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Review

The endocannabinoid signalling system: Biochemical aspects

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Abstract

Knowledge of the endogenous cannabinoid system has expanded greatly during the past years. After the discovery of the cannabinoid receptors, of their endogenous agonists and of the proteins for their synthesis and inactivation, significant progress has been made towards the understanding of the role of the endocannabinoid system in vital functions. Subsequently, an increasing number of papers has been published on the biochemistry and pharmacology of endocannabinoids. This article overviews the endocannabinoid signalling system with focus on its biochemical aspects. In particular we review the mechanisms for the biosynthesis and inactivation of the endocannabinoids, as well as the various molecular targets for some of the endocannabinoids described so far.

Keywords: Anandamide; 2-Arachidonoylglycerol; Cannabinoid receptors; Anandamide transporter; Fatty acid amide hydrolase

Contents

1. Introduction

The finding, in the early 1990s, of specific G-protein-coupled receptors for the psychoactive component of *Cannabis sativa* (−)-Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1971), led to the discovery of a whole endogenous signaling system now known as the *endocannabinoid system*. This consists of the cannabinoid receptors, endocannabinoids and the proteins for their synthesis and inactivation.

Cannabinoid receptors are seven-transmembrane-domain proteins coupled to G-proteins of the $G_{i/o}$ type. Mammalian tissues contain at least two types of cannabinoid receptors, CB_1 and CB_2. CB_1 receptors, cloned in 1992, are mostly expressed in the central nervous system but also in most peripheral tissues including immune cells, the reproductive

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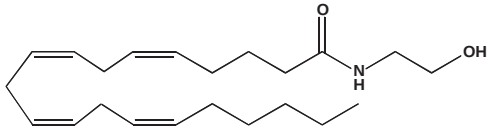
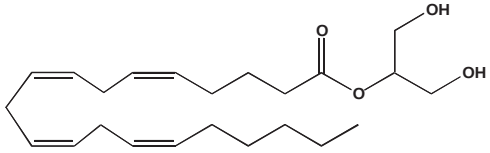
system, the gastrointestinal tract and the lung, while CB_2 receptors, cloned in 1993, are most abundant in the immune system, i.e. in tonsils, spleen, macrophages and lymphocytes (B-cells and natural killer cells) (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993). Inside the brain, CB_1 distribution accounts for the pharmacological properties reported for THC and psychotropic cannabinoids. CB_1 and CB_2 receptors share 44% overall identity and 68% identity within the transmembrane domains. Both receptors are coupled to pertussis toxin-sensitive inhibition of cAMP formation, implicating $G_{i/o}$ proteins as transducers, and to stimulation of p42/p44 mitogen-activated protein kinase activity (Vogel et al., 1993; Bouaboula et al., 1995). CB_1, but not CB_2, receptors signal also via ion channels by inhibiting N- and P/Q-type calcium channels and by activating A-type and inwardly rectifying potassium channels (Mackie and Hille, 1992; Mackie et al., 1995; McAllister et al., 1999). Furthermore, CB_1 activation stimulates phosphatidylinositol 3-kinase and protein kinase B (Gomez del Pulgar et al., 2000; Molina-Holgado et al., 2002).

By definition, endocannabinoids are endogenous compounds capable of binding to and functionally activating these two receptors (Di Marzo and Fontana, 1995). Anandamide (AEA), the first endogenous ligand to be reported at the end of 1992, is the amide between arachidonic acid and ethanolamine, and it acts as a partial CB_1 agonist (Devane et al., 1992) and only as a weak CB_2 agonist (Fig. 1). This compound belongs to the family of the *N*-acyl-ethanolamines (NAEs) already known for their pharmacological properties; other members of this family, such as homo-γ-linolenylethanolamide (HEA) and docosatetraenylethanolamide (DEA), are produced by neurons and bind to CB_1 receptors. In the past 10 years, other endocannabinoids, all derived from arachidonic acid, were identified. First came the finding of 2-arachidonoylglycerol (2-AG), the arachidonate ester of glycerol, which activates both CB_1 and CB_2 receptors (Mechoulam et al., 1995; Sugiura et al., 1995), and, more recently, 2-arachidonylglyceryl ether (noladin, 2-AGE), a selective CB_1 agonist, *O*-arachidonoyl-ethanolamine (virhodamine, OAE), a partial CB_2 agonist and a CB_1 antagonist, and *N*-arachidonoyl-dopamine (NADA), a selective CB_1 agonist and a potent agonist of vanilloid receptors, were discovered (Hanus et al., 2001; Porter et al., 2002; Bisogno et al., 2000; Huang et al., 2002) (Fig. 1). While the physiological role of virhodamine, NADA and 2-AGE has not been clarified yet, the endocannabinoids AEA and 2-AG, since their finding, have been implicated in a wide range of physiological and



