglycerols (DAGs) (Fig. 3), which, like other diacylglycerols, are produced from phospholipid metabolism and remodelling and, ultimately, by the stimulation of G protein-coupled receptors (GPCRs). This observation raises the possibility that the biosynthesis of 2-AG may be regulated independently from that of AEA, and requires different conditions. Several stimuli have been shown to lead to the formation of 2-AG in intact neuronal and non-neuronal cells, including lipopolysaccharides (in macrophages), ethanol or glutamate (in neurons), carbachol or thrombin (in endothelial cells), endothelin (in astrocytes), platelet-activating factor (in macrophages), etc. (Bisogno et al. 1997b; Stella et al. 1997; Sugiura et al. 1998; Mechoulam et al. 1998a; Bisogno et al. 1999b; Di Marzo et al. 1999a; Basavarajappa et al. 2000; Berdyshev et al. 2001; Stella and Piomelli 2001; Liu et al. 2003; Walter and Stella 2003; and Sugiura et al. 2002, for review), but only seldom have the pathways for 2-AG biosynthesis been investigated. In most cases, the DAGs necessary for 2-AG biosynthesis are obtained from the hydrolysis of 2-arachidonate-containing phosphoinositides (PIs), catalysed by the PI-selective phospholipase C or other phospholipases of this type (Di Marzo et al. 1996b; Stella et al. 1997; Kondo et al. 1998; Berdyshev et al. 2001; Stella and Piomelli 2001; Liu et al. 2003), whereas in the case of ionomycin-stimulated neuroblastoma cells and cultured rat microglial cells, DAGs appear to be formed from the hydrolysis of 2-arachidonate-containing phosphatidic acid (PA), catalysed by a PA phosphohydrolase (Bisogno et al. 1999b; Carrier et al. 2004). Regarding the conversion of DAGs into 2-AG, this requires a *sn*-1-selective DAG lipase (Bisogno et al. 1997b;

Stella et al. 1997). Two *sn*-1 DAG lipase isozymes (DAGLα and DAGLβ) have been cloned and enzymatically characterized (Bisogno et al. 2003). They are located in the plasma membrane, are stimulated by Ca^{2+}, appear to possess a catalytic triad typical of serine hydrolases, and, like NAPE-PLD, do not appear to be particularly selective for 2-arachidonate-containing DAGs. Nevertheless, several lines of evidence (Bisogno et al. 2003) suggest that they are responsible for the formation of the endocannabinoid 2-AG in intact cells:

- Over-expression of DAGLα and DAGLβ in COS cells results in significantly higher levels of 2-AG produced following stimulation with ionomycin, but not in higher 2-AG basal levels.
- The expression of DAGLα and DAGLβ in several cell lines correlates with their ability to produce 2-AG following stimulation with ionomycin.
- Inhibition of DAGLα and DAGLβ activity with tetrahydrolipstatin in COS cells and cell lines stimulated with ionomycin results in the impaired production of 2-AG.
- The distribution of the mRNAs encoding for DAGLα correlates with the relative abundance of 2-AG in rodent tissues and organs (Kondo et al. 1998).
- Finally, the two enzymes exhibit a pattern of subcellular expression in nervous tissues that fits with the proposed role of 2-AG either as a mediator of neurite growth, during brain development (Williams et al. 2003) or as a retrograde signal mediating depolarization-induced suppression of neurotransmission and heterosynaptic plasticity in the adult brain (Chevaleyre and Castillo 2003; Wilson and Nicoll 2002, for review). In fact, the enzymes are located on axons during development and post-synaptically in adult neurons (Bisogno et al. 2003).

However, DAG-independent biosynthetic pathways for 2-AG have also been proposed (Sugiura et al. 2002, for review), although their relevance to the regulation of the endocannabinoid signal has not yet been investigated. Noteworthy is the enzymatic hydrolysis of a particular type of lysophosphatidic acid, 2-arachidonoyl-*sn*-glycero-3-phosphate (Nakane et al. 2002).

2.3 Biosynthesis of Other Putative Endocannabinoids

Very little is known about the biosynthesis of the three most recently proposed endocannabinoids, 2-AGE, virodhamine and NADA. Regarding 2-AGE (noladin ether), this compound was previously identified in pig brain (Hanus et al. 2001) and in some rat tissues and brain areas (Fezza et al. 2002) by using mass-spectrometric (MS) methods coupled to chromatographic separations. However, a recent study cast some doubt on the actual existence of 2-AGE in mammalian brain tissue (Oka et al. 2003). At the time of this study it was already known that (1) the only acyl ethers to have been detected in animals before the discovery of 2-AGE were 2-acyl ethers (e.g. alkenyl ethers such as platelet activating factor and plasmalogens); (2) there was no evidence for the existence of any enzyme catalysing the formation

of 2-alkenyl glyceryl ethers from the corresponding fatty acyl alcohols; and (3) although similar enzymes had been previously identified, these had a stringent specificity for the *sn*-1 position of glyceryl ethers with short-medium chain, saturated fatty acids (Nagan and Zoeller 2001). Oka et al. (2003), using MS techniques, could not confirm the presence of 2-AGE in the brain of several mammalian species including pig and rat. These contradictory data might be explained by the use of different extracting procedures, or with the possibility of a "false-positive" MS signal, i.e. an endogenous compound structurally related but not identical to 2-AGE (i.e. with the same molecular weight and similar mass spectrometric fragmentation pattern), which cannot be picked up by all MS techniques. This compound, however, cannot be the 2-AGE isomer 1-arachidonyl glyceryl ether, which can be distinguished from 2-AGE simply on the basis of its chromatographic properties. Clearly, if the existence of 2-AGE were to be confirmed by future studies carried out in other laboratories using exactly the same procedures used by Hanus et al. (2001) and Fezza et al. (2002), some as-yet-unknown biosynthetic pathway, different from that leading to plasmalogens, may exist for this compound. Neuroblastoma N18TG2 intact cells are not capable of converting arachidonate-containing phospholipids into 2-AGE when stimulated with ionomycin, i.e. under conditions where high levels of 2-AG are produced (Fezza et al. 2002). This might suggest a Ca^{2+}-independent or a non-phospholipid-mediated pathway for the formation of this putative endocannabinoid in neurons.

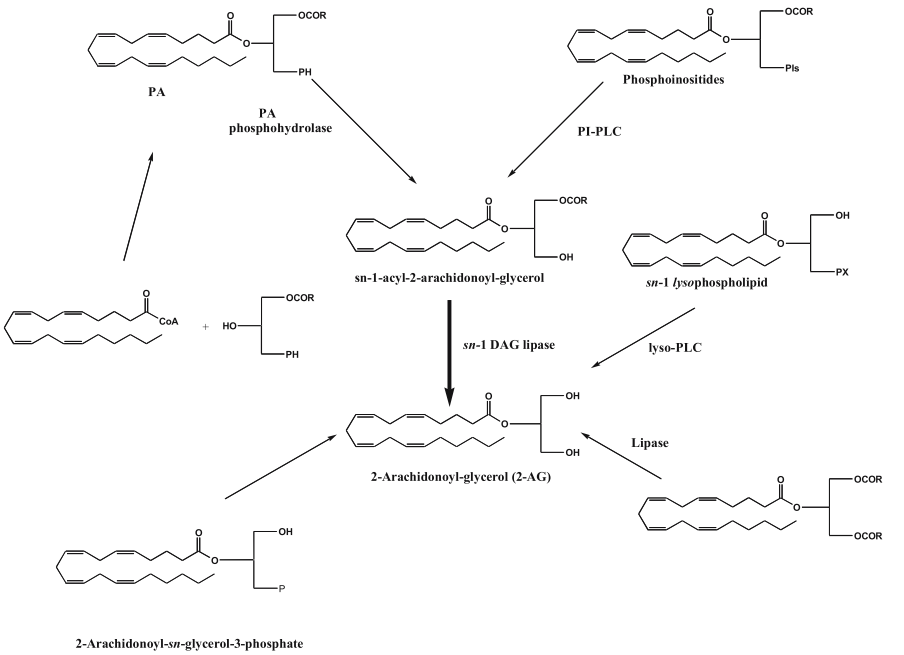

Fig. 3. Major biosynthetic pathways and enzymes for the endocannabinoid 2-arachidonoyl-glycerol (2-AG). *DAG*, di-acyl-glycerol lipase; *PA*, phosphatidic acid; *PI*, phosphoinositide; *PLC*, phospholipase C. P represents a phosphate group

