Biosynthesis and Fate of Endocannabinoids

Maria Grazia Cascio and Pietro Marini

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Abstract

Since the discovery of the two cannabinoid receptors, CB_1 and CB_2, several molecules, commonly defined as endocannabinoids, able to bind to and functionally activate these receptors, have been discovered and characterized. Although the general thought was that the endocannabinoids were mainly derivatives of the n-6 fatty acid arachidonic acid, recent data have shown that also derivatives (ethanolamides) of n-3 fatty acids may be classified as endocannabinoids. Whether the n-3 endocannabinoids follow the same biosynthetic and metabolic routes of the n-6 endocannabinoids is not yet clear and so warrants further investigation. In this review, we describe the primary biosynthetic and metabolic pathways for the two well-established endocannabinoids, anandamide and 2-arachidonoylglycerol.

Keywords

2-arachidonoylglycerol • Anandamide • Biosynthesis • Degradation • Endocannabinoids • Uptake

M.G. Cascio (✉) • P. Marini
School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK
e-mail: m.cascio@abdn.ac.uk

R.G. Pertwee (ed.), *Endocannabinoids*, Handbook of Experimental Pharmacology 231, DOI 10.1007/978-3-319-20825-1_2

Abbreviations

2-AG	2-Arachidonoylglycerol
2-AGE	2-Arachidonoylglyceryl ether
AA	Arachidonic acid
Abh4	Alpha/beta hydrolase 4
ABHD	Alpha/beta hydrolase domain
AEA	Anandamide
Asp	Aspartic acid
CB	Cannabinoid
Cis	Cysteine
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
DHA	Docosahexaenoic acid
DHEA	Docosahexaenoyl-ethanolamide
DTT	Dithiothreitol
EMT	Endocannabinoid membrane transporter
EPA	Eicosapentaenoic acid
EPEA	Eicosapentaenoyl-ethanolamide
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
FLAT	FAAH-like anandamide transporter
GpAEA	Glycerophospho-arachidonoylethanolamide
GPR	G-protein coupled receptor
GSH	Glutathione
HEK	Human embryonic kidney
His	Histidine
Hsp	Heat shock protein
LOX	Lipoxygenase
Lys	Lysine
MAFP	Methylarachidonoylfluorophosphonate
MGL	Monoacylglycerol lipase
NAAA	*N*-acylethanolamine-selective acid amidase
NADA	*N*-arachidonoyldopamine
NAE	*N*-acyl-ethanolamine
NAGly	*N*-arachidonoylglycine
NAM	*N*-arachidonoylmaleimide
NAPE	*N*-acylphosphatidylethanolamine
NArS	*N*-arachidonoylserine
NAT	*N*-acyltransferase
OEA	Oleoyethanolamide
OLDA	*N*-oleoyl dopamine

pAEA	Phosphoanandamide
PE	Phosphatidylethanolamine
PEA	Palmitoylethanolamide
PHARC	Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, cataract
PL	Phospholipase
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
SEA	Stearoylethanolamide
Ser	Serine
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid

1 Endocannabinoids and Endocannabinoid-Like Compounds

The discovery, in 1988, of a high-affinity, stereoselective and pharmacologically distinct cannabinoid receptor in rat brain tissue (Devane et al. 1988), led to a continuous search for natural endogenous ligands. Since then, several molecules have been identified and collectively named as "endocannabinoids". Endocannabinoids are defined as derivatives (amides, esters and ethers) of a long-chain polyunsaturated fatty acid (PUFA), mainly arachidonic acid (AA), capable of binding and functionally activating the cannabinoid receptors (Di Marzo et al. 2004). Although anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids (an *N*-acylethanolamine and a monoacylglycerol, respectively), other endogenous compounds that may also bind to the cannabinoid receptors have been discovered and suggested to be endocannabinoids: *N*-dihomo-γ-linolenoyl ethanolamine and *N*-oleoyl dopamine (OLDA) (Pertwee 2005), 2-arachidonoylglycerol ether (noladin ether, 2-AGE) (Hanus et al. 2001), *O*-arachidonoylethanolamine (virodhamine) (Porter et al. 2002), and *N*-arachidonoyldopamine (NADA) (Huang et al. 2002). It is now well established that the two most studied endocannabinoids (AEA and 2-AG) do not interact only with cannabinoid CB_1 and CB_2 receptors, and exhibit instead a degree of promiscuity that applies also to the less-studied arachidonic acid-derived endocannabinoids (Zygmunt et al. 1999; Hanus et al. 2001; Huang et al. 2002; Porter et al. 2002; Rozenfeld and Devi 2008; den Boon et al. 2012). Anandamide (AEA), from the Sanskrit word *ananda*, which means *bliss*, was the first endogenous cannabinoid identified (Devane et al. 1992; Hanus 2007). This is the ethanolamide of arachidonic acid and behaves as a partial agonist at both cannabinoid CB_1 and CB_2 receptors (Pertwee et al. 2010). Interestingly, it is now well established that AEA possesses an ability to interact also with other receptors, such as the transient receptor potential vanilloid 1 (TRPV1) (Zygmunt et al. 1999) and the peroxisome proliferator-activated receptor (PPAR) family (O'Sullivan 2007). Indeed, some of the effects of AEA are non-CB_1/non-CB_2 receptor mediated (Breivogel et al. 2001;