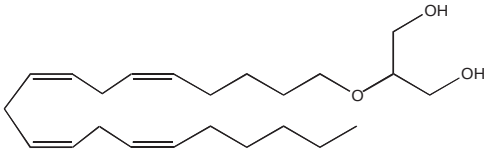
Anandamide

Partial CB_1 agonist, weak CB_2 agonist



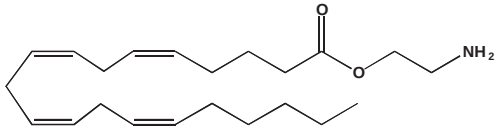
2-Arachidonoyl-glycerol

Full CB_1 and CB_2 agonist



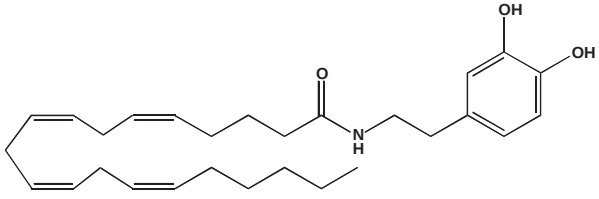
Noladin

Selective CB_1 agonist



Virhodamine

Partial CB_2 agonist and a CB_1 antagonist



N-Arachidonoyl-Dopamine

CB_1 agonist and potent TRPV1agonist

Fig. 1. Chemical structures of the endocannabinoids and their binding and functional properties at cannabinoid receptors.

pathological processes. The full characterization of most of the proteins involved in AEA and 2-AG metabolism, i.e. of the enzymes responsible of their biosynthesis and degradation, will open a new area of research aimed at developing potential therapeutic strategies for the pharmacological treatment of diseases in which the endocannabinoid system seems to be involved. The purpose of this article is to overview the endocannabinoid signalling system in order to provide information as complete and as updated as possible regarding its biochemical aspects.

2. Biosynthesis of endocannabinoids

AEA and 2-AG are not stored in resting cells but, unlike other mediators, they are synthesized and released only "on demand", i.e. when and where necessary, following physiological or pathological stimuli, in a way depending upon Ca^{2+}-dependent phospholipid remodeling (Di Marzo and Deutsch, 1998). Furthermore, the synthesis of AEA and 2-AG is associated with the formation of non-cannabimimetic, or weakly cannabinoid receptor active, related compounds, i.e. of cannabinoid receptor-inactive *N*-acyl-ethanolamines and 2-acyl-glycerols, respectively, which have been suggested, among other things, to potentiate the effects of endocannabinoids ("entourage compounds") (Ben-Shabat et al., 1998). No conclusive data on the biosynthetic mechanisms underling the formation of noladin, virodhamine and NADA have been reported so far.

3. Biosynthesis of AEA

The family of the *N*-acyl-ethanolamines (NAEs), to which AEA belongs, has been long investigated before

