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Endocannabinoids: synthesis and degradation

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Abstract Endocannabinoids were defined in 1995 as endogenous agonists of cannabinoid receptors, i.e. of the G protein-coupled receptors for cannabis's psychoactive principle, Δ^9-tetrahydrocannabinol. Although there appear to be several endocannabinoids, only two of such endogenous mediators have been thoroughly studied so far: anandamide and 2-arachidonoylglycerol (2-AG). A general strategy seems to apply to the biosynthesis and degradation of anandamide and 2-AG, although the levels of these two compounds appear to be regulated in different, and sometimes even opposing, ways. "Endocannabinoid enzymes", that is to say enzymes that catalyse endocannabinoid biosynthesis or degradation, have been identified and in some cases cloned, and will be described in this review together with their possible pharmacological targeting for therapeutic purposes. The cellular and subcellular localization and the modes for the regulation of the expression and activity of these enzymes play an important role in the functions played by the endocannabinoids under physiological and pathological conditions.

Endocannabinoids

Two subtypes of G protein-coupled receptors for cannabis's psychotropic component, Δ^9-tetrahydrocannabinol (THC), have been cloned to date, the cannabinoid CB_1 and CB_2 receptors (Howlett et al. 2004). Yet, five different types of endogenous agonists for these cannabinoid receptors have been identified so far (Fig. 1). These compounds, named endocannabinoids by analogy with THC (Di Marzo and Fontana 1995), are all derived from long-chain polyunsaturated fatty acids. In particular: (1) the anandamides are amides of ethanolamine with polyunsaturated fatty acids with at least 20 carbon atoms and three 1,4-diene double bonds. The C20:4 homologue in this series, *N*-arachidonoylethanolamine (AEA) (Devane et al. 1992), also known simply as anandamide, has been most studied.

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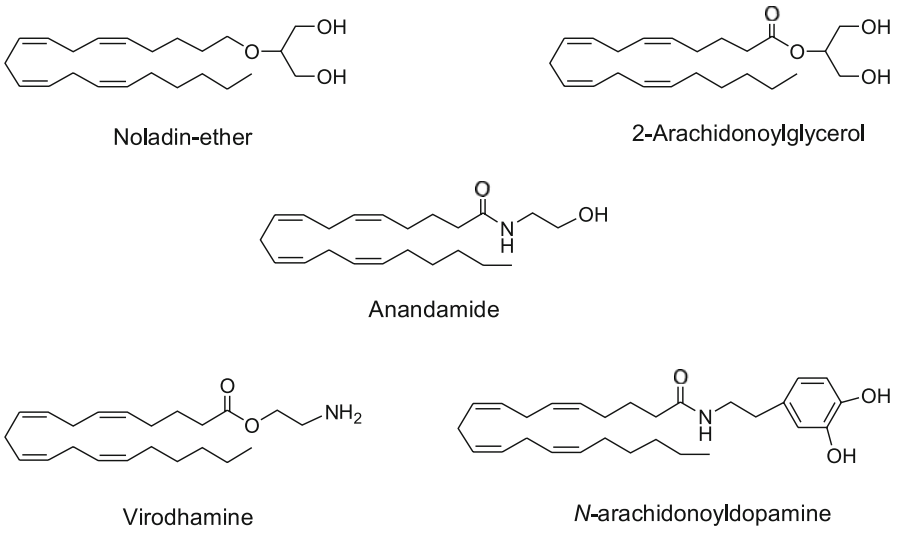

Fig. 1 Chemical structures of the putative endocannabinoids identified to date

We now know that AEA, apart from cannabinoid receptors, can interact with several other plasma membrane proteins (Di Marzo et al. 2002; van der Stelt and Di Marzo 2004); (2) 2-arachidonoyl glycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995) is considered by some researchers the only selective endogenous cannabinoid receptor agonist (Sugiura and Waku 2002); and (3) 2-arachidonyl glyceryl ether (noladin ether) (Hanus et al. 2001), *O*-arachidonoyl-ethanolamine (virodhamine) (Porter et al. 2002) and *N*-arachidonoylethanolamine (NADA) (Bisogno et al. 2000; Huang et al. 2002) have been described only recently, and their pharmacological activities as endocannabinoids have not yet been fully assessed. Therefore, most endocannabinoids isolated to date are derived from arachidonic acid, which is known to serve as a biosynthetic precursor for many other mediators, the eicosanoids. This structural peculiarity of the endocannabinoids, and the high susceptibility of some of them to be hydrolysed enzymatically to arachidonic acid, raise the possibility that some of the pharmacological actions of these compounds are due to this polyunsaturated fatty acid and its many metabolites. Furthermore, as will be discussed below particularly for AEA and 2-AG, the endocannabinoids can in principle be oxidized, prior to their hydrolysis, by enzymes of the arachidonate cascade, thus generating new series of eicosanoids whose pharmacological properties have not been fully investigated. Finally, the fact that all endocannabinoids are, like THC, lipophilic molecules strongly influences their signalling. In fact, it is now becoming clear that these compounds act as local autocrine or paracrine mediators, and that they are potentially bound to serum albumin in the blood (Bojesen and Hansen 2003).

Commonalities between anandamide and 2-AG anabolic and catabolic reactions

A scenario is now emerging indicating that, although different endocannabinoids share the same molecular targets, their levels are regulated in entirely different ways. This seems to apply particularly to AEA and 2-AG, whose biosynthetic and degradative pathways are