Virodhamine, which seems to accompany AEA in all tissues analysed (Porter et al. 2002), might be biosynthetically related to AEA, since the non-enzymatic transformation of NAEs into the corresponding *O*-acyl esters, and vice versa, in the presence of bases or acids, has been reported (Markey et al. 2000). Given the seemingly opposing activity of the two compounds at their receptors (*virodha* in Sanskrit means "opposing"), with virodhamine being an antagonist at cannabinoid CB_1 receptors and a partial agonist at CB_2 receptors, and anandamide a possible antagonist at cannabinoid CB_2 receptors and a partial agonist at CB_1 receptors, this possibility might give rise to an interesting interplay between the two compounds under those pathological conditions (i.e. inflammation) that cause a local decrease of pH.

Original evidence for the formation of NADA from arachidonic acid and dopamine or tyrosine (Huang et al. 2002) suggested a biosynthetic pathway common to that of the recently discovered arachidonoyl amino acids (Huang et al. 2001), i.e. from the direct condensation between arachidonic acid and dopamine, or, alternatively, from the condensation between arachidonic acid and tyrosine followed by the transformation of *N*-arachidonoyl-tyrosine into NADA by the enzymes catalysing dopamine biosynthesis from tyrosine. Preliminary data have shown, however, that NADA cannot be produced from either *N*-arachidonoyl-tyrosine or *N*-arachidonoyl-L-DOPA either in vitro, in brain homogenates, or in vivo, and that the lipid formed from tyrosine and arachidonic acid is not NADA (M.J. Walker and V. Di Marzo, unpublished observations). Clearly, further studies are needed to understand the biosynthetic mechanism for this putative endocannabinoid.

2.4 Inhibitors of Endocannabinoid Biosynthesis

Partly owing to the fact that the NAPE-PLD for AEA and the two DAGLs for 2-AG have been cloned only very recently, no selective inhibitors of endocannabinoid biosynthesis have been developed to date. However, several non-specific inhibitors have been shown to prevent the formation of either AEA or 2-AG. For the former compound, Cadas et al. (1997) showed that several non-selective hydrolase inhibitors, and particularly the PLA_2 inhibitor (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP), could block the activity of crude preparations of the Ca^{2+}-dependent *trans*-acylase. Regarding 2-AG, Bisogno et al. (1999b) found that the PLA_2 inhibitor, oleoyl-oxyethyl-phosphoryl-choline, and the blocker of acylCoA-dependent synthase, thimerosal, could oppose ionomycin-induced formation of 2-AG in intact neurons, possibly by inhibiting the formation of PA precursors. Furthermore, the DAG lipase inhibitor RHC80267 was also found to block 2-AG release from DAGs (Stella et al. 1997; Bisogno et al. 1997b, 1999b). More importantly, the lipase inhibitor tetrahydrolipstatin (orlistat) was recently shown to inhibit the two DAGLs, DAGLα and DAGLβ, at concentrations (IC_{50}=60–250 nM) lower than those previously found to be required to inhibit other lipases (Bisogno et al. 2003). Clearly the chemical structure of this compound (Fig. 3), which is marketed by Roche as an anti-obesity drug, might serve as a template for the development of more selective DAGL inhibitors.

2.5 Endocannabinoid Release

After their biosynthesis, AEA and 2-AG are immediately released into the extracellular medium. This occurs via an unknown mechanism, which, however, several pieces of evidence suggest is one that is dependent on the same putative membrane transporter proposed to facilitate the opposite process, i.e. endocannabinoid cellular uptake (see below). In particular:

- Cells loaded with radiolabelled AEA release this compound through a temperature-dependent and pharmacologically inhibitable mechanism (Hillard et al. 1997).
- AEA biosynthesized de novo inside the cell is released into the extracellular medium via a process that can be inhibited by selective inhibitors of AEA cellular uptake, with subsequent increase of intracellular AEA levels (Ligresti et al. 2004).
- Endocannabinoids have been proposed to act as retrograde messengers for both short- and long-term forms of synaptic plasticity, such as depolarization-induced suppression of excitatory or inhibitory neurotransmission (DSE or DSI) and long-term depression (LTD; see Wilson and Nicoll 2002, for review). It is thought that endocannabinoids are released from the post-synaptic cell following its depolarization, and then act retrogradely on CB_1 receptors on presynaptic neurons to inhibit neurotransmitter release. In one model of this phenomenon, inhibitors of the putative endocannabinoid membrane transporter (EMT), injected into the post-synaptic neuron, have been found to inhibit LTD (Ronesi et al. 2004).

Once released, extracellular endocannabinoids act mostly, and with varying selectivity, on cannabinoid receptors, possibly including subtypes other than CB_1 and CB_2. However, endocannabinoids such as AEA and/or NADA may also act, prior to their release, on intracellular sites on ion channels, such as those on vanilloid TRPV1 (transient receptor potential vanilloid type 1) receptors and T-type Ca^{2+} channels (see below). In this case, release represents a possible way to inactivate, rather than facilitate, the action of endocannabinoids.

3 Endocannabinoid Metabolic Fate

3.1 Cellular Uptake

When incubated with intact cells in vitro, all endocannabinoids are rapidly ($t_{1/2} \leq 5$ min) cleared away from the extracellular medium (Di Marzo et al. 1994; Ben-Shabat et al. 1998; Beltramo and Piomelli 2000; Bisogno et al. 2001; Fezza et al. 2002; Huang et al. 2002). It has been suggested that this process depends on the presence of one or more membrane transporters, the putative EMT (see

above). This hypothesis is supported by evidence that the transport process is saturable and exhibits sensitivity to temperature, selectivity for unsaturated (particularly polyunsaturated) long-chain fatty acid amides and sensitivity to synthetic inhibitors (Di Marzo et al. 1994; Beltramo et al. 1997; Hillard et al. 1997; Bisogno et al. 1997a). Since this process only transports AEA down transmembrane concentration gradients, it can also: (1) mediate AEA release, and (2) act in the absence of other sources of energy and, therefore, function independently of Na^+- and ATP (Hillard and Jarrahian 2000). However, the putative EMT has not been isolated, and its molecular biology remains uncharacterized. This lack of information, together with the following observations, suggested to some authors that AEA membrane transport might simply occur through passive diffusion driven by intracellular enzymatic hydrolysis:

- Endocannabinoids are lipophilic compounds, and such compounds often do not need a membrane transporter to cross the plasma membrane (although there are several exceptions to this rule).
- The presence in the cell of an active AEA-hydrolysing enzyme, fatty acid amide hydrolase (FAAH) (see below), strongly enhances AEA cellular uptake (Deutsch et al. 2001).
- Inhibitors of AEA intracellular metabolism often (but not always) also inhibit AEA transport into the cell (Deutsch et al. 2001; Glaser et al. 2003).
- Under certain experimental conditions, AEA accumulation into the cell is not saturable (Glaser et al. 2003), whereas, in the absence of a cell monolayer, the plastic ware used in studies of AEA cellular uptake can mimic the AEA sequestration process in terms of temperature sensitivity (Fowler et al. 2004).