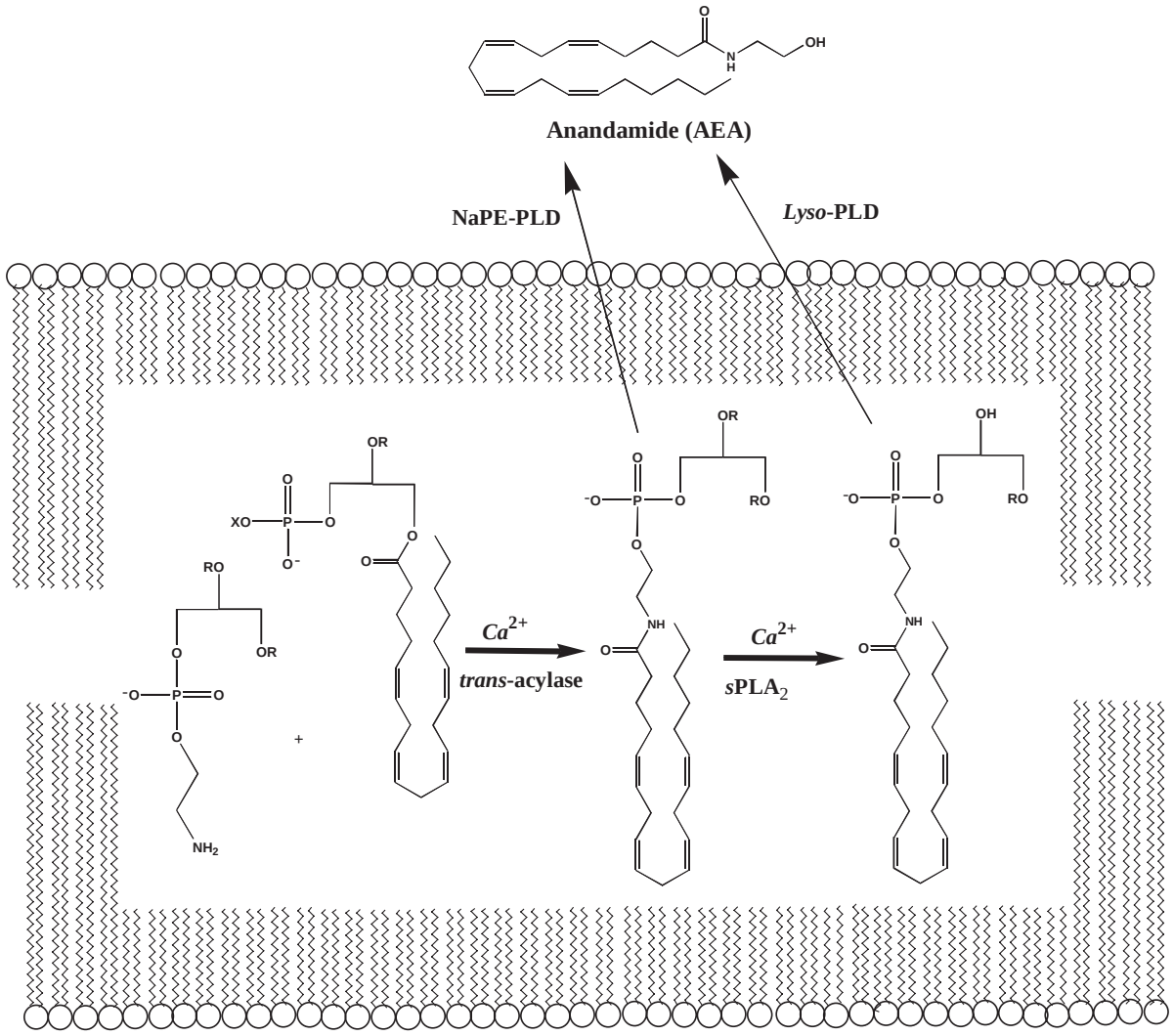


Fig. 2. Major biosynthetic pathways for the endocannabinoid anandamide. NaPE: *N*-arachidonoylphosphatidylethanolamine, PLD: phospholipase D, sPLA$_2$: secretory phospholipase A$_2$ of group IB.

the identification of AEA, and these studies led to the conclusion that those compounds are biosynthesized via a phospholipid-dependent pathway consisting of the enzymatic hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamines (NAPEs) (Schmid et al., 1983, 1990, 1996; Schmid and Berdyshev, 2002; Hansen et al., 1998). The enzyme catalyzing this reaction was identified as a phospholipase D selective for NAPEs (NAPE-PLD), which exhibits catalytic properties different from other PLD enzymes. The phospholipid precursors of NAEs are in turn produced from the enzymatic transfer of an acyl group from the *sn*-1 position of phospholipids to the *N*-position of phosphatidylethanolamine (PE), catalyzed by a Ca^{2+}-dependent *trans*-acylase (Fig. 2). This pathway does not appear to be able to generate a large amount of AEA because the levels of arachidonic acid esterified at the *sn*-1 position of phospholipids are usually very low. This is in agreement with the observation that AEA levels are generally lower than those of the other NAEs in most of the tissues analyzed so far. In fact, both the NAPE-PLD and the *trans*-acylase do not appear to be selective for a particular fatty acid moiety. In particular, the catalytic properties of the NAPE-PLD have been studied using partially purified enzyme preparations. The enzyme has been shown to be dependent upon the concentration of Ca^{2+}, to be stimulated by polyamines, and to be definitively different from the known PLD enzymes, since, among other things, NAPE-PLD does not catalyze the trans-phosphatidylation reaction typical of PLDs (Ueda et al., 2001a; Liu et al., 2002; Petersen and Hansen, 1999). Very recently an enzyme responsible for NAEs formation has been cloned from the mouse, rat and human and classified as a member of the zinc metallo-hydrolase family of the β-lactamase fold (Okamoto et al., 2004). The recombinant enzyme, overexpressed in COS-7 cells, was found to be mostly present in the microsomial fraction, and to be responsible for the formation of AEA and other NAEs from their corresponding NAPEs at comparable rates. The NAPE-PLD does not recognize phosphatidylcoline and phoshatidylethanolamine as substrates and it is widely distributed in mouse organs, with highest concentrations in the brain, kidney and testis. The amino acid sequence reported for the NAPE-PLD does not show homology with those of other PLDs, such as the mammalian PLD_1 and PLD_2 or the glycosylphosphatidylinositol-specific PLD. Although several pieces of evidence point to this route as the mechanism mostly responsible for AEA biosynthesis in intact cells, a pathway for NAE formation independent of the NAPE-PLD was recently reported. According to this pathway, *N*-acyl-PE is first hydrolysed to *N*-acyl-lyso-PE and a free fatty acid by an enzyme member of group IB secretory phospholipase A2, and NAE is then released from *N*-acyl-lyso-PE by a lyso-PLD-like enzyme (Sun et al., 2004) (Fig. 2).