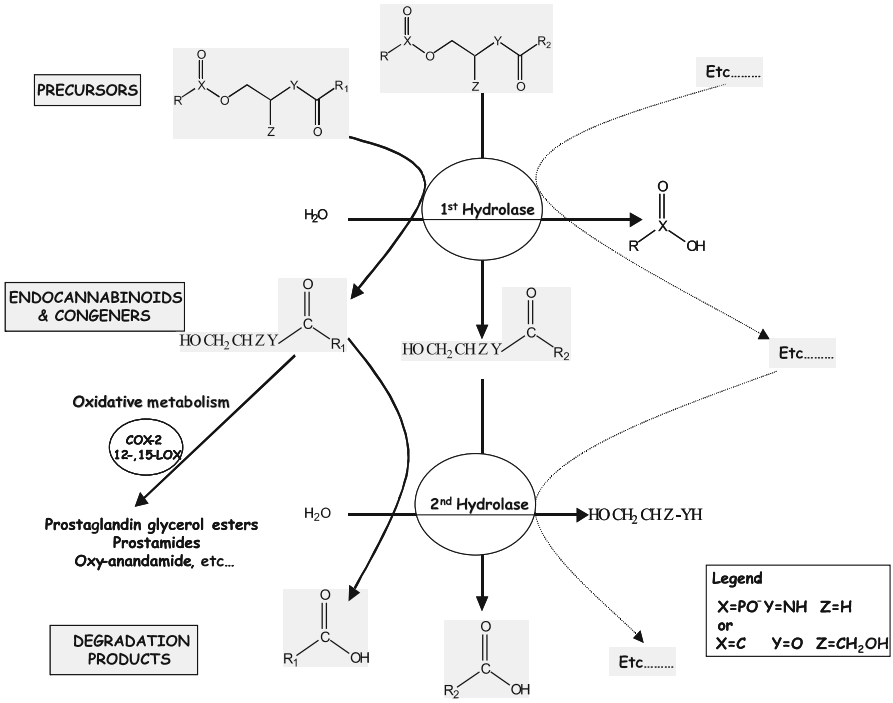

Fig. 2 Commonalities between anandamide and 2-arachidonoylglycerol metabolic pathways. A family of direct biosynthetic precursors, produced from phospholipid remodelling, are all converted into endocannabinoids (R_1=arachidonyl chain) or their cannabinoid receptor-inactive congeners (R_2, etc.) through the catalytic action of a first hydrolytic enzyme (the *N*-acyl-phosphatidylethanolamine-selective phospholipase D—in case of anandamide and other *N*-acylethanolamines—and the *sn*-1-selective diacylglycerol lipases in the case of 2-AG and other 2-acylglycerols). Endocannabinoids and congeners are then degraded to the corresponding fatty acids through the catalytic action of a second hydrolytic enzyme, again common to the same family of compounds [fatty acid amide hydrolase for the *N*-acylethanolamines and possibly the 2-acylglycerols too, and monoacylglycerol lipase(s), which are specific for the 2-acylglycerols]. Members of the same family of congeners (the *N*-acylethanolamines and the 2-acylglycerols) are known to interfere with each other inactivation by substrate competition (Ben-Shabat et al. 1998). Oxidation reaction may serve as "draining reactions" for the potential accumulation of non-polyunsaturated congeners that act also independently from cannabinoid receptors. *COX-2* cyclooxygenase-2, *LOX* lipoxygenases

the ones that have been most studied and utilize entirely different biosynthetic enzymes and precursors. Yet, there are several commonalities between the ways AEA and 2-AG are biosynthesized and degraded (Fig. 2). Since these compounds are eicosanoids, it was reasonable to expect them to be biosynthesized "on demand" and to be immediately released from cells, like with prostaglandins and leukotrienes, and unlike "pre-formed" chemical mediators. Indeed, AEA and 2-AG are produced following the enhancement of intracellular Ca^{2+} concentrations, be it caused by Ca^{2+} influx following, e.g. cell depolarization, or by mobilization of intracellular Ca^{2+} stores subsequent to stimulation of $G_{q/11}$ protein-coupled receptors. Accordingly, as will be discussed herein, the enzymes catalysing the last step of AEA and 2-AG biosynthesis are all Ca^{2+}-sensitive, and extracellular and intracellular Ca^{2+} appear to be differentially involved in the biosynthesis of AEA and 2-AG.

Another common feature of AEA and 2-AG is that they are produced following the hydrolysis of glycero(phospho)lipid precursors, which in turn originate from the remodelling of other membrane phospholipids. Since their most important biosynthetic precursors belong

to two families of lipids, the *N*-acylphosphatidylethanolamines and the diacylglycerols, respectively, and the biosynthesizing enzymes do not appear to be selective for one member of these families over the others, AEA and 2-AG are usually found to occur together with some of their congeners, the *N*-acylethanolamines and the 2-acylglycerols, respectively. The relative amount of a certain congener will reflect the relative amount of its ultimate biosynthetic precursor, and in particular the amount of the corresponding fatty acid esterified on the *sn*-1 and -2 position of phospholipids, respectively. In the case of 2-AG, this compound is the most abundant in its family, and most of its congeners do not seem to possess any biological activity other than that of being capable of potentially enhancing 2-AG life-span or activity at cannabinoid receptors (Ben-Shabat et al. 1998). On the other hand, AEA is one of the least-abundant congeners in its family, and a few of its cognate compounds possess pharmacological actions independent of cannabinoid receptors. The meaning of this seemingly non-selective synthesis of AEA has been debated.

Also the enzymes catalysing AEA and 2-AG hydrolysis are capable of recognizing to some extent as substrates all the unsaturated long-chain congeners of the two endocannabinoids. This means that cannabinoid receptor-inactive congeners can potentially inhibit the degradation of the endocannabinoids by acting as competing substrates, much in the same way the biosynthetic precursors of these congeners can inhibit the conversion of endocannabinoid biosynthetic precursors into endocannabinoids (Fig. 2). A further complication arises from the fact that almost all the enzymes catalysing endocannabinoid biosynthesis and inactivation are hydrolytic enzymes belonging to the large family of serine/cysteine hydrolases. As a consequence, it is difficult to design inhibitors that are selective for each of these enzymes, and this seems particularly true for 2-AG. Although similar to their congeners in their capability of being inactivated by hydrolytic enzymes, AEA and 2-AG are unique in their families because they can be recognized also by some enzymes of the arachidonate cascade, particularly cyclooxygenase-2 (COX-2), and 12- and 15-lipoxygenases (Kozak and Marnett 2002; van der Stelt et al. 2002). These enzymes, if active in the same cell and cell compartment, will catalyse "draining" reactions that may lead to the accumulation of non-polyunsaturated endocannabinoid congeners over polyunsaturated ones, as well as of endocannabinoid oxidation products. The final result would be a shift from cannabinoid receptor stimulation to activation of other molecular targets, i.e. those of saturated or monounsaturated *N*-acylethanolamines and of prostaglandin-ethanolamides and -glycerol esters (see next sections) (Figs. 2, 3). Such a networking of chemical signals provides a typical example of the functional plasticity of lipid mediators, and emphasizes the need of investigating the exact mechanisms regulating endocannabinoid levels in tissues, which will be the subject of the following sections.

Anandamide biosynthesis: one or more pathways?

A study published soon after its discovery suggested that AEA is produced in cell homogenates from the ATP-independent direct condensation of arachidonic acid and ethanolamine (Devane and Axelrod 1994). However, given the high stability of amide bonds, this process must overcome a transition state with very high energy; in fact, it has never been shown to occur in intact cells and can be observed in cell-free systems only when using non-physiological concentrations of arachidonic acid and ethanolamine. Indeed, the enzyme catalysing this most likely non-physiological process is the same that catalyses

AEA hydrolysis (see below), but it works in reverse because of the high concentrations of what would normally be the reaction products (Kurahashi et al. 1997; Arreaza et al. 1997).

Since 1994, it has become widely accepted that AEA is instead produced "on demand" from the hydrolysis of a pre-formed membrane phospholipid precursor, *N*-arachidonoylphosphatidylethanolamine (NArPE) (Di Marzo et al. 1994). This biosynthetic pathway is identical to that through which previously identified long-chain *N*-acyl-ethanolamines are produced from the hydrolysis of the corresponding *N*-acylphosphatidylethanolamines (NAPEs) (Schmid et al. 1990). Measurable levels of NArPE, although relatively low compared to other NAPEs, were found in brain and testis, together with an enzymatic activity capable of converting it into AEA (Di Marzo et al. 1996; Sugiura et al. 1996; Cadas et al. 1997). This enzyme was deemed to be very similar, or identical, to the phospholipase D (PLD)-like enzyme identified in the 1980s in H. Schmid's laboratory (Natarajan et al. 1986; Schmid et al. 1996), which, unlike other PLDs, exhibited no transphosphatidylating activity and low affinity for other phospholipids, and was characterized by little selectivity for any particular NAPE (Sugiura et al. 1996; Petersen and Hansen 1999). The enzyme catalysing the conversion of NAPEs into the corresponding *N*-acylethanolamines was, therefore, termed NAPE-selective PLD (NAPE-PLD). With the recent cloning and expression of the protein (Okamoto et al. 2004), it could also be confirmed that the NAPE-PLD is: (1) chemically and enzymatically distinct from other PLD enzymes; (2) stimulated by Ca^{2+}; and (3) almost equally efficacious with most NAPEs, and hence responsible for the formation of other biologically active *N*-acylethanolamines, such as the C16:0, C18:0 and C18:1 congeners, which all possess pharmacological activity independent of cannabinoid receptors. The amino acid sequence of the enzyme, in fact, revealed no homology with the cloned PLD enzymes and showed it to be a member of the β-lactamase fold of the zinc-metallo-hydrolase family of enzymes (Okamoto et al. 2004). Over-expression of the enzyme in cells led to higher cellular levels of AEA and correspondingly lower levels of NArPE, thus supporting its role in the biosynthesis of this endocannabinoid (Okamoto et al. 2005). Furthermore, it was recently shown that the NAPE-PLD is a fundamental determinant of uterine anandamide levels during mouse pregnancy, and that its expression correlates spatially and temporally with its activity and with anandamide levels, which in turn control embryo implantation (Guo et al. 2005).