Monory et al. 2002). 2-Arachidonoylglycerol (2-AG) is the arachidonate ester of glycerol that was isolated from peripheral tissues. This molecule can activate both CB_1 and CB_2 receptors with similar potency and efficacy (Mechoulam et al. 1995; Sugiura et al. 1995) as well as γ-aminobutyric acid receptors (Sigel et al. 2011). 2-Arachidonoy-glyceryl ether (noladin ether) binds to CB_1 receptors, and very weakly to CB_2 receptors, and also affects AEA uptake (Fezza et al. 2002; Páldyová et al. 2008). Recently, its classification as an endocannabinoid has been questioned because of its very low concentration in the brain (Oka et al. 2003). Virodhamine (from the Sanskrit word *virodha*, which means *opposite*) is the ester of arachidonic acid, and it has been reported to behave as a full agonist at CB_2 receptors and as both a partial agonist/antagonist at CB_1 receptors and a weak inhibitor of AEA uptake (Porter et al. 2002). This molecule can also interact with PPAR-α receptors (Sun et al. 2006) and GPR55 receptors (Sharir et al. 2012). *N*-arachidonoyl-dopamine (NADA), like AEA, behaves both as an endovanilloid and an endocannabinoid (Bisogno et al. 2000; Huang et al. 2002). It also interacts with PPAR-γ receptors (O'Sullivan 2007) and can antagonize the melastatin type-8 (TRPM8) cation channel (De Petrocellis et al. 2007).

We recently discovered that in addition to n-6 long-chain PUFA endocannabinoids, the ethanolamides of two n-3 fatty acids derived mainly from fish oils in the human diet, DHA (C22:6) and EPA (C20:5), should also be classified as endocannabinoids (Brown et al. 2010; Cascio 2013). These n-3 fatty acid ethanolamides are docosahexaenoyl-ethanolamide (DHEA) and eicosapentaenoyl-ethanolamide (EPEA), both of which bind to and partially activate CB_1 and CB_2 receptors and are produced both in vivo and in vitro after administering fish oil or individual n-3 long-chain PUFA (Sugiura et al. 1996; Bisogno et al. 1999; Berger et al. 2001; Brown et al. 2010, 2011; Maccarrone et al. 2010; Cascio 2013). These n-3 endocannabinoids show anti-inflammatory properties in macrophages and adipocytes (Fezza et al. 2014) and can inhibit cell growth in breast cancer by triggering autophagy via PPAR-γ receptors (Fezza et al. 2014). Interestingly, we recently reported evidence that both DHEA and EPEA possess cannabinoid receptor-dependent and -independent anti-proliferative effects in androgen receptor-positive and -negative prostate cancer cell lines (Brown et al. 2010).

Finally, endocannabinoids are produced together with cannabinoid receptor-inactive saturated and mono- or di-unsaturated compounds that are defined as endocannabinoid-like compounds. These compounds have been reported to exert their cannabimimetic effects by acting as "entourage molecules" that prevent endocannabinoids being degraded by specific metabolic enzymes (Cascio 2013). Palmitoylethanolamide (PEA) possesses both anti-inflammatory and analgesic activity, likely mediated by the TRPV1 and PPAR-α receptors (Costa et al. 2008; Ho et al. 2008; Di Cesare et al. 2013; Esposito et al. 2014), and it also interacts with GPR55 receptors (Moriconi et al. 2010). Stearoylethanolamide (SEA) produces anti-inflammatory, immunomodulatory as well as anorexic effects (Maccarrone et al. 2002; Dalle Carbonare et al. 2008; Ghafouri et al. 2013). Oleoyethanolamide (OEA) can activate both GPR119 and GPR55 receptors (Overton et al. 2008; Moriconi et al. 2010), and it regulates food intake in rodents by a mechanism that

involves the activation of PPAR-α receptors (Rodríguez de Fonseca 2004). Oleamide is an unsaturated fatty acid amide isolated from the cerebrospinal fluid of sleep-deprived cats (Cravatt et al. 1995) which behaves as a full cannabinoid CB_1 receptor agonist (Leggett et al. 2004). Other compounds that have been recently classified as endocannabinoids-like compounds are *N*-arachidonoylglycine (NAGly) and *N*-arachidonoylserine (NArS). NAGly interacts with both GPR18 and GPR92 receptors (Kohno et al. 2006; Oh et al. 2008; Fezza et al. 2014; McHugh et al. 2014) and behaves as a FAAH inhibitor (Cascio et al. 2004). The chemical structures of the endocannabinoids can be found in this volume in Pertwee "Endocannabinoids and Their Pharmacological Actions".