4. Biosynthesis of 2-arachidonoylglycerol

In unstimulated tissues and cells the levels of 2-AG are higher than those of AEA, although they are probably overestimated due, for example, to the rapid increase of 2-AG formation that follows rat decapitation (Sugiura et al., 2001). This simple observation suggests that only a part of 2-AG found in tissues is used to activate cannabinoid receptors. In fact this endocannabinoid is an important precursor and/or degradation product of phospho-, di- and tri-glyceride pathways. Several stimuli have been shown to cause 2-AG formation in intact cells, such as lipopolysaccharides, endothelin, platelet-activating factor, ionomycin, carbachol, thrombin, etc. (Bisogno et al., 1997a; 1999; Liu et al., 2003; Basavarajappa et al., 2000; Stella et al., 1997; Sugiura et al., 1998; 2002; Di Marzo et al., 1999; Berdyshev et al., 2001; Mechoulam et al., 1998; Stella and Piomelli, 2001; Walter and Stella, 2003). In most cases, 2-AG is produced from the hydrolysis of diacylglycerols containing arachidonate in the 2 position (DAGs), catalysed by a DAG lipase selective for the *sn*-1 position. DAGs, in turn, can be produced from the hydrolysis either of phosphoinositides (PI), catalysed by a PI-selective phospholipase C (PI-PLC), as in macrophages, platelets and cortical neurons, or of phosphatidic acid (PA), catalysed by a PA phosphohydrolase, in mouse neuroblastoma cells N18TG2 and in a rat microglial RTMGL1 cell line (Di Marzo et al., 1996; Stella et al., 1997; Kondo et al., 1998; Berdyshev et al., 2001; Stella and Piomelli, 2001; Liu et al., 2003; Bisogno et al., 1999; Carrier et al., 2004) (Fig. 3). Regarding the enzymatic conversion of DAGs into 2-AG, two *sn*-1 DAG lipase isozymes (DAGLα and DAGLβ) have been cloned, enzymatically characterized and proposed to be responsible for the formation of the endocannabinoid 2-AG in intact cells (Bisogno et al., 2003). Based on their amino acid sequences, it was possible to show that both enzymes contain a lipase-3, and a Ser-lipase motif, and to suggest the presence of four transmembrane-spanning domains, with the amino terminus on the citosolic side. Both proteins, transfected in COS-7 cells, are mostly localized in the plasma membrane, and exhibit optimal activity at pH=7. Their pattern of expression correlates with the proposed function of the 2-AG either as a mediator of neurite growth, during brain development, or as retrograde messenger mediating depolarization-induced suppression of inhibitory or excitatory neurotransmission (DSI or DSE), in the adult brain. In fact, the enzymes are located in axonal tracts during brain development, in order to produce 2-AG to promote axonal growth and guidance; in the adult brain, instead, they disappear from the growth cone and are transferred post-synaptically to produce and release 2-AG which acts backwards on CB_1 receptors on pre-synaptic neurons to inhibit neurotransmitter release cells (Bisogno et al., 2003; Williams et al., 2003; Chevaleyre and Castillo, 2003; Wilson and Nicoll, 2002).