However, several observations still strongly, albeit indirectly, support the existence of an EMT, or at least of some specific intracellular process distinct from FAAH for bringing about the cellular uptake of endocannabinoids (for a more detailed review see Hillard and Jarrahian 2003):

- Several cell types can be found that can rapidly take up AEA from the extracellular medium even though they do not express FAAH; furthermore, synaptosomes from transgenic mice lacking FAAH can still take up AEA efficiently and in a saturable manner (Ligresti et al. 2004);
- Several compounds have been developed that are capable of inhibiting AEA cellular uptake without inhibiting AEA enzymatic hydrolysis via FAAH (Di Marzo et al. 2001b, 2002c; De Petrocellis et al. 2000; Lopez-Rodriguez et al. 2001; Ortar et al. 2003); indeed, the chemical prerequisites necessary for fatty acid amide derivatives to inhibit AEA uptake are so stringent that there can be no doubt that this process is mediated by a specific protein (Piomelli et al. 1999; Ligresti et al. 2004).
- FAAH inhibitors enhance, and anandamide uptake inhibitors inhibit, anandamide accumulation into some cells (Kathuria et al. 2003).

- Substances that inhibit the EMT *enhance* responses to exogenous AEA that are elicited at extracellular sites (i.e. at CB_1 receptors) and *inhibit* those that are elicited at intra-cellular targets (i.e. TRPV1 receptors, see De Petrocellis et al. 2001)—if these compounds were simply acting by inhibiting FAAH, they should enhance AEA effects in both cases.
- A selective EMT inhibitor can modify the distribution of de novo biosynthesized AEA between the intracellular and extracellular milieu, without altering its total amounts (Ligresti et al. 2004).
- 2-AGE and NADA, two endocannabinoids that are resistant and refractory to enzymatic hydrolysis, respectively, are still taken up by cells in a temperature-dependent way; their uptake is inhibited competitively by AEA (Huang et al. 2002; Fezza et al. 2002), although none of the specific EMT inhibitors mentioned above has ever been tested on the cellular uptake of these compounds.
- Lipopolysaccharide inhibits FAAH expression without affecting AEA cellular uptake (Maccarrone et al. 2001); conversely, nitric oxide, peroxynitrite and superoxide anions stimulate AEA cellular re-uptake (Maccarrone et al. 2000a), while acute or chronic ethanol inhibits this process (Basavarajappa et al. 2003), without affecting FAAH activity.

These data suggest that, although intracellular hydrolysis does greatly influence the rate of AEA facilitated diffusion, the uptake process is likely to be mediated by a mechanism subject to regulation and distinct from the one catalysing AEA hydrolysis.

3.2 Enzymatic Hydrolysis

3.2.1 Anandamide Hydrolysis

The hydrolysis of AEA is catalysed by FAAH, an enzyme originally purified and cloned from rat liver microsomes (Cravatt et al. 1996), that also catalyses the hydrolysis of other long-chain NAEs and, in vitro, of 2-AG. Since the hydrolysis products do not activate cannabinoid receptors, this reaction represents a true inactivation mechanism. FAAH is probably the same enzyme identified in the 1970s and 1980s as a NAE-hydrolysing enzyme (see Natarajan et al. 1984, for an example). It also catalyses the hydrolysis of arachidonoyl methyl ester, and hence it is possible that virodhamine is also a substrate, although this possibility has not been tested yet. Finally, FAAH also catalyses the hydrolysis of long-chain primary fatty acid amides, such as the putative sleep-inducing factor oleamide (Maurelli et al. 1995; Cravatt et al. 1996). The structural and kinetic properties of FAAH have been widely reviewed in the literature (Bisogno et al. 2002; Cravatt and Lichtman 2003, for recent reviews) and will be described in more detail in other chapters of this volume. In brief, the enzyme has an alkaline optimal pH and is found in intracellular

membranes; what was originally thought to be the hydrophobic domain responsible for this localization is instead important for the formation of active oligomers, whereas its localization on intracellular membranes might be regulated by an SH3 (Src homology region 3) consensus proline-rich sequence also necessary for enzymatic activity. Furthermore, judging from the recently elucidated X-ray structure of FAAH crystals in complex with its substrate (Bracey et al. 2002), one more domain may exist conferring the enzyme with the ability to associate with the plasma membrane. The catalytic amino acid of FAAH has been identified as Ser241, and two other residues of the amidase consensus sequence, Ser217 and Cys249, contribute to its enzymatic activity through a catalytic mechanism different from that of other amidases and *Ser* hydrolases (Patricelli and Cravatt 2000). The promoter region on the FAAH gene has been identified (Puffenbarger et al. 2001; Waleh et al. 2002), and is up-regulated by progesterone and leptin (Maccarrone et al. 2003a,b), and down-regulated by estrogens and glucocorticoids (Waleh et al. 2002).

Finally, transgenic FAAH-deficient mice have been developed. They are more responsive to exogenously administered AEA (Cravatt et al. 2001), and their brains contain 15-fold higher levels of AEA than wild-type mice. The phenotype of these mice is characterized also by higher susceptibility to kainate-induced seizures (Clement et al. 2003) and by lower sensitivity to some painful stimuli (Cravatt et al. 2001), which suggests that inhibition of FAAH might lead to the development of novel analgesics.

Another amidase has been characterized whose molecular size, substrate selectivity, optimal pH and tissue distribution are very different from those of FAAH (Ueda et al. 2001b; Ueda 2002, for a review). This enzyme appears to be located in lysosomes and might play a major role in the inactivation not so much of AEA as of its anti-inflammatory and analgesic congener, *N*-palmitoylethanolamine, which lacks activity at both CB_1 and CB_2 receptors (see Lambert et al. 2002, for review).

3.2.2
2-Arachidonoylglycerol Hydrolysis

Although FAAH can catalyse 2-AG hydrolysis both in cell-free homogenates and in some intact cells (Di Marzo et al. 1998a; Ligresti et al. 2003), 2-AG levels are not increased in FAAH knockout mice (Lichtman et al. 2002). This finding, together with previous reports on the existence of additional hydrolases for 2-AG degradation in porcine brain, in rat circulating platelets and macrophages, and in mouse J774 macrophages (Di Marzo et al. 1999a,b; Goparaju et al. 1999), suggests that FAAH may not be uniquely responsible for 2-AG inactivation under physiological conditions in vivo. The additional 2-AG hydrolases are known as monoacylglycerol lipases (MAGLs), are usually found in both membrane and cytosolic fractions, and also recognize other unsaturated monoacylglycerols, such as, for example, mono-oleoyl-glycerol, which is in fact a competitive inhibitor of 2-AG hydrolysis (Ben-Shabat et al. 1998; Di Marzo et al. 1998a). In rat circulating macrophages and platelets, 2-AG hydrolase activity was found to be lower following lipopolysaccha-

ride treatment (Di Marzo et al. 1999a). A MAGL with enzymatic properties and subcellular distribution very similar to these roughly characterized enzymes was cloned in the 1990s from mouse (Karlsson et al. 1997), and more recently from man and rat (Karlsson et al. 2001; Ho et al. 2002; Dinh et al. 2002). Evidence for its participation in 2-AG degradation was provided for the rat enzyme that, as previously found for FAAH (Egertova et al. 1998), is expressed in brain regions with high cannabinoid CB_1 receptor density, such as the hippocampus, but, unlike FAAH, occurs in pre-synaptic neurons and is likely to be expressed in the same neurons as CB_1 receptors (Dinh et al. 2002). This finding supports the role of rat MAGL in the degradation of that pool of 2-AG that acts as an endocannabinoid retrograde synaptic signal.