Whether or not the direct conversion of NArPE into AEA is the only pathway through which the endocannabinoid is produced in cells still needs final demonstration through the use, for example, of small-interfering RNA (siRNA) technology or of transgenic mice lacking the NAPE-PLD. In fact, although the precursor-product relationship between NArPE and AEA has been substantiated by several studies, including the almost identical distribution of the two molecules in the brain (Bisogno et al. 1999a) and their similar rates of enhancement during brain development (Berrendero et al. 1999), there are additional potential pathways for the conversion of NArPE into the endocannabinoid. Evidence was recently reported in support of another route converting NAPEs into 2-lyso-NAPEs via the action of a group IB soluble phospholipase A_2, and then of 2-lyso-NAPEs into *N*-acylethanolamines via a selective lysoPLD (Sun et al. 2004). In cell-free homogenates this pathways applies to both AEA and its 16:0 congener. Finally, preliminary data suggest that in RAW 264.7 macrophages stimulated with lipopolysaccharide, AEA can be produced from the phosphatase-catalysed hydrolysis of 2′-phospho-AEA, which in turn would derive from phospholipase C-catalysed hydrolysis of NArPE (Liu et al. 2005).

In conclusion, more than one pathway—as is often the case with lipid mediators—may underlie the formation of AEA in cells, depending on the stimulus and on its capability to induce elevations of intracellular Ca^{2+} and/or activate different intracellular hydrolytic en-

zymes. In this context, it is interesting to note that basal AEA levels in mice are under the control of G_q/G_{11} proteins, since transgenic mice lacking these two proteins exhibit significantly reduced brain levels of this endocannabinoid (N. Wettschureck, H.H.H. Tsubokawa, M. van der Stelt, A. Moers, H. Krestel, S. Petrosino, G. Shutz, V. Di Marzo, S. Offermanns, submitted). On the other hand, if these animals are stimulated with kainic acid, which causes neuronal depolarization and Ca^{2+} influx, their brains produce AEA also in the absence of G_q/G_{11} proteins. This suggests that, depending on the conditions, this endocannabinoid, unlike 2-AG (see the following section) can be synthesized following mobilization of either intracellular or extracellular Ca^{2+} alone. Irrespective of the biosynthetic route, AEA always seems to be synthesized inside the cell, to be released into the extracellular milieu only afterwards. In fact, all the biosynthetic enzymes implicated in AEA biosynthesis are intracellular. Since this compound can efficaciously interact with other proteins, in some cases (e.g. as with the vanilloid TRPV1 receptor) using intracellular binding sites, one could foresee that its activation of cannabinoid receptors occurs in some cells after that AEA has influenced the activity of other proteins. Finally, it will be crucial to assess the cellular localization of AEA-synthesizing enzymes, and particularly to verify if, as in the case of 2-AG-synthesizing enzymes, they are post-synaptic in the adult brain and hence in agreement with the possible function as retrograde messengers suggested for endocannabinoids (see below).

2-AG biosynthesis: perhaps less complicated than originally thought

Unlike AEA, 2-AG exhibits selectivity for cannabinoid receptors (Di Marzo et al. 2002) and is also more abundant in almost all tissues analysed so far (Sugiura et al. 2002) (although not, for example, in blood, Maccarrone et al. 2002). Furthermore, this compound, again unlike AEA, behaves as a full agonist in most assays of CB_1 and CB_2 receptor functional activity (McAllister and Glass 2002). Therefore, pharmacological manipulation of the biosynthesis of 2-AG is likely to produce a strong impact on cannabinergic signalling. However, since the first studies on the biosynthesis of this endocannabinoid in intact cells (Bisogno et al. 1997; Stella et al. 1997), it was clear that 2-AG is synthesized through more than one pathway. Furthermore, this lipid is at the crossroads of several metabolic pathways involving glycerophospholipids, triacylglycerols and *sn*-1,2-diacylglycerols (DAGs). However, 2-arachidonate-containing DAGs were immediately identified as the most likely biosynthetic precursors for 2-AG when this compound is used as an extracellular ligand of cannabinoid receptors (Bisogno et al. 1997). In these cases, DAGs appear to be produced in turn from the hydrolysis either of phosphoinositol-bis-phosphate (PIP_2), catalysed by the PIP_2-selective phospholipase C (Stella et al. 1997; Kondo et al. 1998; Berdyshev et al. 2001; Liu et al. 2003) or of phosphatidic acid (PA), catalysed by the PA-selective phosphohydrolase (Bisogno et al. 1999b; Carrier et al. 2004; Oka et al. 2005), and they are converted into 2-AG by the action of *sn*-1-selective DAG lipases (DAGL), which have remained uncharacterized until a couple of years ago. Only recently, two *sn*-1-selective DAGL isoforms, DAGLα and DAGLβ, have been cloned (Bisogno et al. 2003). The molecular characterization and expression of these two enzymes into host cells allowed for the discovery of some of their important features: (1) They contain the typical lipase-3 and Ser-lipase signature sequences; (2) within the Ser-lipase signature sequence, two highly conserved amino acid residues, Ser443 and Asp495—which are normally used to form the catalytic triad of this type of enzyme—were found to be necessary for DAGL activity through site-directed mutagenesis studies; (3) near their N-terminus, the enzymes contain four hydrophobic, and possi-

bly *trans*-membrane, domains, which probably explain why the DAGLs were observed to be localized to the plasma membrane; (4) both DAGLs are stimulated by Ca^{2+} and inhibited by glutathione; (5) although both enzymes exhibit strong selectivity for DAGs over phospholipids, monoacylglycerides, triacylglycerols and fatty acid amides, they do not appear to prefer DAGs with any particular fatty acyl chain in the 2 or *sn*-1 position. Interestingly, DAGLα appeared to be more abundant in the adult brain and DAGLβ in the developing brain. More importantly, the cellular localization of two enzymes shifts during brain development, as they appear to be co-localized with CB_1 in neuronal axons of the peri-natal nervous system, and "move" to a location complementary to CB_1, i.e. to post-synaptic neurons, in the adult brain (Bisogno et al. 2003). This "localization shift" reflects the proposed roles for 2-AG as an autocrine endocannabinoid in axonal guidance (Williams et al. 2003), and as a retrograde messenger in the control of synaptic plasticity in the adult brain (Chevaleyre and Castillo 2003; Melis et al. 2004a).