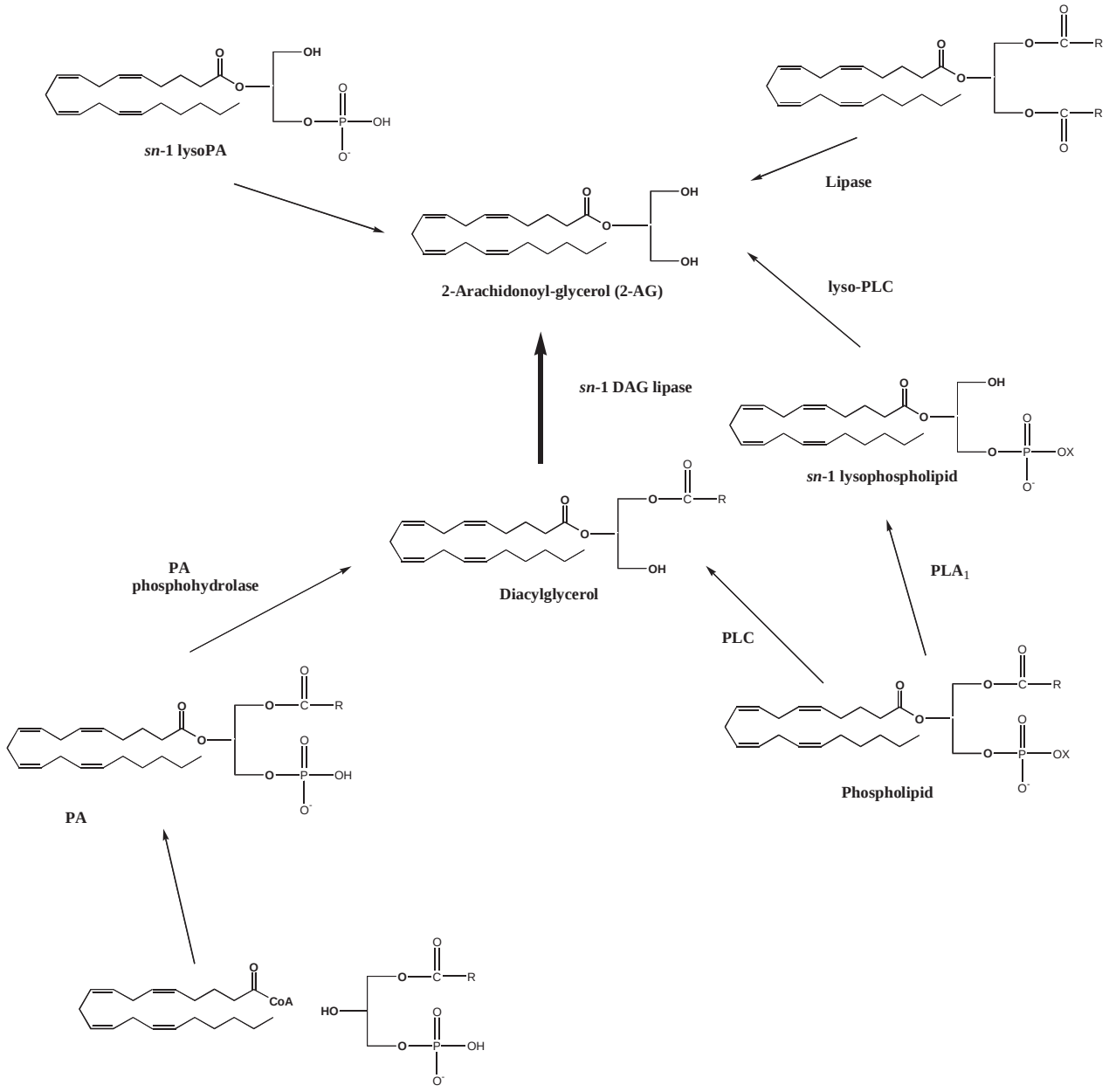


Fig. 3. Biosynthetic pathways for the endocannabinoid 2-arachidonoylglycerol. PA: phosphatidic acid, PLC: phospholipase C, PLA_1: phospholipase A_1.

Like with the enzymes involved in AEA biosynthesis, the two DAG lipases do not appear to be selective for 2-arachidonate-containing DAGs.

5. Inactivation of endocannabinoids

The endocannabinoids, as any other endogenous mediator of physiological and pathological responses, need mechanisms for their rapid removal from their molecular targets and subsequent degradation. Because they are lipophilic compounds, the endocannabinoids can diffuse through the cell membrane. In order to be rapid, selective and subject to regulation, the diffusion of the endocannabinoids through the plasma membrane needs to be facilitated by a carrier or to be driven by a mechanism capable of rapidly reducing their intracellular concentration, or both. Indeed, AEA appears to be taken up by several cells types at least in part via a facilitated transport mechanism, known as the anandamide membrane transporter (AMT). In fact, anandamide cellular uptake is saturable, temperature-dependent and sensitive to synthetic inhibitors as expected from a protein-mediated process (Di Marzo et al., 1994; Beltramo et al., 1997; Bisogno et al., 1997b; Hillard et al., 1997; Maccarrone et al., 1998, 2000a,b). The AMT has not been isolated or cloned yet, but indirect evidence suggests the possibility that it can also mediate 2-AG, noladin, virodhamine and NADA uptake (Huang et al., 2002; Fezza

et al., 2002; Wilson and Nicoll, 2002). Some authors have reported evidence against the existence of the AMT, suggesting that the enzyme mostly responsible for AEA hydrolysis, the fatty acid amide hydrolase (FAAH), by reducing the intracellular concentration of AEA and possibly by even associating transiently with the plasma membrane, may be uniquely responsible of AEA cellular uptake (Bracey et al., 2002; Glaser et al., 2003). On the other hand, several data are in agreement with a facilitated transport independent of FAAH (Table 1) (Hillard and Jarrahian, 2003): (1) cells line types that do not express FAAH are still able to rapidly take up AEA; (2) synthetic compounds that inhibit AEA cellular uptake with no effect on AEA enzymatic hydrolysis have been reported (Di Marzo et al., 2001; 2002a; De Petrocellis et al., 2000; Lopez-Rodriguez et al., 2001; Ortar et al., 2003); (3) saturable AEA accumulation can be still observed in synaptosomes and cells prepared from FAAH-null mice (Ligresti et al., 2004; Fegley et al., 2004); (4) selective AMT inhibitors block AEA activity at cytosolic site of action TRPV1 vanilloid receptors, while enhancing its extracellular CB_1-mediated effects, suggesting that these opposite effects cannot be due to FAAH inhibition (De Petrocellis et al., 2001a); (5) the trans-membrane movement of AEA in cerebellar granule cells is affected by ethanol treatment in a time- and dose-dependent manner, without any effect on FAAH activity (Basavarajappa et al., 2003); (6) the AMT appears to facilitate the uptake of noladin and NADA, which are resistant to FAAH hydrolysis (Huang et al., 2002; Fezza et al., 2002); (7) a selective AMT inhibitor, VDM11, was found to block AEA release without any effect on AEA de novo biosynthesis inside the cells (Ligresti et al., 2004). From these data it is possible to conclude that FAAH activity can influence facilitated AEA and endocannabinoid uptake, but also that other mechanisms different from endocannabinoid intracellular metabolism must intervene to enhance the rate of endocannabinoid membrane transport.