3.3 Other Metabolic Reactions

3.3.1 Re-esterification

The hydrolysis products of both AEA and 2-AG, i.e. arachidonic acid and ethanolamine or glycerol, are immediately recycled into membrane phospholipids to possibly re-enter the biosynthetic pathways of the two endocannabinoids at a later stage. However, 2-AG can be directly esterified into (phospho)glycerides prior to its hydrolysis, via phosphorylation and/or acylation of its two free hydroxyl groups (for a review see Sugiura et al. 2002). This pathway was suggested to occur in mouse N18TG2 neuroblastoma and rat basophilic leukaemia (RBL-2H3) cells and in mouse J774 macrophages (Di Marzo et al. 1998, 1999a,b). Most importantly, direct esterification into membrane phosphoglyceride fractions, and, to a minor extent, into diacylglycerol and triacylglycerol fractions, occurs for 2-AGE (Fezza et al. 2002), which would otherwise be difficult to metabolize, as its ether bond is refractory to enzymatic hydrolysis.

3.3.2 Oxidation and Methylation

The possible enzymatic oxidation of the arachidonoyl moiety of endocannabinoids was hypothesized shortly after the discovery and definition of endocannabinoids in the early 1990s (Fontana and Di Marzo 1995). Support for this hypothesis was soon obtained in the form of evidence for AEA metabolism by cell-free homogenates expressing various lipoxygenases and cytochrome P450 oxidases (Bornheim et al. 1993; Ueda et al. 1995) and, later, also for AEA metabolism by cyclooxygenase-2, but not cyclooxygenase-1 (Yu et al. 1997). In more recent years it was also found that oxidation products of both AEA and 2-AG could be formed easily in intact cells, and that 2-AG is as good a substrate for cyclooxygenase-2 as arachidonic acid (for a review see Kozak and Marnett 2002). The activity of the oxidation products at cannabinoid receptors depended very much on the type of metabolite formed, with

some lipoxygenase products being still capable of binding to both CB_1 and CB_2, and cyclooxygenase-2 products being inactive (Edgemond et al. 1998; Berglund et al. 1999; Maccarrone et al. 2000b; van der Stelt et al. 2002). Indeed, recent pharmacological data point to the existence of distinct, non-cannabinoid receptor, specific molecular targets for *both* prostaglandin-ethanolamides (prostamides), in particular prostamide $F_{2\alpha}$ (Matias et al. 2004), and prostaglandin E_2 glycerol ester (Nirodi et al. 2004). Prostamides, however, are rather stable to further metabolism, except for prostamide E_2, which undergoes slow dehydration/isomerization to prostamide B_2 (Kozak et al. 2001), whereas prostaglandin E_2 glyceryl ester is instead rapidly hydrolysed in rat, but not human, plasma (Kozak et al. 2001). None of these compounds is a substrate for the endocannabinoid transporter or FAAH (Matias et al. 2004; V. Di Marzo and L. Marnett, unpublished data). Regarding lipoxygenase products of AEA and 2-AG, it has been suggested that undefined lipoxygenase products of AEA act via vanilloid TRPV1 receptors (see below) (Craib et al. 2001), although there is no direct evidence for the interaction of hydroxy-anandamides or leukotriene-ethanolamides with these receptors. In contrast, 12- and 5-lipoxygenase products of arachidonic acid are known to interact with TRPV1 receptors (Hwang et al. 2000). The 15-(*S*)-hydroxy-derivative of 2-AG was recently shown to be formed in intact cells and to activate the peroxisome proliferation activator receptor-α (Kozak et al. 2002). Very little data, if any, exist on the further metabolism of AEA and 2-AG lipoxygenase products. Based on evidence available to date, it is possible that oxidation of AEA and 2-AG, while leading to the partial or complete inactivation of their endocannabinoid signal, might produce in some cases compounds active on other molecular targets, and hence represent an unusual example of "agonist functional plasticity".

Apart from its arachidonoyl moiety, the catecholamine moiety of NADA is also likely to be subject to both enzyme-catalysed and non-enzymatic oxidation. However, to date, only the methylation of the 3-hydroxy-group of NADA by catechol-*O*-methyl transferase has been observed (Huang et al. 2002). The reaction product is significantly less active at TRPV1 receptors (Huang et al. 2002), whereas its activity at CB_1 receptors has not been investigated.

3.4 Inhibitors of Endocannabinoid Inactivation

Several selective FAAH inhibitors have been developed (for reviews see Bisogno et al. 2002; Deutsch et al. 2002), some of which have IC_{50} values in the low nanomolar or subnanomolar range of concentrations (Boger et al. 2000; Kathuria et al. 2003) (Fig. 4). The first FAAH inhibitors to be developed, such as the irreversible inhibitor methyl-arachidonoyl-fluoro-phosphonate (MAFP) (Deutsch et al. 1997b; De Petrocellis et al. 1997), and the trifluoromethyl ketones, which are competitive inhibitors, (Koutek et al. 1994), came from the large pool of previously identified PLA_2 inhibitors, and were also found to interfere with CB_1 receptor activity. Others, such as the still widely used palmitylsulphonyl fluoride (AM374) (Deutsch et al. 1997a), appeared to be more selective towards CB_1 receptors but have never