Apart from their cellular localization, two more series of experiments confirm the role of these DAGLs in controlling the levels of 2-AG when this metabolic intermediate acts as an endocannabinoid: (1) the expression/activity of the enzymes reflects the tissue concentrations of 2-AG under certain physiopathological concentrations, i.e. when the brain levels of the endocannabinoid change dramatically when passing from the light to the dark phase of the day (Valenti et al. 2004) or following induction of neuronal damage with a β-amyloid peptide (Di Marzo et al. 2005), or when 2-AG uterine concentrations are up-regulated in animals with defective leptin signalling (Maccarrone et al. 2005); (2) pharmacological inhibition of the DAGLs was found to reduce 2-AG levels in several cell types stimulated with ionomycin (Bisogno et al. 2003) and to inhibit at the same time CB_1 receptor-mediated cellular phenomena such as fibroblast growth factor (FGF)-induced axonal sprouting in the developing brain, or depolarization-induced suppression of excitation in adult dopaminergic neurons (Bisogno et al. 2003; Melis et al. 2004a). It must be underlined, however, that although the inhibitor used in these latter studies, tetrahydrolipstatin, is very potent against the DAGLs (IC_{50} ~100 nM), it is also known to interfere with the activity of other lipases, albeit at higher concentrations. Therefore, results obtained with more selective inhibitors, or using other techniques, such as siRNAs, must be seen before conclusively asserting that these enzymes, and hence DAGs, are always and uniquely responsible for the biosynthesis of the "endocannabinoid 2-AG" in intact cells. Indeed, recent data have shown that the PLC-DAG-DAGL pathway is probably not involved in controlling the basal levels of 2-AG in unchallenged mice. In fact, transgenic mice lacking G_q/G_{11} proteins that mediate metabotropic receptor-induced activation of phosphoinositide-selective PLC (PLCβ) do not exhibit reduced brain levels of this endocannabinoid (Wettschureck et al., submitted). It is possible, therefore, that most of the relatively high basal levels of 2-AG are not used to activate cannabinoid receptors (the tissue concentration of this compound in the rat brain approximates 5 μM, and would cause a permanent activation of CB_1 receptors), and are formed via biosynthetic precursors other than DAGs (Nakane et al. 2002) and/or through the action of PLC isoforms different from PLCβ (Di Marzo et al. 1996). On the other hand, unlike what described above for AEA, if mice are stimulated with kainic acid, their brains cannot synthesize "on demand" 2-AG in the absence of G_q/G_{11} proteins, and subsequently cannot protect themselves against kainate-induced excitotoxicity (N. Wettschureck, H.H.H. Tsubokawa, M. van der Stelt, A. Moers, H. Krestel, S. Petrosino, G. Shutz, V. Di Marzo, S. Offermanns, submitted). This finding: (1) is in agreement with the suggestion that PLCβ serves as a coincidence detector directing extracellular and intracellular Ca^{2+} mobilization into the formation of endocannabinoids, and of 2-AG in particular (Hashimotodani et al. 2005), and (2) is probably due to the fact that, unlike AEA, both intracellular Ca^{2+} and

PLCβ-derived biosynthetic precursors (i.e. DAGs) are necessary for the formation of that population of 2-AG which acts as a brain endocannabinoid, at least under certain conditions. It will be interesting to confirm these data by measuring the brain levels of 2-AG in yet-to-be-developed DAGLα/β "double knockout" mice. Furthermore, it will be important to assess the importance of DAGL-dependent, but PLCβ-independent, pathways, such as the one relying on PA and PA-selective phosphohydrolase for the formation of DAG precursors (Bisogno et al. 1999b; Carrier et al. 2004; Oka et al. 2005), in the formation of the "endocannabinoid 2-AG".

Biosynthesis of NADA and other endocannabinoids: more questions than answers

Partly due to the fact that their roles as endocannabinoids have not yet been conclusively confirmed, little information still exists on the biosynthetic and catabolic pathways of NADA, virodhamine and noladin ether. Regarding the latter compound, after the initial work of Hanus et al. (2001), two more studies have reported contrasting results on its actual occurrence in brain tissue (Fezza et al. 2002; Oka et al. 2003), and this may have somehow hampered the performance of further studies on noladin ether's metabolic pathways. However, it was clearly established that this putative endocannabinoid is not produced in intact mouse neuroblastoma cells from arachidonic acid incorporated into phospholipids and after ionomycin stimulation, i.e. using conditions leading to the biosynthesis of large amounts of 2-AG (Fezza et al. 2002). As to NADA, strong evidence has been reported against this compound being produced from the metabolism of *N*-arachidonoyl-tyrosine and using the same enzymes converting tyrosine into dopamine. In fact, although *N*-arachidonoyl-tyrosine is present in small amounts in the brain, it cannot be efficaciously converted into *N*-arachidonoyl-DOPA by tyrosine hydroxylase either in vitro or in vivo, and injection of synthetic *N*-arachidonoyl-DOPA into rat brain does not result in the formation of NADA. Conversely, measurable amounts of NADA can be obtained in brain homogenates from arachidonic acid and dopamine, whereas in vivo the basal levels of NADA in rat striatum are strongly dependent on this brain area receiving dopamine from the substantia nigra (S.S.-J. Hu, J.S.-C. Chen, S.M. Huang, A. Minassi, T. Bisogno, R. Roskoski, V. Di Marzo, J.M. Walker, submitted). It remains to be established if the formation of NADA from the condensation of arachidonic acid with dopamine occurs directly or via the intermediacy of arachidonoyl-coenzyme A (CoA) or other activated forms of this fatty acid. Preliminary data investigating the possibility that arachidonoyl-CoA is converted into NADA yielded contradictory results (V. Di Marzo, T. Bisogno, F. Fezza and M. Maccarrone, unpublished results).

Finally, regarding virodhamine, whether this compound is produced from anandamide via a non-enzymatic reaction at slightly alkaline pH (Markey et al. 2000) or through an independent pathway has not been investigated yet.

The endocannabinoid membrane transporter: an "unknown" between release and re-uptake

Given the intracellular localization of their biosynthetic and catabolic enzymes, and the extracellular/lipid interface localization of cannabinoid receptor binding sites, AEA and 2-AG need to be transported across the membrane in order to activate the receptors and