Recently, the AEA uptake has been investigated in primary neuronal culture obtained from FAAH(+/+) and (−/−) mice and the effect of UCM707, an inhibitor of AEA uptake inactive against FAAH, has been tested. The authors distinguished the uptake of AEA into different components suggesting that both FAAH and CB_1 cannabinoid receptors participate to the cellular uptake of AEA, and that an additional protein still inhibited by UCM707 also contributed to AEA uptake (Ortega-Gutierrez et al., 2004). Furthermore, some authors have suggested a new model for AEA uptake that might occur via a caveolae/lipid raft-related endocytic process in RBL-2H3 cells (McFarland et al., 2004). Recently, the putative AMT has been suggested to contribute to 2-AG inactivation also in hippocampal slices (Hajos et al., 2004).

6. Hydrolysis

Once inside the cell, the endocannabinoids are degraded through mechanisms depending on their chemical nature (Fig. 4). One enzyme, FAAH, has been identified as mostly responsible of AEA and, in some cases, 2-AG hydrolysis to arachidonic acid and ethanolamine or glycerol, respectively (Cravatt et al., 1996; Cravatt and Lichtman, 2002; Bisogno et al., 2002). FAAH was originally purified and cloned from rat liver, and catalyzes the hydrolysis also of long chain primary fatty acid amides and glycerol esters; its structural and catalytic properties have been fully investigated. FAAH is an integral membrane protein of 597 amino acids, and it has been cloned from a wide range of species with a high degree of conservation between mouse and human. The enzyme contains a short "amidase signature" sequence enriched in serine and glycine residues. Site-directed mutagenesis studies have identified the amino acids residues involved in the catalytic site of the enzyme, and the genomic loci containing human and mouse FAAH

Table 1
Evidence against (right) and in favour (left) of the possibility that the AMT is uniquely due to FAAH and not to a membrane carrier

- Cells line types that do not express FAAH are able to rapidly take up AEA (Di Marzo et al., 1999; Piomelli et al., 1999)
- Saturable AEA accumulation can be still observed in synaptosomes prepared from FAAH-null mice (Ligresti et al., 2004)
- Synthetic compounds selectively inhibit AEA cellular uptake without affecting FAAH activity (Di Marzo et al., 2001, 2002a; De Petrocellis et al., 2000; Lopez-Rodriguez et al., 2001; Ortar et al., 2003)
- Selective AMT inhibitors block AEA activity at cytosolic site of action TRPV1 vanilloid receptors, while enhancing its extracellular CB_1-mediated effects (De Petrocellis et al., 2001a)
- The AMT facilitates the uptake of noladin and NADA, which are resistant to FAAH hydrolysis (Huang et al., 2002; Fezza et al., 2002)
- AEA uptake is mediated by a combination of FAAH-dependent and -independent mechanism (Ortega-Gutierrez et al., 2004)
- AEA uptake, but not FAAH activity are inhibited by acute or chronic ethanol treatment (Basavarajappa et al., 2003)
- AEA release by cells is blocked by a selective AMT inhibitor, VDM11, without affecting de novo biosynthesis of AEA (Ligresti et al., 2004)
- AEA uptake is increased in cells transfected with FAAH cDNA (Deutsch et al., 2001)
- By using very short incubation times in order to minimize FAAH activity, AEA uptake is not always saturable (Glaser et al., 2003)