2 Biosynthesis of the Endocannabinoids

It is generally accepted that endocannabinoids are not stored in cells awaiting release, but are rather synthesized on demand in a Ca^{2+}-dependent manner in response to physiological and pathological stimuli (Di Marzo and Deutsch 1998). However, recent data suggest that AEA can also be stored inside the cell (Oddi et al. 2008). AEA as well as other *N*-acyl-ethanolamines were initially considered as terminal products of *post mortem* tissue degradation, and their physiological role remained controversial until the identification of their biosynthetic and metabolic pathways (Piomelli 2014).

The principal pathway of AEA biosynthesis includes a first step, catalysed by a calcium-dependent *N*-acyltransferase (NAT), in which an acyl chain is transferred from the *sn*-1 position of a glycerophospholipid to the amino group of the hydroxyethyl moiety of phosphatidylethanolamine (PE), and a second step in which the generated *N*-acylphosphatidylethanolamine (NAPE) is hydrolysed to NAE and phosphatidic acid, through a reaction catalysed by a phosphodiesterase of the phospholipase D-type (NAPE-PLD) (Fig. 1). NAPE-PLD, that is chemically and enzymatically distinct from other known PLDs, is a member of the β-lactamase family of zinc-metal hydrolases, is highly conserved in mouse, rat and human, is stimulated by calcium, is highly expressed in the brain as well as in kidney, spleen, lung, heart and liver and is involved in the formation of other, cannabinoid-receptor inactive, *N*-acyl-ethanolamines (C16:0, C18:0 and C18:1) (Petersen and Hansen 1999; Ueda et al. 2001a; Liu et al. 2002; Okamoto et al. 2004). Interestingly, studies performed using NAPE-PLD knockout mice suggested that while an increase in endogenous levels of NAPEs with saturated and monounsaturated *N*-acyl chains was observed, few or no changes were observed in the levels of polyunsaturated NAPEs and NAEs, thus suggesting the existence of alternative AEA biosynthetic pathways (Brown et al. 2013; Cascio 2013; Fonseca et al. 2013). There is evidence too that: (1) AEA is formed from *N*-acyl-lysophosphatidylethanolamine by a lysophospholipase-D-enzyme (lyso-PLD) (Sun et al. 2004) (Fig. 1); (2) AEA is also formed in a pathway in which a crucial role is played by an additional enzyme, α/β-hydrolase 4 (Abh4), which can act on either NAPE or lyso-NAPE to generate glycerophospho-arachidonoylethanolamide (GpAEA), that is subsequently