then to be degraded. The two compounds as well as other putative endocannabinoids are quite lipophilic and therefore there is currently a lively debate regarding the possibility that endocannabinoid uptake by, and release from, cells occurs via facilitated transport across the plasma membrane and not via simple passive diffusion. In the latter case, intracellular metabolism facilitate the uptake by, but would only retard the release from, the cell. The evidence in favour and against the existence of a common carrier, or at least of a specific process, for the bi-directional membrane transport of all endocannabinoids according to the gradient of concentrations across the plasma membrane (Porter et al. 2002; Huang et al. 2002; Beltramo and Piomelli 2000; Bisogno et al. 2001; Hajos et al. 2004; Fezza et al. 2002) has been lately summarized by various authors (Hillard and Jarrahian 2003; McFarland and Barker 2004; De Petrocellis et al. 2004). That fatty acid amide hydrolase (FAAH) cannot account alone for endocannabinoid cellular uptake is supported not only by several indirect data (Bisogno et al. 2005a), but also and most importantly by recent experiments carried out using: (1) cells from FAAH-knockout mice; (2) uptake inhibitors selective vs FAAH; and (3) confocal microscopy to assess the spatial and functional separation between anandamide uptake and hydrolysis (Ligresti et al. 2004; Ortega-Gutierrez et al. 2004; Fegley et al. 2004; Ortar et al. 2003; Oddi et al. 2005). However, the putative endocannabinoid membrane transporter has not been cloned yet, nor has any protein involved in endocannabinoid transport across the membrane been identified to date. Intriguingly, preliminary evidence has been presented recently on the pharmacological characterization of an anandamide membrane transporter (Chesterfield et al. 2005). Using a novel radioligand and a variety of small-molecule inhibitors of AEA uptake, the authors identified a high-affinity plasma membrane binding site whose pharmacology correlated to that of the putative endocannabinoid membrane transporter. It is also possible that endocannabinoid membrane transport is the result of more than one process, perhaps including endocytosis and/or the interaction with more than one protein in more than one subcellular compartment (Hillard and Jarrahian 2003; McFarland and Barker 2004).

The bi-directionality of facilitated endocannabinoid transport mentioned above is suggested, among other things, by the fact that the release of endocannabinoids after their biosynthesis is blocked by the same selective inhibitors that block reuptake (Ligresti et al. 2004; Ronesi et al. 2004). This finding raises an important question: How can substances that block both endocannabinoid uptake and release also elevate the levels of endocannabinoids available for cannabinoid receptor stimulation and subsequently induce beneficial actions in animal models of disorders where endocannabinoids play a protective function? Examples of these disorders include, to date, both central and peripheral disorders (see section “Conclusions: new therapeutic drugs from studies of endocannabinoid synthesis and degradation”). Clearly, if inhibitors of the putative endocannabinoid transporter reach the tissues involved in the disease only after the disease itself has already developed, and hence after the endocannabinoids involved in the disease have been already released, inhibitors of the transporter can prolong endocannabinoid action at cannabinoid receptors. It is also possible that not all cell types are permeable to the endocannabinoid membrane transporter inhibitors developed so far, and that these can only block re-uptake, with no effect on release unless they are previously inserted or allowed to diffuse inside the cell (as shown by Ronesi et al. 2004). In fact, competitive inhibition of release would require that the inhibitors—and not only the endocannabinoid—are inside the cell.

Fatty acid amide hydrolase: AEA degradation and beyond

It was soon clear that AEA is inactivated through the hydrolysis of its amide bond to arachidonic acid and ethanolamine (Di Marzo et al. 1994; Deutsch and Chin 1993), i.e. through the same route previously shown to underlie *N*-acylethanolamine degradation (Schmid et al. 1996). The possibility of using previously obtained information facilitated the identification of the enzyme responsible for this reaction, which was partially purified by Ueda and colleagues (1995), named "anandamide amidohydrolase", and proposed to be identical to *N*-acylethanolamine hydrolase (Schmid et al. 1996). In subsequent studies, it was also observed that this enzyme could also recognize as a substrate oleoylamide (oleamide), a putative sleep factor (Maurelli et al. 1995). This finding allowed for the demonstration that the "oleamide amidase" cloned in 1996 by Cravatt and his collaborators catalysed the hydrolysis also of AEA (Cravatt et al. 1996). Due to its wide selectivity for fatty acid amides, including long-chain *N*-acylethanolamines, primary amides, *N*-acylamino acids and, more recently, *N*-acyltaurines (Fowler et al. 2001; Ueda 2002; Deutsch et al. 2002; Bisogno et al. 2002; Saghatelian et al. 2004), the enzyme was named "fatty acid amide hydrolase" (FAAH). Despite its name, however, FAAH also catalyses quite efficiently the hydrolysis of fatty acyl esters, including 2-AG (Goparaju et al. 1998; Di Marzo et al. 1998) and most probably virodhamine too (Steffens et al. 2005).

Since the several important structural features of FAAH were recently discussed in a comprehensive review (McKinney and Cravatt 2005), they will not be described here. These features were revealed by means of site-directed mutagenesis studies and, more recently, crystallographic X-ray studies carried out on the protein in a complex with a covalent inhibitor (Deutsch et al. 2002; Bracey et al. 2002). On the other hand, several aspects of the function of FAAH under both physiological and pathological conditions were revealed by: (1) the study of the phenotype of transgenic mice lacking the enzyme (the "FAAH-knockout mice") (Cravatt et al. 2001); (2) the design of specific FAAH inhibitors suitable for use in vivo (Bisogno et al. 1998; Kathuria et al. 2003; Lichtman et al. 2004); (3) immunohistochemical studies describing the tissue and cellular distribution of the enzyme and its relationship with CB_1 receptor distribution (Egertova et al. 2003; Gulyas et al. 2004); and (4) the identification of the promoter region of the *Faah* gene and of its regulation by several transcription factors and hormones (Maccarrone et al. 2000b, 2003a, b). As will be discussed herein, we now know that FAAH is an important determinant of endocannabinoid and fatty acid amide levels in several, but not all, physiological and pathological states. In fact, at least in the case of AEA, there is little doubt that FAAH is the major, if not the only, degrading enzyme in the brain since, in this organ, AEA levels are dramatically elevated in FAAH-deficient mice (Cravatt et al. 2001) or following administration of FAAH inhibitors to rats (Kathuria et al. 2003). However, regarding some peripheral organs, such as the duodenum and the liver in rats and mice, there seems to be some discrepancy as to whether or not deletion of the *Faah* gene in mice, or pharmacological inhibition of the enzyme, cause elevation of AEA levels (Fegley et al. 2005; Cravatt et al. 2004). Some studies found that AEA was up-regulated in the liver, kidney and testis of these transgenic mice (Cravatt et al. 2004) as well as in their small intestine (Capasso et al. 2005), where the FAAH inhibitor *N*-arachidonoyl-serotonin administered to wild-type mice also causes an increase of AEA levels (Capasso et al. 2005). By contrast, in another study, the other FAAH inhibitor URB-597, or genetical deletion of *Faah*, did not result in the elevation of small intestine and liver AEA levels (Fegley et al. 2005). Clearly, methodological differences and different pharmacokinetic profiles of the FAAH inhibitors used account for such discrepancies. However, the

possibility that another AEA hydrolysing enzyme may exist in peripheral organs should not be ruled out. This is unlikely to be the recently cloned "*N*-acylethanolamine-hydrolysing acid amidase" (Tsuboi et al. 2005), since this enzyme is specific for long-chain saturated *N*-acylethanolamines. Conversely, it is clearly emerging that FAAH is also involved in the control of fatty acid amides different from AEA. In a recent study (Cravatt et al. 2004), it was elegantly shown how the genetic inactivation of the *Faah* gene, when limited to peripheral tissues, resulted in an anti-inflammatory phenotype that was not due to elevated activation of CB_1 receptors. The authors suggested that other bioactive fatty acid amides that are substrates for FAAH, i.e. oleoylethanolamide, palmitoylethanolamide or *N*-arachidonoylglycine, could be responsible for the elevated threshold to inflammatory pain via peripheral actions.