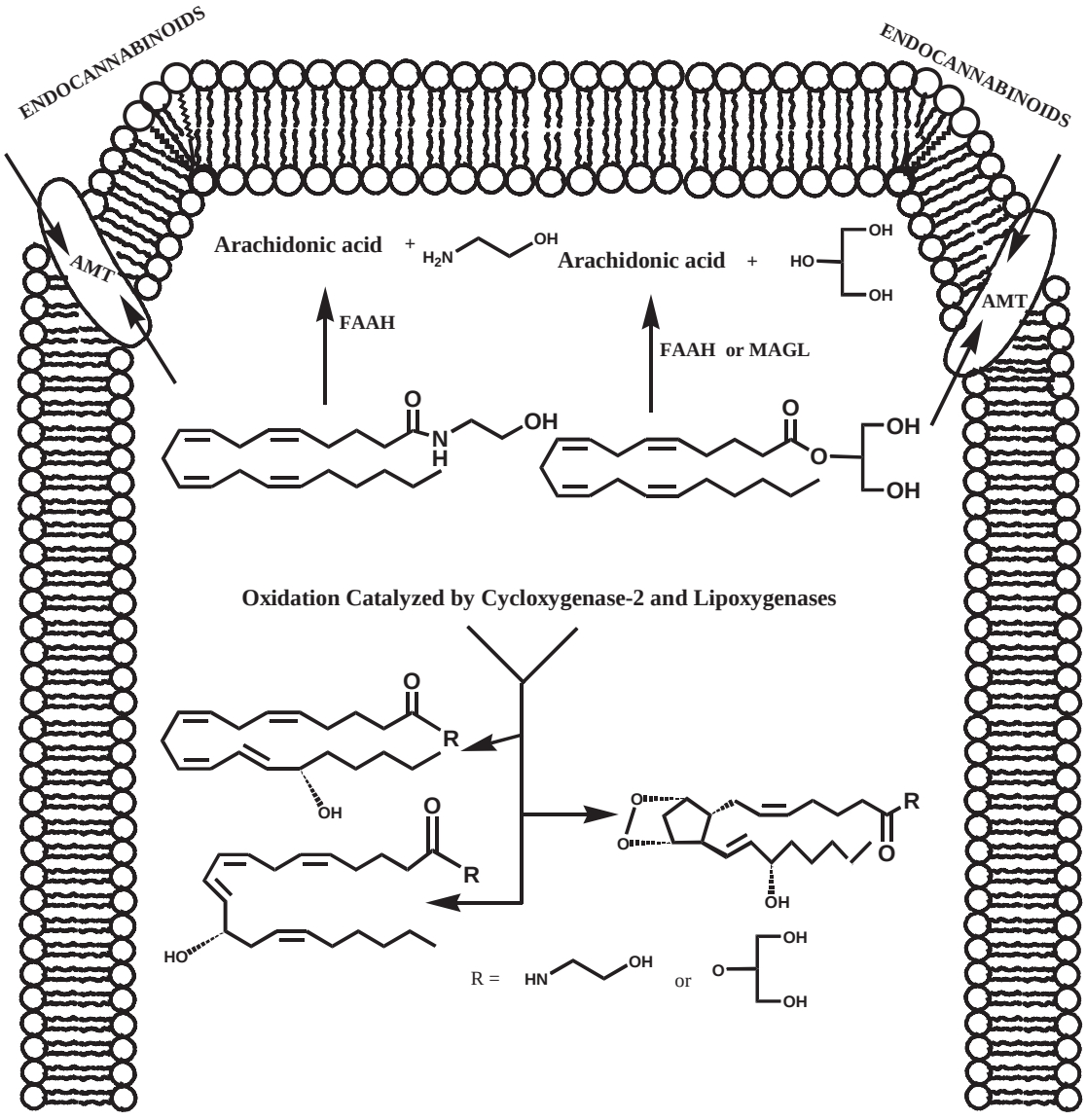


Fig. 4. Mechanisms for endocannabinoids inactivation. FAAH: fatty acid amide hydrolase, MAGL: monoacylglycerol lipase.

genes have been identified (Patricelli and Cravatt, 2000). The promoter region on the FAAH gene has been studied and it is targeted by progesterone and leptin, which up-regulate the enzyme, and by estrogens and glucocorticoid, which instead down-regulate it (Puffenbarger et al., 2001; Waleh et al., 2002; Maccarrone et al., 2003a,b). FAAH is mainly expressed in microsomial membranes and has an alkaline optimal pH. Extensive SAR studies for the interaction of fatty acid long chain derivatives with FAAH have been reported suggesting that both the alkyl chain and the polar "head" of AEA are important for interaction with the active site. Another amidase, seemingly located in lysosomes, and playing a major role in the inactivation of the AEA congener, *N*-palmitoyl-ethanolamine, has been also characterized (Ueda et al., 2001b).

Although FAAH can catalyze 2-AG hydrolysis (Di Marzo and Deutsch, 1998), 2-AG levels, unlike those of AEA, are not increased in FAAH "knockout" mice (Lichtman et al., 2002). This observation is in agreement with the previously reported evidence regarding the existence of other enzymes catalyzing 2-AG inactivation different from FAAH (Di Marzo et al., 1999; Goparaju et al., 1999). Other 2-AG hydrolases, known as monoacylglycerol lipases (MAGLs), and present in both membrane and cytosolic subcellular fractions, can catalyze 2-AG enzymatic hydrolysis. MAGLs recognize as substrates also other unsaturated monoacylglycerols which in some cases compete with 2-AG inactivation (Ben-Shabat et al., 1998; Di Marzo and Deutsch, 1998). A MAG lipase, inactive on AEA and with high homology with other human and mouse MAGLs, has been cloned from human, mouse and, more recently, rat (Karlsson et al., 2001; Ho et al., 2002; Dinh et al., 2002). In rat brain, this MAGL is present with the highest levels in regions where CB_1

cannabinoid receptors are expressed (hippocampus, cortex, anterior thalamus and cerebellum). Furthermore, immunohistochemical studies in the hippocampus suggested a presynaptic localization of the enzyme, supporting the role of rat MAGL in the degradation of 2-AG as retrograde messenger, and supplementing the data showing that the DAGLs responsible for 2-AG production are instead postsynaptic in the adult brain (Dinh et al., 2002; Bisogno et al., 2003). Recent studies have confirmed the complementary localization in the brain for the MAGL and FAAH, pre-synaptic and post-synaptic, respectively, suggesting different roles for the two endocannabinoids in the CNS (Gulyas et al., 2004).