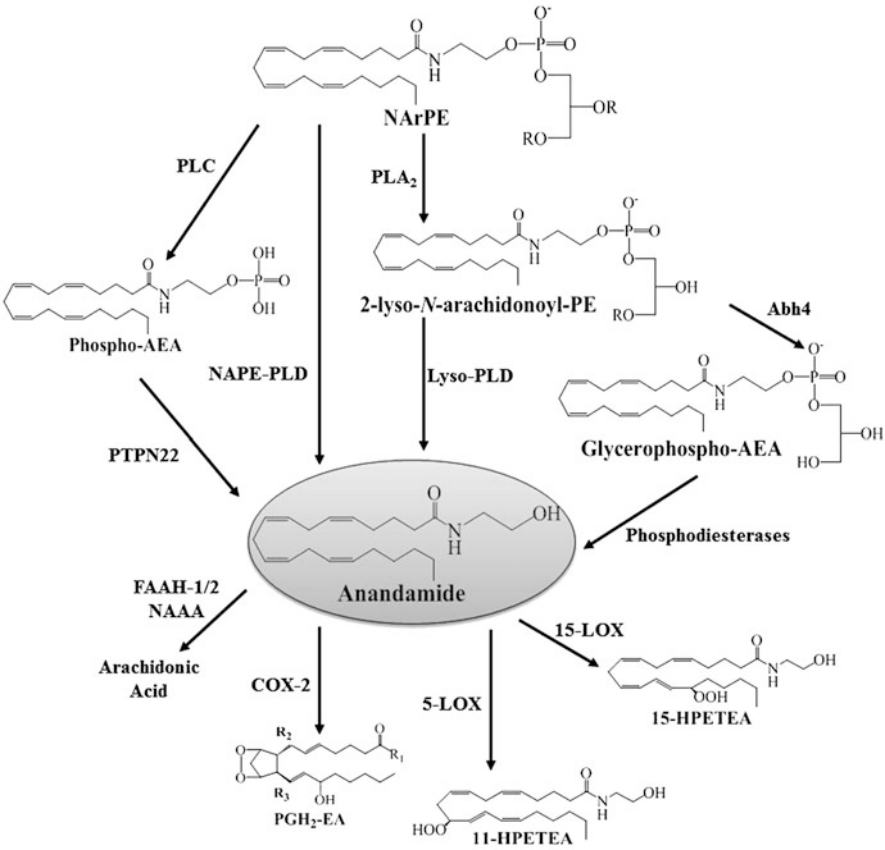

Fig. 1 Schematic representation of anandamide biosynthesis and degradation. *NArPE* *N*-arachidonoylphosphatidyl-ethanolamine, *PLC* phospholipase C, *PTPN22* protein tyrosine phosphatase, *PLA*$_2$ phospholipase A$_2$, *PE* phosphatidyl-ethanolamine, *PLD* phospholipase D, *Abh4* α/β-hydrolase 4, *PG* prostaglandin, *HPETEA* hydroxyperoxyeicosatetraenoylethanolamide, *LOX* lypoxygenase, *COX* cyclooxygenase, *FAAH* fatty acid amide hydrolase, *NAAA* *N*-acylethanolamine-hydrolysing acid amidase, *R*^{1} ethanolamine

converted to AEA in the presence of a phosphodiesterase (Simon and Cravatt 2006) (Fig. 1) and finally (3) that NAPE can also be hydrolysed, by phospholipase C, to phosphoanandamide (pAEA) which, in turn, is dephosphorylated by phosphatases to AEA (Liu et al. 2006) (Fig. 1). Interestingly, an alternative biosynthetic pathway for AEA might also exist that involves direct condensation of free arachidonic acid and ethanolamine, catalysed by an AEA synthase. However, this pathway requires high "non-physiological" concentrations of both substrates (Sugiura et al. 1996; Ueda et al. 1996).

For 2-AG, the most accepted biosynthetic pathway is the hydrolysis of membrane phospholipids that is catalysed by phospholipase C (PLC) and that produces 1,2-diacylglycerol (DAG), which in turn is converted to 2-AG by diacylglycerol