Despite the wide substrate selectivity of FAAH, this enzyme certainly plays a role in regulating the activity of CB_1 receptors, as shown by the several immunohistochemical data pointing to the complementary distribution of the two proteins in the CNS. In fact, CB_1 and FAAH are mostly localized at the pre- and post-synaptic level, respectively, in many (but not all) brain areas (Egertova et al. 2003; Gulyas et al. 2004). In these regions, FAAH may regulate post-synaptic levels of anandamide (Egertova et al. 2003), whereas in regions such as the globus pallidus and substantia nigra pars reticulata, CB_1 receptors are not associated with FAAH expression. In these brain regions the control over endocannabinoid signalling may be less restricted than in regions enriched with FAAH. Finally, in those brain regions where FAAH-immunoreactive neurons occur in the absence of CB_1, the enzyme may be involved in the regulation of endocannabinoid actions at other endocannabinoid receptors (Di Marzo et al. 2002), or of receptors for other FAAH substrates.

2-AG degradation: redundancy again

Unlike AEA, 2-AG can be metabolized via several different chemical reactions. If one looks just at the enzymatic hydrolysis of this compound, apart from FAAH, another hydrolase, the monoacylglycerol lipase (MAGL), was suggested to be involved in 2-AG inactivation in both nervous and immune cells and tissues; and even with this enzymatic activity, there appears to be some heterogeneity (Goparaju et al. 1999; Di Marzo et al. 1999a; Saario et al. 2004). A MAGL enzyme was cloned first from the human and mouse (Karlsson et al. 1997; Ho et al. 2002) and more recently from the rat (Dinh et al. 2002a). Strong evidence for its role in 2-AG degradation, at least in isolated cells, was provided through the use of the siRNAs, which showed how "silencing" of MAGL results in the impairment of 2-AG degradation and in the enhancement of 2-AG, but not AEA, cellular levels (Dinh et al. 2004). However, other monoacylglycerol lipases are likely to exist. In fact, MAGL was found to account for only 50% of the total 2-AG-hydrolysing activity in soluble fractions of rat brain (Dinh et al. 2004); and the membrane-bound and soluble 2-AG hydrolase(s) previously found in rat circulating macrophages and platelets were shown to be sensitive to down-regulation by lipopolysaccharide in different ways (Di Marzo et al. 1999a). This heterogeneity may also explain why, although the cloned MAGL does not recognize AEA as substrate, MAGL activities in rat macrophage membranes and rat cerebellum cytosolic fractions are sensitive to inhibition by high micromolar concentrations of AEA (Di Marzo et al. 1999a; Ghafouri et al. 2004).

Like FAAH and other "endocannabinoid enzymes", the cloned MAGL, whose other general features have been reviewed recently (Dinh et al. 2002b), is also characterized by

poor substrate selectivity: It recognizes as substrates both *sn*-1- and 2-acylglycerols with almost any long-chain fatty unsaturated acid esterified to the glycerol backbone (see also Di Marzo et al. 1999b). Interestingly, this enzyme appears to be distributed in the CNS in the same brain regions as CB_1 receptors and, unlike FAAH, is mostly a pre-synaptic enzyme (Gulyas et al. 2004; Dinh et al. 2002a). Therefore, from studies on the cellular localization of DAGLα and β, it can be extrapolated that MAGL is localized complementarily to these major 2-AG biosynthesizing enzymes in the adult brain (Bisogno et al. 2003), in agreement with the proposed role of 2-AG in CB_1 receptor-mediated retrograde signalling (Chevaleyre and Castillo 2003; Melis et al. 2004a). In fact, 2-AG can be produced in a Ca^{2+}-sensitive way from the post-synaptic neuron, and diffuse towards the pre-synaptic terminal where it: (1) activates CB_1 receptors, leading to inhibition of neurotransmitter release, and (2) is subsequently inactivated through enzymatic hydrolysis. However, our current knowledge of the role of the cloned MAGL in the control of endocannabinoid "tone" is still limited due to the lack of a "knockout" mouse for this enzyme and of selective inhibitors. A recent article reported the development of a series trifluoromethyl ketone and methyl ketone thioether derivatives as possible specific inhibitors of 2-AG hydrolysis (Nithipatikom et al. 2005). Although the authors showed that some of these compounds effectively inhibit 2-AG hydrolysis by cytosolic fractions of prostate carcinoma (PC)-3 cells, and significantly enhance 2-AG levels in these cells, no direct data on the selectivity of these compounds vs FAAH or the DAGLs were reported. In another study, the *N*-ethyl-maleimide derivative of arachidic acid (*N*-arachidonylmaleimide) was reported to potently inhibit 2-AG hydrolysis (IC_{50}=180 nM) (Saario et al. 2005), but again with no data on the possible selectivity of this compound.

While the role of MAGLs in controlling the rate of 2-AG degradation in vivo is becoming widely recognized, a similar function for FAAH is still controversial. In some studies, FAAH genetic inactivation and/or pharmacological inhibition does not result in the elevation of brain 2-AG concentrations, thus prompting that FAAH may not be important in the control of 2-AG levels (Cravatt et al. 2001; Kathuria et al. 2003; Fegley et al. 2005; Lichtman et al. 2002). However, bearing in mind the high redundancy of pathways and enzymes through which 2-AG is metabolized in vitro, this conclusion is probably not entirely warranted. In fact, one alternative pathway or enzyme might compensate for the loss of FAAH and explain why 2-AG levels are not changed following inactivation of this enzyme. It would require the absence or the genetic or pharmacological inactivation of both FAAH and MAGLs—at least—to observe an elevation of 2-AG levels in an animal. Indeed, previous experiments showing that 2-AG levels are elevated following inactivation of MAGL, as opposed to FAAH, were carried out using experimental conditions where little or no detectable FAAH activity is present, i.e. in: (1) homogenates of rat cerebellum (Saario et al. 2004), (2) HeLa cells (Dinh et al. 2004), or (3) mouse brain cytosolic fractions (Dinh et al. 2004). Instead, a parsimonious interpretation of the available data is that MAGL is not the sole hydrolytic enzyme for 2-AG. In fact, data are now emerging according to which pharmacological blockade of FAAH does produce a significant elevation of 2-AG as well as AEA tissue levels, for example when: (1) a metabolically stable, non-competitive FAAH inhibitor such as *N*-arachidonoylserotonin (Bisogno et al. 1998) is administered systemically and sub-chronically (de Lago et al. 2005); or (2) FAAH inhibitors are administered locally and chronically (Bifulco et al. 2004; Bisogno et al. 2005b); (3) 2-AG levels are measured in peripheral organs (Capasso et al. 2005). Therefore, it is likely that both FAAH and MAGLs might play an important function in the enzymatic hydrolysis of 2-AG. The subsequent formation of arachidonic acid may not only represent the inactivation of an endocannabinoid

signal, but also the formation of arachidonic acid derivatives with altogether different biological functions (Jarai et al. 2000; Kojima et al. 2002; Gauthier et al. 2005).

Finally, to underscore further redundancy as a hallmark of 2-AG inactivation pathways, strong evidence also exists for the direct esterification of this compound into neutral glycerolipids and phospholipids (Di Marzo et al. 1999a). The relevance of these pathways, which were already known when 2-AG was considered a mere intermediate in glycerol(phospho)lipid metabolism, to the regulation of the cannabinergic signal still need to be assessed.