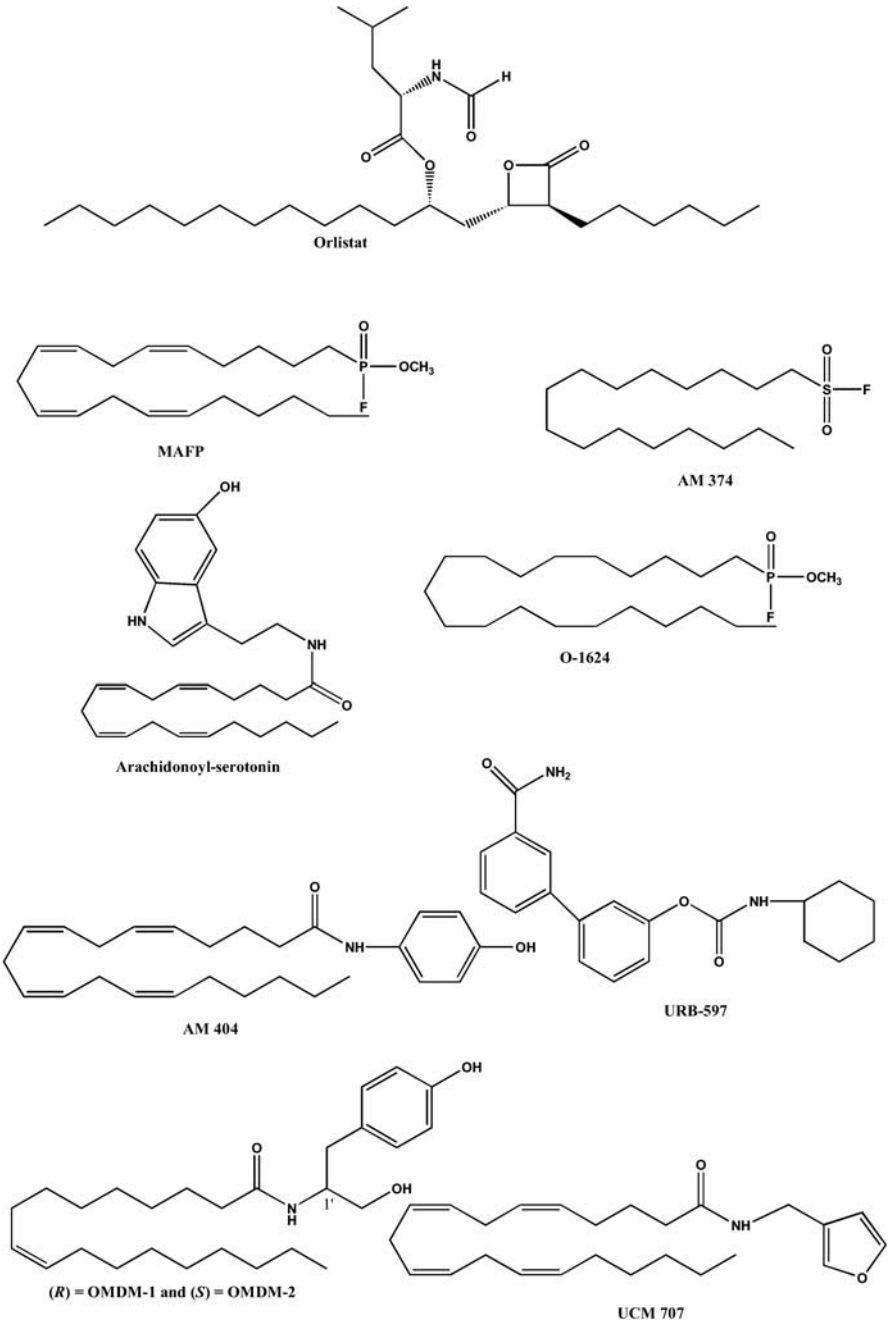

Fig. 4. Chemical structures of inhibitors of endocannabinoid biosynthesis or inactivation

been tested against PLA_2 enzymes. Among the FAAH inhibitors developed so far, particularly noteworthy are:

- *N*-arachidonoyl-serotonin (AA-5-HT, Bisogno et al. 1998), which is not particularly potent (IC_{50} values in the low μM range), but was tested against CB_1 and CB_2 receptors and PLA_2 enzymes and found to be inactive, and is suitable for use in vivo (V. Di Marzo, unpublished observations); so far, it has not been possible to enhance its inhibitory potency by chemical modification (Fowler et al. 2003).
- Several ultra-potent compounds developed by Boger and co-workers (Boger et al. 2000, 2001), whose use in vivo, however, has not been reported as yet.
- A series of MAFP analogues, one of which, O-1624, is quite potent and selective vs CB_1 receptors and was found to enhance anandamide levels after intrathecal administration to mice (Martin et al. 2000).
- A series of alkylcarbamic acid aryl esters, which were found to have very interesting structure–activity relationships against FAAH (Tarzia et al. 2003). One of these compounds, URB-597, is very potent and very selective for FAAH, although it was not tested against PLA_2. It is suitable for in vivo use, as its administration to rats causes a strong elevation of brain AEA levels with corresponding analgesic activity and anxiolytic actions (Kathuria et al. 2003).

With regard to inhibitors of the putative EMT, the development of a very potent and selective inhibitor has been hindered so far by the lack of any molecular data on this elusive protein. The prototypical EMT inhibitor, AM404 (Beltramo et al. 1997, Fig. 4), exhibits IC_{50} values in the 1- to 10-μM range of concentrations and has been widely used in vivo in laboratory animals. However, it has now been established that this compound can also inhibit FAAH and stimulate TRPV1 vanilloid receptors (Jarrahian et al. 2000; Zygmunt et al. 2000; De Petrocellis et al. 2000; Ross et al. 2001) and that both these properties, together with inhibition of EMT, can explain why AM404 can enhance AEA levels in vivo, since TRPV1 stimulation leads to enhanced AEA biosynthesis (Di Marzo et al. 2001d; Ahluwalia et al. 2003a). Therefore, great care is needed when using this compound in vivo. Recently, several compounds have been developed that are more potent as EMT inhibitors than as FAAH inhibitors or TRPV1 agonists:

- VDM11 and VDM13 (De Petrocellis et al. 2000) have been used as pharmacological tools in vitro, for example to demonstrate the action of AEA on TRPV1 at an intracellular site (De Petrocellis et al. 2001; Andersson et al. 2002). VDM11 has also been used to demonstrate anti-proliferative endocannabinoid tone in colorectal carcinoma cells in vitro (Ligresti et al. 2003), and to investigate the role of endocannabinoids in retrograde signalling during long-term depression (Ronesi et al. 2004). Finally, VDM11 has been used successfully in many in vivo studies, for example in the gastrointestinal system following i.p. administration (Pinto et al. 2002; Mascolo et al. 2002; Izzo et al. 2003). Interestingly, VDM11 was recently shown to also block endocannabinoid release (Ligresti et

al. 2004; Ronesi et al. 2004). The major drawback of VDM11 and VDM13 is that, like AM404, they are not very stable metabolically and can be hydrolysed to arachidonic acid by brain homogenates (Ortar et al. 2003).

- UCM-707 was developed from several other "head" analogues of AEA and found to be very potent on the EMT on some cells (Lopez-Rodriguez et al. 2003a,b), but not others (Ruiz-Llorente et al. 2004; Fowler et al. 2004). Apart from being more potent as an AEA uptake inhibitor than as a TRPV1 agonist or FAAH inhibitor, this compound is very suitable for in vivo use (de Lago et al. 2002), and has been successfully employed to help demonstrate that AEA plays a role in neuroprotection against kainate-induced seizures in mice (Marsicano et al. 2003).
- OMDM-1 and OMDM-2 are the first selective inhibitors of AEA cellular uptake to be developed from a fatty acid other than arachidonic acid, i.e. oleic acid (Ortar et al. 2003). For this reason, and also because it is more stable to hydrolysis in rat brain homogenates, OMDM-2 appears to exert a more long-lasting inhibition of spasticity in mice with experimental allergic encephalomyelitis (de Lago et al. 2004b), and to improve several motor and immunological parameters of the disorder (C. Guaza and V. Di Marzo, unpublished observations).

Although both basic and applied research with AEA transport inhibitors has already produced several interesting results of relevance to the endocannabinoid system, the isolation and cloning of the putative EMT remains an important objective since, if such a protein really exists, the identification of its molecular features should lead to the development of even more potent inhibitors.

4
Pharmacology of Endocannabinoids

4.1
Endocannabinoid Molecular Targets: Beyond CB_1 and CB_2 Receptors

By definition (Di Marzo and Fontana 1995), endocannabinoids act primarily at cannabinoid CB_1 and/or CB_2 receptors, and they do so with varying affinity and efficacy. AEA, NADA and 2-AGE are more selective for CB_1, with the following rank of affinity: AEA$\geq$2-AGE>NADA. 2-AG has almost the same affinity for both receptors, and its K_i varies considerably according to the experimental conditions. Several assays have been used to examine the functional activity of endocannabinoids and to compare it with that of synthetic cannabinoids and THC (Pertwee 1997), and those used most often are:

- The GTP-γ-S binding assay, which provides an indirect measure of the ability of ligands to induce coupling of receptors to G-proteins.
- The cyclic adenosine monophosphate (cAMP) assay, in which the ability to inhibit forskolin-induced cAMP production is measured.