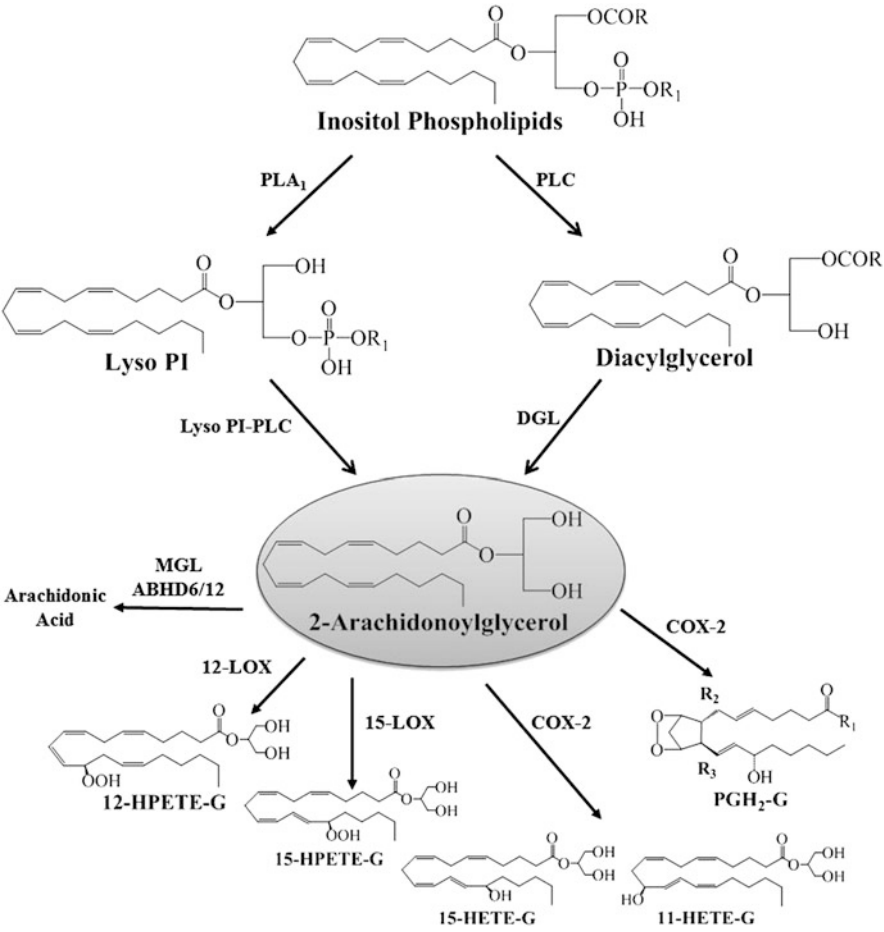

Fig. 2 Main pathways for 2-arachidonoylglycerol biosynthesis and degradation. *PLC* phospholipase C, *PLA1* phospholipase A_1, *PI* phospatidyl-inositol, *DGL* diacylglycerol lipase, *HETE-G* hydroxyeicosatetraenoyl-glycerol, *HPETE-G* hydroxyperoxyeicosatetraenoyl-glycerol, *LOX* lypoxygenase, *COX* cyclooxygenase, *MGL* monoacylglycerol lipase, *ABHD* α/β-hydrolase domain, *R1* glycerol

lipase (DGL) (Bisogno et al. 2003) (Fig. 2). DGL exists in two closely related forms designated α and β, that are both active at pH 7, both stimulated by calcium and glutathione (GSH) and both inhibited by inhibitors of Ser/Cis-hydrolases, such as *p*-hydroxy-benzoate-mercuric and $HgCl_2$ but not by phenylmethylsulphonyl fluoride (Bisogno et al. 2003). Both enzymes are also inhibited by RHC80267, which is able to block the formation of 2-AG by intact cells (Bisogno et al. 2003). Pharmacological studies have revealed that during neuronal development, localization of DGLα and DGLβ changes from pre- to post-synaptic elements, i.e. from axonal tracts in the embryo to dendritic fields in the adult, suggesting a different need for 2-AG

synthesis from the pre- to the post-synaptic compartment during brain development (Bisogno et al. 2003; Williams et al. 2003). Furthermore, there is evidence too that DGLα plays an essential role in the regulation of retrograde synaptic plasticity and neurogenesis (Gao et al. 2010; Tanimura et al. 2010; Savinainen et al. 2012). Like AEA, 2-AG can also be synthesized via other pathways. However, the physiological importance of these proposed pathways is not yet clear.

3 Uptake of the Endocannabinoids: Proposed Mechanisms

Once released into the extracellular space, endocannabinoids exert the majority of their effects by acting, as retrograde messengers, at CB_1 cannabinoid receptors present on the surface of presynaptic nerve terminals (Piomelli 2014). So far, it is not clear how the endocannabinoids access their metabolic enzymes. Indeed, while monoacylglycerol lipase (MGL, the main metabolic enzyme of 2-AG) is localized pre-synaptically, fatty acid amide hydrolase (FAAH, the main metabolic enzyme of AEA) is localized post-synaptically, thus at a certain distance from the site of action of AEA (Piomelli 2014). To explain the mechanism(s) by which AEA would be taken up by cells, several interesting hypotheses have been proposed (Fowler 2012, 2013). Briefly, AEA is a lipophilic molecule and as such it could easily diffuse through the cell membrane. However, a simple diffusion through the membrane would cease once the equilibrium in the AEA gradient between the extracellular and intracellular environment is reached, unless this equilibrium is prevented by intracellular metabolism induced by FAAH (Fowler 2012, 2013). However, although FAAH may, of course, influence the uptake of AEA (at least the speed with which this process takes place), the uptake is clearly distinct from FAAH. Indeed, (1) compounds able to selectively inhibit the cellular uptake of AEA, but not FAAH, have been identified (De Petrocellis et al. 2000; Di Marzo et al. 2001, 2002; López-Rodríguez et al. 2001; Ortar et al. 2003); (2) inhibitors of FAAH increase, and inhibitors of AEA uptake decrease, the accumulation of AEA within cells (Kathuria et al. 2003); (3) cells that do not express FAAH rapidly internalize AEA (Di Marzo et al. 1999; Deutsch et al. 2001); (4) NADA and noladin, although they are not FAAH substrates, are rapidly internalized by cells (Fezza et al. 2002; Huang et al. 2002) and (5) a saturable AEA accumulation was observed in synaptosomes and cells prepared from genetically modified mice that do not express FAAH (Fegley et al. 2004; Ligresti et al. 2004).

In addition, since endocannabinoid uptake is rapid, temperature-dependent, selective for anandamide over other acylethanolamides and saturable, the hypothesis that AEA uptake may occur through a facilitated transport mechanism has also been proposed (Di Marzo et al. 1994; Hillard and Jarrahian 2000; Fezza et al. 2008). Unfortunately, the protein responsible for this transport, better known as "Endocannabinoid Membrane Transporter" or EMT, has not yet been cloned and its existence is supported only by indirect evidence. More recent studies have shown the existence of carrier proteins that would facilitate diffusion of AEA through the plasma membranes. Examples are the fatty acid binding proteins FABP5 and FABP7, but not FABP3 (Kaczocha et al. 2009), the heat shock protein

70 (Hsp70) and albumin (Oddi et al. 2009), and the most recently identified FAAH-like anandamide transporter (FLAT). FLAT is a dimeric protein lacking the membrane-anchoring domain of the FAAH dimer, and its overexpression in HEK-293 cells increases AEA uptake (Fu et al. 2012). Other hypotheses have also been proposed (Oddi et al. 2008; Di Pasquale et al. 2009; Fowler 2013).