Endocannabinoid oxidation and its possible role

Due to the presence of an arachidonate moiety in their chemical structure, endocannabinoids are in principle easily oxidized through the action of the same enzymes that catalyse the oxidation of arachidonic acid. Indeed, both anandamide and 2-AG are substrates for some enzymes of the arachidonate cascade, in particular the 12- and 15-lipoxygenases, cytochrome p450 oxygenases and COX-2 (Fig. 3). These reactions have been found to occur in intact cell systems only in a few cases, and never in vivo, and their biological meaning is still a matter for speculation (Kozak and Marnett 2002). While lipoxygenase products (Fig. 3) are usually still capable of binding to cannabinoid receptors to some extent and/or to inhibit FAAH (van der Stelt et al. 2002), the prostanoid derivatives of both AEA and 2-AG, obtained from the further metabolism of COX-2-derived, but not COX-1-derived, endoperoxide products of the two compounds, are inactive on all cannabinoid and prostanoid receptors and, in some cases, appear to act instead at novel and yet-to-be-characterized receptors (Ross et al. 2002; Nirodi et al. 2004; Matias et al. 2004). Among these compounds, it is worthwhile mentioning prostaglandin $F_{2\alpha}$-ethanolamide, which can be obtained from prostaglandin $F_{2\alpha}$ through the action of COX-2 and prostaglandin F synthase (Koda et al. 2004), and prostaglandin E_2-glycerol ester, which is formed from COX-2 and prostaglandin E synthase (Kozak et al. 2002a). Interestingly, these prostaglandin-ethanolamides (or prostamides) and prostaglandin-glycerol esters are not subject to FAAH or MAGL-catalysed hydrolysis (Matias et al. 2004; V. Di Marzo and L.J. Marnett, unpublished observations). To date, the evidence in favour of the existence of these compounds in vivo is limited—for prostamides—to data obtained by injecting AEA in FAAH-deficient mice (Weber et al. 2004), or by treating primary amnion tissue explants with endocannabinoids (Glass et al. 2005). We have obtained preliminary mass-spectrometric evidence for the presence of a prostamide $F_{2\alpha}$-like compound in several murine tissues from untreated animals, with the highest concentrations in the eye and lungs (V. Di Marzo, I. Matias, J. Chen and D. Woodward, unpublished data).

While the structural bases of COX-2 interaction with AEA have been thoroughly examined (Kozak et al. 2003), indirect evidence for the participation of this enzyme in the inactivation of the endocannabinoid signal in the hippocampus has been reported. In fact, in this brain area, inhibitors of COX-2, but not FAAH, potentiate both short-term and long-term endocannabinoid-mediated synaptic plasticity (Kim and Alger 2004; Slanina and Schweitzer 2005).

Regarding AEA metabolites obtained from lipoxygenases, it has been suggested, so far only on the basis only of pharmacological evidence, that they might be capable of stimulating vanilloid TRPV1 (Craib et al. 2001). Indeed, a recent study demonstrated the possible action of the leukotriene B_4 derivative of AEA at these receptors (McHugh et al. 2005). Re-

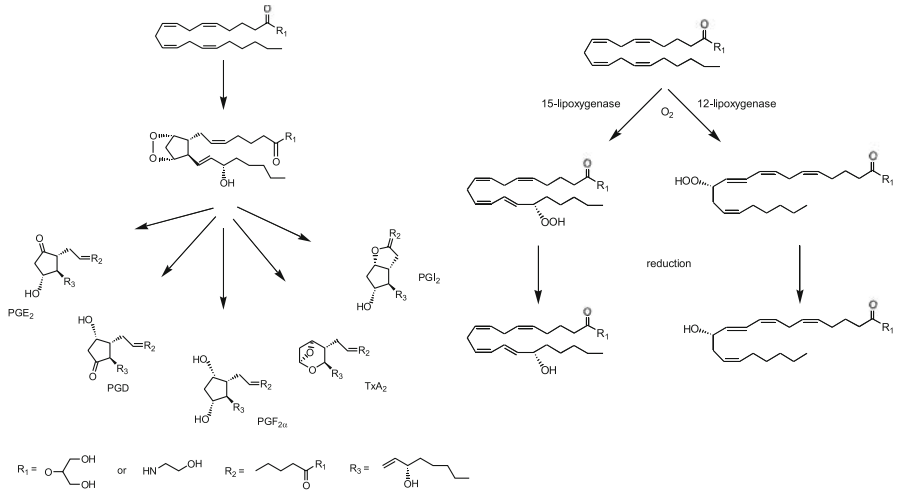

Fig. 3 Enzymatic oxidation of endocannabinoids. The oxidation of anandamide and 2-AG has been suggested to occur through the catalytic action of either cyclooxygenase-2 or 12- and 15-lipoxygenases. The oxidation products of the former, but not latter, reaction are totally inactive on cannabinoid receptors, but may bind to yet-to-be-identified receptors

cent evidence suggested that also the 15-lipoxygenase derivative of 2-AG (Fig. 3), but not 2-AG itself, interacts with peroxisome proliferator-activated receptor (PPAR)-α (Kozak et al. 2002b).

Regulation of endocannabinoid levels: what is the target, biosynthesis or degradation?

The endocannabinoids represent a typical example of mediators whose levels are controlled through the regulation not only of biosynthetic but also of catabolic enzymes (Table 1). Furthermore, these compounds are produced from biosynthetic precursors that in turn derive from phospholipids whose levels are potentially influenced by the diet and by its relative contents in ω3 and ω6 polyunsaturated fatty acids, in particular arachidonic and docosahexaenoic acids (AA and DHA). Indeed, diets defective or rich in DHA will increase or decrease, respectively, the brain levels of 2-AG (Watanabe et al. 2003), whereas milk deprived of both AA and DHA will lead to lower brain levels of AEA in newborns (Berger et al. 2001).

There are several examples of regulation of AEA levels via opposite regulation of FAAH activity and, in particular, expression (Maccarrone et al. 2000b, 2003a, b) under both physiological and pathological conditions. Also the MAGL is subject to regulation, resulting in corresponding opposing changes in 2-AG levels (Di Marzo et al. 1999a; Witting et al. 2004; Maccarrone et al. 2005), although the promoter region of the *Magl* gene has not been identified yet. In some cases, endocannabinoid levels can be regulated by targeting in opposite ways both biosynthetic and degradative enzymes, as in the case of 2-AG in astrocytes following P2X7 receptor stimulation (Witting et al. 2004), and of AEA in mouse uterus during embryo uterine implantation (Maccarrone et al. 2000b; Guo et al. 2005). There are also cases in which the expression and/or activity of both biosynthetic and the degradative enzyme are up-regulated, and these changes lead nevertheless to the up-regulation of endocannabinoid levels, as is the case with rat brain 2-AG when passing from the light to the dark phase of the

Table 1 Possible mechanisms underlying the regulation of endocannabinoid levels. The regulatory events that lead to elevation or decrease of endocannabinoid levels are shown in normal type or in italics, respectively. Examples are mostly limited to physiological conditions, and many more examples of each type of regulation have been found during pathological conditions