- Assays that measure the ability of ligands to inhibit voltage-activated Ca^{2+} channels or to stimulate the activity of G protein-coupled inwardly rectifying K^+ channels (GIRK).
- An assay that measures the ability of ligands to inhibit electrically evoked contractions of the mouse vas deferens.

Just as the affinity constant of AEA, 2-AGE and NADA depends on the type of membrane preparation and radioligand used to carry out the binding assay (for an example see Appendino et al. 2003), so too their efficacy depends very much on the type of functional assay used. For example, both noladin and NADA are more potent than AEA at inducing Ca^{2+} transients in neuroblastoma cells via CB_1 receptors (Sugiura et al. 1999; Bisogno et al. 2000). Noladin and 2-AG are equipotent, and much more potent than AEA at inhibiting voltage-activated Ca^{2+} channels in rat sympathetic neurons previously injected with cDNA encoding human CB_1 (Guo and Ikeda 2004). Indeed, AEA behaves as a partial agonist at CB_1 in most assays of functional activity, and is almost functionally inactive on CB_2 (see McAllister and Glass 2002 for review). Virodhamine acts as an antagonist for CB_1 and a partial agonist for CB_2, thus behaving in an opposite way to AEA (Porter et al. 2002). 2-AG appears to be equipotent and a full agonist at both receptor subtypes (McAllister and Glass 2002), although its affinity constants at both targets are lower than those of AEA (Mechoulam et al. 1995).

To add further complexity to this scenario, there is now increasing evidence, based on pharmacological and biochemical data, for the existence of non-CB_1, non-CB_2 GPCRs that respond to physiologically relevant concentrations of endocannabinoids and their congeners, and of AEA in particular (for comprehensive reviews see Di Marzo et al. 2002b and Pertwee 2004). These putative receptors can be grouped into three categories:

- "WIN-55,212-2/AEA/vanillyl-fatty acid amide" receptors: the first example of such sites of action was detected in murine astrocytes (Sagan et al. 1999). Through this, or a very similar receptor, AEA inhibits adenylyl cyclase and, possibly, gap-junction-mediated Ca^{2+} signalling in astrocytes (Venance et al. 1995). Indeed, a GPCR with a distribution different from CB_1 receptors and sensitive to both AEA and WIN-55,212-2, but not to other cannabinoid receptor agonists, was described in several brain areas of CB_1 knockout mice (Di Marzo et al. 2000a; Breivogel et al. 2001; Monory et al. 2002). Still to be clarified is whether this proposed receptor is similar to the putative site of action that mediates the inhibitory effect of WIN-55,212-2 on glutamate release in the mouse hippocampus (Hajos et al. 2001) and is sensitive to capsaicin (Hajos and Freund 2002). This in turn, might be the same receptor as the one that has been postulated to mediate some of the actions of fatty acid–vanillamine amides, such as arvanil and its analogues (Di Marzo et al. 2001b,d; 2002c; Brooks et al. 2002).
- "AEA/abnormal-cannabidiol receptors": another possible GPCR for AEA and for the non-psychotropic cannabinoid, abnormal-cannabidiol (abn-cbd), has been

detected in vascular endothelial cells. This putative receptor mediates the local vasodilator (but not the systemic hypotensive) effects of AEA, and is blocked by both cannabidiol and a synthetic analogue, O-1918 (Jarai et al. 1999; Offertaler et al. 2003). It is coupled to guanylyl cyclase and p42/44 mitogen-activated protein kinase and protein kinase B/Akt. Interestingly, this novel endothelial receptor seems to be activated also by NADA (O'Sullivan et al. 2004). A receptor sensitive to abn-cbd has been proposed to mediate microglial cell migration (Walter et al. 2003), but this site of action, unlike the one in endothelial cells, was also activated by 2-AG.

– "Saturated NAE receptors": one other GPCR, for *N*-palmitoylethanolamine, has been proposed to explain some of the analgesic and anti-inflammatory actions of this AEA congener (Calignano et al. 1998). A receptor for *N*-palmitoylethanolamine has been proposed to occur also in microglial cells (Franklin et al. 2003) and shown to be different from that proposed to mediate the central effects of another saturated AEA congener, *N*-stearoylethanolamine (Maccarrone et al. 2002b).

In addition, several channels that transport Ca^{2+} and K^+ across the cell membrane are targeted directly by sub-micromolar concentrations of AEA (Di Marzo et al. 2002b). These are:

– TASK-1 K^+ channels (Maingret et al. 2001), which are inhibited by AEA.
– T-type Ca^{2+} channels (Chemin et al. 2001), which are also blocked by AEA, apparently acting at an intracellular site.
– Vanilloid TRPV1 receptors, the sites of action of capsaicin, the pungent component of "hot" red peppers (Szallasi and Blumberg 1999), which in contrast are activated by AEA and NADA (Zygmunt et al. 1999; Smart et al. 2000; Huang et al. 2002). In this case the effect clearly requires the activation of an intracellular domain of the protein (De Petrocellis et al. 2001; Jordt and Julius 2002), a mechanism that explains the significantly higher potency with which AEA and NADA induce TRPV1-mediated currents when injected directly into the neuron (Premkumar et al. 2004; Evans et al. 2004).

In heterologous expression systems, the potency of AEA for inducing typical TRPV1-mediated effects (e.g. cation currents, Ca^{2+}-influx and cell depolarization) is at least fivefold lower than its average potency at CB_1 receptors. However, recent data (recently reviewed by Di Marzo et al. 2001a; 2002a; Ross 2003; van der Stelt and Di Marzo 2004) indicate that the potency of AEA and NADA at TRPV1 receptors increases by up to 10- to 15-fold in some pathological states. In fact, the number of TRPV1-mediated pharmacological effects, in vitro and in vivo, being reported in the literature for AEA is increasing by the day. A recent study showed that elevated levels of endocannabinoids acting at TRPV1 cause ileitis in toxin A-treated rats (McVey et al. 2003). Evidence for a role for AEA and TRPV1 in store-operated Ca^{2+}-entry into sensory neurons has also been found (M. van der Stelt and V. Di Marzo, manuscript in preparation). Furthermore, as AEA often exerts opposing actions, depending on whether it acts on CB_1 or TRPV1 receptors, blockade of CB_1 receptors

may reveal that TRPV1-mediated effects of AEA can be exerted at concentrations lower than originally thought (Ahluwalia et al. 2003b). Finally, there are in vitro preparations, such as the rat mesenteric artery, where the efficacy and potency of AEA and NADA at TRPV1 are comparable to those of capsaicin (Zygmunt et al. 1999; O'Sullivan et al. 2004). Thus, many authors now agree that TRPV1 and CB_1 receptors may be considered as ionotropic and metabotropic receptors for the same class of endogenous fatty acid amides, including so far AEA and NADA (Di Marzo et al. 2002a,b). A further recent development in this area of research has been the demonstration that THC and a second plant cannabinoid, cannabinol, but not AEA, activate the ANKTM1 receptor, which is another type of transient receptor potential (TRP) channel and appears to be the primary molecular target for mustard oils in some sensory efferents (Jordt et al. 2004). In contrast, AEA and 2-AG, after their hydrolysis to arachidonic acid and conversion to epoxygenase derivatives, activate TRPV4 channels (Watanabe et al. 2003). These TRP channel-mediated actions seem to be important, for example, in the control of small artery dilation (see below), and indicate a partial overlap between the ligand recognition pre-requisites of cannabinoid receptors and some TRP channels.