Several synthetic compounds that are able to inhibit the cellular uptake of AEA have been developed so far, some examples being AM404, VDM11, UCM707, OMDM1, OMDM2 and LY21832110 (Pertwee 2014). These compounds have been reported to possess, at least in animals, promising pharmacological properties for the treatment of cancer, pain, multiple sclerosis, Parkinson's disease, Huntington disease and anxiety (Pertwee 2014). Interestingly, AM404 has also been reported to be effective against nicotine-seeking behaviour and obsessive compulsive disorders (Pertwee 2014).

4 Degradation of the Endocannabinoids

Two main metabolic pathways have been identified so far: one hydrolytic and the other oxidative (Figs. 1 and 2). AEA is mainly hydrolyzed by FAAH (Cravatt et al. 1996; Giang and Cravatt 1997; Bracey et al. 2002), while 2-AG is mainly hydrolyzed by MGL (Dinh et al. 2002) and also by FAAH. In addition to these two enzymes, an *N*-acylethanolamine-selective acid amidase (NAAA) (Ueda et al. 1999) and, more recently, a second FAAH (FAAH-2) (Wei et al. 2006), as well as two other enzymes ABHD6 and ABHD12, (Blankman et al. 2007) have been reported to participate in the degradation of several endocannabinoids. Both AEA and 2-AG can also be degraded by enzymes of the arachidonate cascade, such as cyclooxygenase-2 (COX-2), lipoxygenases (LOXs) as well as cytochrome P450 enzymes, to produce the corresponding hydroxy- (in the case of lipoxygenases) and epoxy- (in the case of cytochrome P450 monooxidases) derivatives or to produce prostamides and prostaglandin glycerol esters (in the case of cyclooxygenases and prostaglandin synthases) (Piscitelli and Di Marzo 2012) (Figs. 1 and 2). While both hydroxy- and epoxy-endocannabinoids have been reported to act at both cannabinoid CB_1 and CB_2 receptors as well as at the vanilloid receptors, TRPV1 (hydroxy-endocannabinoids) and TRPV4 (epoxy-endocannabinoids), both prostamides and prostaglandin-glycerol esters are inactive at cannabinoid receptors. It has been suggested that they act at new, not yet identified, receptors (Piscitelli and Di Marzo 2012). Below, we report a brief description of the enzymes involved in the hydrolysis of endocannabinoids.

4.1 FAAH and NAAA

FAAH, which was first cloned by Cravatt et al. (1996), is an integral membrane protein widely distributed in various tissues of rat (Desarnaud et al. 1995; Cravatt et al. 1996; Katayama et al. 1997), mouse (Sun et al. 2005), and human (Giang and

Cravatt 1997). This enzyme, active at pH 8–10, is mainly localized on microsomal membranes and contains 597 amino acids, with a short "amidase" sequence enriched in glycine and serine residues. The isolation of FAAH was made possible by the previous development of potent transition state inhibitors, one of which was used to carry out affinity chromatography purification (Cravatt et al. 1996; Petrosino and Di Marzo 2010). A covalent inhibitor, instead, was used to facilitate the formation of crystals of a slightly modified, soluble form of FAAH and to obtain its structure by X-ray crystallography (Bracey et al. 2002; Petrosino and Di Marzo 2010). FAAH catalytic triad is composed of Ser-Ser-Lys, in which Ser241 plays a critical role as both acid and base in the hydrolytic cycle, whereas Lys142 is the activator of Ser241, and Ser217 participates in the catalytic mechanism of FAAH by facilitating the nucleophile attack and the exit of the leaving group (Petrosino and Di Marzo 2010). Importantly, it has been reported that the promoter region of the FAAH gene is up-regulated by progesterone and leptin and down-regulated by estrogens and glucocorticoids (Puffenbarger et al. 2001; Waleh et al. 2002; Maccarrone et al. 2003a, b). Ergetova and co-workers (Egertová et al. 1998) analysed the distribution of FAAH in rat brain and compared its cellular localization with CB_1-type cannabinoid receptors using immunocytochemistry. High concentrations of FAAH were detected in the cerebellum, hippocampus and neocortex, which are enriched with cannabinoid receptors. Immunocytochemical analysis of these brain regions revealed a complementary pattern of FAAH and CB_1 expression with CB_1 immunoreactivity occurring in fibres surrounding FAAH-immunoreactive cell bodies and/or dendrites (Egertová et al. 1998). In the cerebellum, FAAH was expressed in the cell bodies of Purkinje cells and CB_1 was expressed in the axons of granule cells and basket cells, neurons which are presynaptic to Purkinje cells (Egertová et al. 1998).