	Anandamide	2-AG	Reference(s)
Dietary availability of PUFA precursors	Increased brain levels in piglet and mouse pup brain following increased AA and DHA in milk	Increased and *decreased* brain levels in mice following decreased or increased DHA in diet, respectively	Berger et al. 2001; Watanabe et al. 2003
Regulation of biosynthesis (Mostly exerted by regulating NAPE-PLD and DAGL expression/activity for anandamide and 2-AG, respectively)	In mouse uterus during embryo implantation	In microglial cells after P2X7 receptor stimulation; *In rat brain when passing from light to dark phase of the day*; *In rat hypothalamus following systemic administration of leptin (upstream of DAGL)*; *In the mouse uterus (by leptin)*	Guo et al. 2005; Witting et al. 2004; Valenti etal. 2004; Di Marzo et al. 2001; Maccarrone et al. 2005
Regulation of catabolism (Exerted by regulating FAAH and MAGL expression/activity for anandamide and 2-AG, respectively)	*In the mouse uterus (by leptin)*; *In lymphocytes stimulated with leptin or progesterone*; *In mouse uterus during embryo implantation (by progesterone)*	In macrophages stimulated with LPS; In microglial cells after P2X7 receptor stimulation	Maccarrone et al. 2000b, 2003a, 2005; Di Marzo et al. 1999a; Witting et al. 2004
Regulation of re-uptake (Via the putative membrane transporter)	*In the mouse uterus (by leptin)*	No direct evidence	Maccarrone et al. 2005

AA arachidonic acid, 2-AG 2-arachidonoyl glycerol, DHA docosahexaenoic acid, FAAH fatty acid amide hydrolase, DAGL sn-1-selective diacylglycerol lipase, MAGL monoacylglycerol lipase, NAPE-PLD N-acylphosphatidylethanolamine-selective phospholipase D

day (Valenti et al. 2004) or following induction of neuronal damage with a β-amyloid peptide (Di Marzo et al. 2005). These concurrent changes of the anabolic and catabolic enzymes probably occur when the stimulation of endocannabinoid levels needs to be transient, since they result in an overall increase of endocannabinoid turnover. Finally, there are examples of the same stimulus leading to the same change in the tissue concentrations of AEA and

2-AG, but through different regulatory strategies. This is the case of leptin, which causes down-regulation of AEA levels in the blood and uterus via up-regulation of FAAH expression, whereas it provokes a decrease of 2-AG levels by enhancing its degradation in the uterus and by inhibiting its biosynthesis in both the hypothalamus and the uterus (Di Marzo et al. 2001; Maccarrone et al. 2005). Finally, the possibility that COX-2 over-expression during certain pathological states, such as inflammation and colon carcinoma, may contribute to regulating endocannabinoid levels has not been investigated yet.

Although the endocannabinoid membrane transporter has not been cloned to date, this putative protein too has been shown to be subject to regulation. In particular, nitric oxide stimulates AEA cellular uptake in many cell types, whereas chronic treatment of cells with ethanol inhibits it (Maccarrone et al. 2000a; Basavarajappa et al. 2003). In either case, no effect on FAAH activity was found, whereas leptin inhibits both AEA reuptake by, and FAAH activity in, mouse uterine tissue (Maccarrone et al. 2005).

In conclusion, several possibilities exist for the regulation of endocannabinoid levels, including the one described in the second section of this article and utilizing the concomitant formation of cannabinoid receptor-inactive endocannabinoid congeners capable of acting as competitive substrates for biosynthetic or hydrolytic enzymes. Such a variety of mechanisms indicates that the concentrations of AEA and 2-AG near their molecular targets, and hence the activity of the cannabinoid receptors, are tightly regulated and, therefore, that this signalling system must play a fundamental role under various conditions.

Conclusions: new therapeutic drugs from studies of endocannabinoid synthesis and degradation

The recent development of Rimonabant (Sanofi-Aventis, Paris, France), a selective cannabinoid CB_1 receptor antagonist/inverse agonist, as an efficacious anti-obesity agent with possible use also against the metabolic syndrome (Van Gaal et al. 2005), and the future marketing in Canada of Sativex (GW Pharmaceuticals, Salisbury, UK), a pharmaceutical preparation—based on a *Cannabis* extract—against neuropathic pain in multiple sclerosis, might one day be followed by the use of therapeutic drugs that also manipulate the levels rather than the action of endocannabinoids, thereby influencing the tone of cannabinoid receptors indirectly. For example, given the strong weight of endogenous 2-AG in the activation of cannabinoid receptors, inhibitors of the DAGL might produce a blockade of endocannabinoid signalling similar to that caused by receptor antagonists, and hence, like the latter compounds, find an application in those disorders where, at least in animal models, excessive endocannabinoid signalling appears to contribute to the progress or the symptoms of the disease, i.e Parkinson's disease and levodopa-induced dyskinesia (Di Marzo et al. 2000; van der Stelt et al. 2005; Fernandez-Espejo et al. 2005), Alzheimer's disease (Mazzola et al. 2003; Di Marzo et al. 2005), obesity and metabolic syndrome (Di Marzo and Matias 2005), penile erection (Melis et al. 2004b), nicotine, cocaine and alcohol dependence (Cohen et al. 2002; Soria et al. 2005), and relapse of cocaine and heroin abuse (De Vries et al. 2001; Fattore et al. 2003). On the other hand, inhibitors of endocannabinoid re-uptake and enzymatic hydrolysis might represent an alternative to the use of direct CB_1 and CB_2 receptor agonists, which are more likely to exert undesired psychotropic and immune-suppressive effects, respectively, and to undergo tolerance. Examples of disorders that could be treated with such inhibitors include, to date, both central (experimental allergic encephalomyelitis, kainate-induced excitotoxicity, etc.) and peripheral (cholera-toxin-induced intestinal hypersecretion, cancer, hyperten-

sion, etc.) disorders (Bifulco et al. 2004; Baker et al. 2001; Marsicano et al. 2003; Izzo et al. 2003; Batkai et al. 2004; Mestre et al. 2005). But in principle, in all the diseases—or in all the distinct phases of a certain disease—where endocannabinoids are produced "on demand" to exert a protective function irrespective of their molecular targets, inhibitors of their inactivation should produce therapeutic effects.

Finally, the exact knowledge of the molecular targets of some endocannabinoid metabolic products, such as prostamides and prostaglandin glycerol esters, might lead to new therapeutic agents. Indeed, the 17-phenyl-derivative of prostamide $F_{2\alpha}$, bimatoprost (Lumigan, Allergan, Irvine, CA), is already marketed as an anti-glaucoma agent. This compound exhibits very low affinity for and potency at the FP receptor, although it exerts a powerful contraction of the isolated feline iris sphincter and potently reduces the intraocular pressure in ocular normotensive dogs (Woodward et al. 2001). Indeed, prostamide $F_{2\alpha}$, and hence possibly bimatoprost, may act on an as-yet-uncharacterized novel receptor, since they both exert a strong contraction of feline lung parenchyma at low nanomolar concentrations, and have an entirely different pharmacological profile from prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in all the other available assays for FP receptor-mediated activity, while exerting no activity in a wide range of binding assays for other known prostanoid receptors (Woodward et al. 2001, 2003). The very recent report of a selective antagonist of prostamide, but not $PGF_{2\alpha}$, contractile action on cat iris strips strongly suggests the existence of a specific receptor for these putative COX-2 metabolites of anandamide and their synthetic analogs (Woodward et al. 2005). Based on this experience, it can be foreseen that the full understanding of endocannabinoid synthesis and degradation will lead to more therapeutic drugs for a variety of disorders that still await an efficacious and safe treatment.

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