4.2 Endocannabinoid Pharmacological Actions: Some Major Differences from THC

The pharmacology of endocannabinoids overlaps with that of THC to a great extent. However, important qualitative and quantitative differences have been observed between the pharmacological actions in vivo of THC and, for example, AEA. Together with the high metabolic instability of endocannabinoids, the observation that some of these compounds can activate receptors different from CB_1 and CB_2 can certainly explain some of these differences. This is particularly true for the four behavioural actions that, when assessed together in mice, have been used to characterize a compound as *cannabimimetic* in vivo, i.e. the ability to: (1) inhibit an acute pain response in the "tail flick" or "hot plate" tests; (2) induce immobility on a "ring"; (3) inhibit spontaneous locomotion in an open field; and (4) reduce body temperature. Although activity in this "mouse tetrad" is exhibited by all CB_1 receptor agonists, particularly if they possess a cannabinoid-like chemical structure, it is now accepted that a compound may still exhibit activity in all four tests and yet not act via these receptors (see Wiley and Martin 2003 for a recent critical discussion of this concept). For example, AEA-vanilloid "hybrid" compounds that potently stimulate TRPV1, but not CB_1, receptors are also very potent and efficacious in the tetrad (Di Marzo et al. 2002c), and each of the activities assessed in this way can also be elicited by capsaicin in either mice or rats. Indeed, AEA, unlike THC, is still active in at least three of the four tetrad tests when these are carried out in transgenic mice lacking a functional CB_1 receptor (Di Marzo et al. 2000a), or when these receptors are blocked with SR141716A (rimonabant) (Adams et al. 1998). However, the activity of AEA in these tests has never been assessed using TRPV1-knockout mice. Therefore, the possibility that the effects of this endocannabinoid on the tetrad in CB_1-knockout mice are mediated by these

receptors has not been addressed experimentally. Interestingly, a recent study showed that AEA, if administered i.p. to Wistar rats, can cause hypolocomotion via TRPV1 receptors (de Lago et al. 2004a). Indeed, given the ability of AEA to interact with several other receptors (see previous section), and the possible lack of specificity of the tetrad of tests, the fact that this compound can exert central actions even in the absence of CB_1 receptors cannot be regarded any longer as surprising, although the search for the possible alternative target(s) responsible for these actions in vivo is far from being concluded.

The local vasodilator actions, and the effects (or lack of effects) on the release from sensory neurons of nociceptive neuropeptides, represent two other examples of pharmacological differences between THC and endocannabinoids (Randall et al. 2002). THC appears to be either inactive or weakly active in isolated artery preparations, depending on the absence or presence on capsaicin-sensitive perivascular neurons of novel THC receptors (Wagner et al. 1999; Zygmunt et al. 1999; Zygmunt et al. 2002), recently identified as ANKTM1 channels (Jordt et al. 2004). AEA does not activate ANKTM1 but nevertheless produces vasodilation through several complex, concurrent mechanisms (see Ralevic et al. 2003 and Hiley and Ford 2004, for recent reviews) that, for example, involve the participation of endothelial abn-cbd-sensitive receptors, TRPV1 receptors on perivascular neurons and K^+ and Ca^{2+} channels, etc., as well as the possible formation of arachidonate metabolites. The potent vasodilator effect of NADA is also complex (O'Sullivan et al. 2004). Thus, it is mediated by TRPV1 channels, abn-cbd-sensitive receptors and CB_1 receptors, with the relative contribution made by each of these varying according to whether experiments are performed with the superior mesenteric artery or with small mesenteric vessels. Finally, while the vasodilator actions of 2-AG in such preparations have been found to depend solely on its hydrolysis to arachidonic acid and subsequent conversion to cyclooxygenase products (Járai et al. 2000), recent data suggest that 2-AGE (noladin) acts via a novel non CB_1/CB_2 $G_{i/o}$-linked receptor (Ralevic et al. 2004).

Apart from resulting in qualitatively and quantitatively different vasodilator effects, the difference in the abilities of AEA, NADA and THC to stimulate the release of nociceptive/vasodilator neuropeptides (i.e. substance P and calcitonin gene-related peptide) via TRPV1 receptors explains why THC, which does not activate TRPV1, is never pro-nociceptive, whereas AEA and, particularly, NADA can produce hyperalgesic effects (Ahluwalia et al. 2003a; Price et al. 2004). Interestingly, NADA can be anti-nociceptive when administered systemically in vivo, possibly due to its agonist activity at CB_1 receptors (Bisogno et al. 2000), and induces nocifensive reactions when administered locally (Huang et al. 2002; Price et al. 2004).

Finally, neuroprotection is another area in which endocannabinoids and THC produce qualitatively and quantitatively different effects both in vitro and in vivo (see van der Stelt et al. 2003; Walter and Stella 2004, for recent reviews). Apart from its actions on CB_1 receptors, THC, but not anandamide, was also found to behave as an anti-oxidant in vivo (Hampson et al. 1998; Marsicano et al. 2002). Conversely, AEA exerts neuroprotective effects against excitotoxicity that are not uniquely mediated by CB_1 receptors (van der Stelt et al. 2001; Veldhuis et al. 2003).

5
New Drugs from the Endocannabinoid System

5.1
Regulation of Endocannabinoid Levels Under Pathological Conditions

Although we now know that the effects of endocannabinoids and exogenously administered THC can be both qualitatively and quantitatively different, the fact that the symptoms of many ailments have been reported to be alleviated by THC and *Cannabis* provided the rationale to test whether pathological alterations of endocannabinoid signalling can be causative of pathological states, or of their signs. There is now increasing evidence that endocannabinoid levels undergo significant changes in several animal models of both acute and chronic disorders. In particular, they appear to be transiently elevated in specific brain areas during several pathological conditions of the CNS, i.e. following insults or stressful stimuli, such as:

- Glutamate excitotoxicity, in the hippocampus (Marsicano et al. 2003)
- Food deprivation, in the hypothalamus and limbic forebrain (Kirkham et al. 2002)
- Exposure to an aversive memory, in the basolateral amygdala (Marsicano et al. 2002)
- Administration of a painful stimulus, in the periaqueductal grey (Walker et al. 1999)

In these cases, endocannabinoid signalling is enhanced to minimize the impact of the insult or of the stressful stimulus, respectively by:

- Protecting neurons from damage, via feed-back inhibition of glutamatergic neuron activity (Marsicano et al. 2003)
- Reinforcing appetite, via inhibition of anorexic signals (Kirkham et al. 2002; Di Marzo et al. 2001c; Cota et al. 2003)
- Suppressing aversive memories, via inhibition of γ-aminobutyric acid (GABA)-ergic signalling (Marsicano et al. 2002)
- Producing central analgesia, by suppressing the activity of nociceptive circuits (Walker et al. 1999)

Findings that CB_1 receptors appear to contribute significantly to protection from stroke in animals (Parmentier-Batteur et al. 2002), and that 2-AG is protective in a model of head trauma (Panikashvili et al. 2001), support the hypothesis that endocannabinoids have a neuroprotective role. In fact, endocannabinoid signalling is also elevated in animal models of neurodegenerative diseases, such as:

- In reserpine- or 6-hydroxy-dopamine-treated rats (two models of Parkinson's disease) at the level of the basal ganglia (Di Marzo et al. 2000b; Maccarrone et al. 2003c)

- In β-amyloid-treated rats (a model of Alzheimer's disease), in the hippocampus (authors' own unpublished results)
- In mice with chronic relapsing experimental allergic encephalomyelitis (CREAE, a model of multiple sclerosis), in the spinal cord (Baker et al. 2001)