FAAH is also able to metabolize other fatty acid amides such as *N*-arachidonoyldopamine and a large number of mono-unsaturated and saturated compounds (Ueda 2002; Fegley et al. 2005; Ho and Hillard 2005; Lo Verme et al. 2005). Examples are PEA (De Petrocellis et al. 2001; Ueda et al. 2001b; Ueda 2002; Lo Verme et al. 2005), oleoylethanolamide, *N*-arachidonoylserine and *N*-arachidonoylglycine, the latter two of which have also been reported to be FAAH inhibitors (Sheskin et al. 1997; Bradshaw and Walker 2005; Ho and Hillard 2005). Recently, a second isoform of FAAH, FAAH-2, has been identified. It shows ~20 % sequence similarity with FAAH at the amino acid level and is expressed in several species, including human, primates, frog, chicken, pufferfish and zebrafish, but not in rodents (Wei et al. 2006). FAAH-1 and FAAH-2 are located on the cytosolic and luminal sides of intracellular membranes, respectively. Both FAAH enzymes have distinct tissue distribution. Indeed, FAAH-2 was detected in the heart and ovary, but not in the brain, small intestine or testis, which are known to express FAAH-1. However, FAAH-1 and FAAH-2 were both detected in the prostate, lung, kidney and liver (Wei et al. 2006). FAAH is also involved in the hydrolysis of 2-AG (Di Marzo and Deutsch 1998), although it has been observed that levels of 2-AG, unlike those of AEA, are not increased in FAAH-knockout mice (Lichtman et al. 2002). Interestingly, recent reports have shown that FAAH is involved in the production of

symptoms of a variety of disorders and that FAAH inhibitors may be effective at ameliorating acute, inflammatory, visceral and neuropathic pain as well as osteoarthritic pain and hyperalgesia induced by bladder inflammation (Pertwee 2014). Importantly, unlike direct CB_1 agonists, FAAH inhibitors produce antinociception in mice at doses that do not induce hypomotility, hypothermia, catalepsy and hyperphagia or signs of physical or psychological dependence (Pertwee 2014). A few examples of FAAH inhibitors are URB597, OL135, O-1887, URB532, AM374 (palmitylsulphonyl fluoride), *N*-arachidonoylglycine and *N*-arachidonoyl serotonin, JNJ1661010 and CAY10401, AM3506 and AM5206, ST4070, PF3845 and PF04457845 (Pertwee 2014).

One other enzyme involved in AEA hydrolysis is NAAA. This enzyme is a cysteine hydrolase belonging to the *N*-terminal nucleophile hydrolase superfamily, is present in cellular lysosomes or in the Golgi apparatus of cells, is active only at acidic pH and shows higher selectivity for PEA than for AEA (Brown et al. 2013; Ueda et al. 2013). Millimolar concentrations of dithiothreitol (DTT) as well as non-ionic detergents such as Triton X-100 and Nonidet P-40 are required to promote its full activity (Brown et al. 2013; Ueda et al. 2013). NAAA is highly expressed in a number of blood cell lines, as well as in macrophages in various rodent tissues. In humans, NAAA mRNA is expressed most abundantly in prostate followed by leukocytes, liver, spleen, kidney and pancreas (Ueda et al. 2010). Prostate cancer cell lines like PC3, LNCaP and DU-145 also express high levels of NAAA (Ueda et al. 2010). Interestingly, due to its selectivity towards PEA, selective NAAA inhibitors that can increase local levels of endogenous PEA are expected to be anti-inflammatory and analgesic drugs (Petrosino et al. 2010; Ueda et al. 2013).

4.2 MGL, ABHD6 and ABHD12

MGL is a serine hydrolase responsible for about 85 % of the 2-AG hydrolyzing activity of mouse brain (Blankman et al. 2007). This enzyme of about 303 amino acids is present in both membrane and cytosolic subcellular fractions and can recognize other unsaturated monoacylglycerols also as substrates, which in some cases compete with 2-AG inactivation (Ben-Shabat et al. 1998; Di Marzo and Deutsch 1998). MGL is sensitive to sulphydryl-specific reagents, and comparison models strongly suggest that cysteine residues present near its binding site play a role in the catalytic mechanism (Saario et al. 2005), although the catalytic triad of this enzyme also involves Ser122, Asp239 and His269 (Karlsson et al. 1997). The distribution of MGL was studied in rat, and it was shown to be ubiquitous (Karlsson et al. 1997). Specifically, MGL mRNA was reported to be present in adrenal gland, heart, adipose tissue, kidney, ovary, testis, spleen, lung, liver, skeletal muscle and brain (particularly in hippocampus, cortex, thalamus and cerebellum, where CB_1 receptors are highly expressed) (Dinh et al. 2002). Ultrastructural localization studies show that MGL is mainly pre-synaptic and often co-localizes with CB_1 receptors in the axon terminals (Savinainen et al. 2012). The complimentary

localization in the brain for MGL and FAAH, pre-synaptic and post-synaptic, respectively, has suggested different roles for the two main endocannabinoids in the central nervous system (Gulyas et al. 2004). Several MGL inhibitors have been developed so far. Methylarachidonoylfluorophosphonate (MAFP) inhibits MGL irreversibly but lacks selectivity, since it inhibits most metabolic serine hydrolases (Saario et al. 2004; Savinainen et al. 2010, 2012). *N*-arachidonoylmaleimide (NAM) selectively, but only partially (85 %), inhibits MGL (Saario et al. 2005; Blankman et al. 2007; Savinainen et al. 2012). Other MGL inhibitors include the non-competitive/irreversible inhibitors, URB602 and JZL184, and the reversible inhibitor, OMDM169 (Petrosino and Di Marzo 2010). Like FAAH inhibitors, MGL inhibitors have been found to have potential therapeutic applications, as indicated, for example, by results obtained from experiments using animal models of acute, visceral, inflammatory, neuropathic or bone cancer pain (Pertwee 2014). Interestingly, MGL inhibitors have also been reported to be efficacious against signs of breast, ovarian, skin and prostate cancer in animal models (Pertwee 2014). Recent data have shown that MGL inhibitors such as JZL184 and URB602 can protect neurons from β amyloid peptide-induced neurodegeneration and apoptosis, suggesting a therapeutic potential for the treatment of Alzheimer's disease (Pertwee 2014). Unfortunately, it has been reported that JZL184 shares the ability of direct CB_1 agonists to induce both physical and psychological dependence in mice as well as tolerance to their antinociceptive effects (Schlosburg et al. 2010; Ghosh et al. 2013; Pertwee 2014).

2-AG metabolism is also catalysed by two integral membrane proteins, α/β-hydrolase domain containing protein-6 (ABHD6) and -12 (ABHD12). Both enzymes belong to the α/β-hydrolase superfamily, with the postulated catalytic triad serine-aspartic acid-histidine (Savinainen et al. 2012). ABHD6, in neurones, is localized at sites of 2-AG generation, including post-synaptic dendrites of principal glutamatergic neurones as well as some GABAergic interneurons (Savinainen et al. 2012). ABHD12 is highly expressed in microglia, macrophages and osteoclasts (Fiskerstrand et al. 2010). Interestingly, it was observed that mutations in the ABHD12 gene are causally linked to a neurodegenerative disease called PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract) (Fiskerstrand et al. 2010; Savinainen et al. 2012).

5 Conclusions

Over the past 20 years, substantial progress has been made in the understanding of the endocannabinoid system. In particular, new molecules have been classified as endocannabinoids (e.g., the ethanolamides of two omega-3 fatty acids), and new targets other than cannabinoid CB_1 and CB_2 receptors have been identified and held accountable for some of the effects of the endocannabinoids. Moreover, substantial progress has also been made in the identification as well as in the characterization of the enzymes responsible for both the biosynthesis and the metabolism of the main endocannabinoids, AEA and 2-AG. It still remains to be established whether these

enzymes catalyse the formation or degradation of other, less studied, endocannabinoids. In addition, several molecules have been developed that are able to interact more or less selectively or more or less potently with enzymes or uptake processes of the endocannabinoid system. Many of these molecules, such as FAAH and MGL inhibitors as well as endocannabinoid uptake inhibitors, have been discovered, albeit only in animal models, to possess notable therapeutic potential for the treatment of diseases such as cancer, pain, neurodegenerative diseases and so on. Unfortunately, some of these molecules, such as MGL inhibitors, have also been shown to share the ability of direct CB_1 cannabinoid receptor agonists to cause physical and psychological dependence. This problem still needs to be overcome. Finally, as recently and elegantly discussed by Piomelli (Piomelli 2014), one important question about the endocannabinoids that still remains unresolved is how such lipophilic molecules are able to cover the distance between their site(s) of action and the site(s) of their enzymatic degradation. This distance is quite short for 2-AG, whose main metabolic enzyme (MGL) is localized presynaptically, and thus close to the pre-synaptic CB_1 receptors on which 2-AG acts, but longer for anandamide, which after acting on presynaptic CB_1 receptors must travel trans-synaptically in order to be metabolized by FAAH, which is primarily postsynaptic. The research on the understanding of the endocannabinoid system never ends.

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