The possible function of this up-regulated signalling, as suggested by pharmacological studies, is presumably to counteract neuronal hyperactivity and local inflammation, and hence damage, or, in the case of multiple sclerosis, to inhibit tremor and spasticity (Baker et al. 2000). However, the progressive nature of some disorders appears to result in a permanent, as opposed to transient, hyperactivation of the endocannabinoid system. This phenomenon appears to even contribute to the development of symptoms typical of Parkinson's disease and Alzheimer's disease, i.e. inhibition of motor activity and loss of memory, respectively, which in fact can be antagonized by CB_1 blockers (Di Marzo et al. 2000b; Mazzola et al. 2003). Furthermore, these effects may result, in some cases, in a compensatory down-regulation of CB_1 receptor expression (Silverdale et al. 2001; Berrendero et al. 2001). In contrast, in animal models of Huntington's chorea there is a loss of CB_1-expressing fibres from the basal ganglia even at the early stages of the disorder, and this results in reduced levels of both endocannabinoids and CB_1 receptors. This decrease in endocannabinoid signalling may contribute to the hyperkinesia typical of the first phase of the disease (Lastres-Becker et al. 2001; Denovan-Wright and Robertson 2000).

The endocannabinoid system is implicated in the physiological control of food intake and energy balance, not only after food deprivation but also in animal models of genetic obesity in which it appears to become overactive at the level of both the hypothalamus and adipocytes (Di Marzo et al. 2001c; Bensaid et al. 2003). This possibly explains why, following treatment of mice and rats with rimonabant, a transient inhibition of food intake and a more persistent reduction of fat mass are observed (Ravinet-Trillou et al. 2003), and why CB_1 "knockout" mice show a reduced susceptibility to obesity in response to a fat diet (Ravinet-Trillou et al. 2004).

Endocannabinoids also participate in pathological conditions of the cardiovascular, immune, gastrointestinal and reproductive systems. Enhanced macrophage and/or platelet endocannabinoid levels are found in rats during hemorrhagic and septic shock, or following liver cirrhosis and experimental myocardial infarction, and cause the hypotensive state typical of these conditions (Wagner et al. 1997; Varga et al. 1998; Batkai et al. 2001; Wagner et al. 2001). Anandamide levels and/or cannabinoid CB_1 receptor expression levels are also enhanced in three mouse models of intestinal disorders, i.e.:

- Small intestine inflammation (Izzo et al. 2001)
- Cholera toxin-induced intestinal hyper-secretion and diarrhoea (Izzo et al. 2003)
- Peritonitis-induced paralytic ileus (Mascolo et al. 2002)

While enhanced signalling at CB_1 receptors contributes to the production of reduced intestinal motility typical of paralytic ileus, in small intestine inflammation and cholera toxin-induced hyper-secretion and diarrhoea it affords tonic protection against the symptoms of the disorders.

Again, by acting preferentially at cannabinoid CB_1 receptors, anandamide plays a dual function in mouse embryo implantation, by stimulating it at low concentrations and inhibiting it at higher ones (Wang et al. 2003). Indeed, impaired anandamide hydrolysis in the blood of pregnant women leads to high levels of this compound correlating with premature abortion or failure of implanted in vitro-fertilized oocytes (Maccarrone et al. 2000c, 2002a).

Finally, enhanced endocannabinoid signalling is found in some human malignancies as compared to the corresponding healthy tissues (Ligresti et al. 2003; Schmid et al. 2002b), as well as in human cancer cells that are exhibiting a high degree of invasiveness (Sanchez et al. 2001; Portella et al. 2003). This observation, together with the finding that stimulation of either CB_1 or CB_2 receptors causes blockage of the proliferation of cancer cells or induction of their apoptosis in vitro (Ligresti et al. 2003; Galve-Roperh et al. 2001), and inhibition of cancer growth, angiogenesis and metastasis in vivo (Galve-Roperh et al. 2001; Bifulco et al. 2001; Portella et al. 2003; Casanova et al. 2003), suggests that endocannabinoids may afford some protection against tumoural growth and spread.

In summary, altered endocannabinoid signalling accompanies several central and peripheral disorders, the effect of this being to counteract symptoms and, maybe, even disease progression. In some cases, a hyperactive or a defective endocannabinoid system contributes to the production of symptoms. In view of the parallelism found between many experimental models and the corresponding clinical disorders (see Di Marzo et al. 2004 for a review), these findings suggest that substances that either prolong the half-life of endocannabinoids or prevent their formation or action may have therapeutic uses.

5.2 Potential Therapeutic Use of Inhibitors of Endocannabinoid Metabolic Fate

As pointed out in this chapter, endocannabinoids appear to be produced "on demand" to play, in many cases, a protective role "when and where needed". This observation provides one more rationale for the design of novel substances that, by retarding the inactivation of endocannabinoids when they are being produced with a protective function, might be exploited therapeutically. Promising results in preclinical studies have already been published with inhibitors of endocannabinoid metabolism in animal models of:

- Acute pain (Martin et al. 2000; Kathuria et al. 2003), particularly with FAAH inhibitors
- Epilepsy (Marsicano et al. 2003), with the uptake inhibitor UCM-707

- Multiple sclerosis (Baker et al. 2001; de Lago et al. 2004b; C. Guaza and V. Di Marzo, unpublished observations), with both uptake and FAAH inhibitors
- Parkinson's disease (Maccarrone et al. 2003c), with both uptake and FAAH inhibitors
- Anxiety (Kathuria et al. 2003), with URB-597, a potent FAAH inhibitor
- Cholera toxin-induced intestinal hypersecretion and diarrhoea (Izzo et al. 2003), with the uptake inhibitor VDM11

Unlike the direct stimulation of cannabinoid receptors with systemic agonists, this approach is likely to influence endocannabinoid levels mostly in those tissues where there is an ongoing production of these compounds, and hence it is less likely to result in undesired side-effects.

6 Concluding Remarks

As can be surmised from the data reviewed in this chapter, considerable progress has been made in little more than 10 years towards the understanding of those mechanisms underlying the regulation of the "cannabinergic" signal, particularly if one takes into consideration the fact that the cloning of the first cannabinoid receptor was only reported in 1990, and the first endocannabinoid identified only 2 years later. Apart from being conserved in all vertebrate phyla, the endocannabinoid system is also present, possibly with some major differences in the structure of receptors and in their function, in most invertebrates (McPartland 2004), thus corroborating the concept of its participation in vital functions. Although great breakthroughs in endocannabinoid biochemistry and pharmacology have been achieved in little more than a decade, several other milestones need to be met. In particular, it will be necessary:

- To understand the regulation at the molecular level of the enzymes catalysing anandamide and 2-AG biosynthesis and inactivation
- To assess the role as endocannabinoids of virodhamine, NADA and 2-AGE, find their biosynthetic pathways and clarify their regulation
- To establish transgenic mice lacking functional genes for endocannabinoid biosynthesis, and to study their phenotype
- To isolate and clone the putative EMT
- To develop selective and potent inhibitors of endocannabinoid biosynthesis and of 2-AG degradation that can be used in vivo
- To clone the novel receptors proposed for AEA and to establish their actual participation in endocannabinoid pharmacological actions in vivo

– To carry on identifying those disorders that can be caused, at least in part, by a malfunctioning endocannabinoid system, or whose onset, progress and/or symptoms are counteracted tonically by the endocannabinoids

Once these further tasks have been achieved, and the regulation of the endocannabinoid system under both physiological and pathological conditions is fully understood, it will be possible to assess whether endocannabinoid-based medicines with clear advantages over other established therapeutic drugs could be developed in the future.

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