7. Other mechanisms of inactivation

Ethanolamine, arachidonic acid and glycerol, the hydrolysis products of AEA and 2-AG, are recycled into membrane phospholipids in order to be used again, at least in part, in the biosynthetic pathways of the two endocannabinoids. Furthermore 2-AG, unlike AEA, can be re-esterified into phospholipids also before being enzymatically hydrolyzed, and this re-esterification occurs through mechanisms involving phosphorylation or acylation of its hydroxyl groups (Sugiura et al., 2002). This metabolic pattern may became very important for the inactivation of noladin, whose ether bond cannot be enzymatically hydrolyzed (Fezza et al., 2002). Because of the presence of arachidonate moiety, the possibility that endocannabinoids can also be susceptible to oxidative mechanisms catalyzed by lipoxygenases, cyclooxygenases and cytochrome *P*450 oxidases has been investigated (Kozak and Marnett, 2002). Regarding the lipoxygenase products of AEA and 2-AG, they can be formed through the action of 12- and 15-, but not 5-lipoxygenases (Kozak and Marnett, 2002; van der Stelt et al., 2002). The 12-hydroxy-derivative of AEA still binds to cannabinoid receptors while the 15-hydroxy-derivative does not but it inhibits FAAH (Edgemond et al., 1998; van der Stelt et al., 2002). Unidentified hydroxy-derivatives of AEA have been suggested to act, like AEA, via vanilloid TRPV1 receptors (Craib et al., 2001). The 15-hydroxy-derivative of 2-AG was recently shown to be formed in eukaryotic cells, and its potential biological actions as a peroxisome proliferator-activated receptor (PPAR) α, but not γ, agonist was also investigated (Kozak et al., 2002). It has been also established that AEA and 2-AG can be enzymatically transformed into the corresponding prostaglandin ethanolamines (prostamides) and prostaglandin glyceryl esters, respectively, through the action of cyclooxygenase-2 and, subsequently, of several prostaglandin synthases. Studies on the metabolism and possible interactions with cannabinoid and prostaglandin receptors of these compounds have been published (Woodward et al., 2001; Ross et al., 2002). No specific molecular targets have been identified for either prostamides or prostaglandin glyceryl esters. Very recently, we reported that prostamides stimulate cat iris contraction by a mechanism not due to transformation into prostaglandins, activation of prostanoid receptors, enhancement of endogenous AEA levels, or gating of TRPV1 vanilloid receptors, suggesting the interaction with novel receptors functionally expressed in the cat iris (Matias et al., 2004). Likewise, prostaglandin E_2 glycerol ester was recently shown to activate with high potency a novel G-protein-coupled receptor (GPCR) (Nirodi et al., 2004). Few investigations of P450-mediated endocannabinoid metabolism have been reported. These studies reported the production of monoxigenated AEA-derivatives through the activation of murine hepatic P450s (Bornheim et al., 1993, 1995). Finally, NADA, due to its chemical structure containing arachidonic and a cathecolamine moiety, can be, at least in theory, a substrate for oxidation. However, to date, only the methylation of the 3-hydroxy-group of NADA by cathecolamine *O*-methyl transferase has been observed (Huang et al., 2002).

8. Inhibitors

The knowledge of the mechanisms underlying the biosynthesis and inactivation of the endocannabinoids contributed to a better understanding of the effects mediated by cannabinoid receptors when they are activated by their endogenous ligands, and opened the way to the hypothesis that compounds able to regulate endocannabinoid metabolism might become potential therapeutic agents for the treatment of diseases where the endocannabinoid system is involved. Since AEA and 2-AG biosynthetic enzymes have been identified only recently, no selective inhibitor for these proteins has been developed so far. However, non-specific inhibitors have been shown to inhibit the formation of either AEA or 2-AG in intact cells. In particular, serine hydrolase inhibitors can counteract AEA formation in cortical neurons and 2-AG formation in N18TG2 cells (Cadas et al., 1997; Bisogno et al., 1999). Furthermore RHC80267, (1,6-bis-(cyclohexyloximinocarbonylamino)-hexane), a non-selective DAG lipase inhibitor, blocks 2-AG formation in intact cells (Stella et al., 1997; Bisogno et al., 1999). More importantly, tetrahydrolipstatin, THL, a general lipase inhibitor, was found to inhibit the recently cloned DAGLα and DAGLβ with very high potency (Bisogno et al., 2003) (Fig. 5).

Regarding the AMT, AM404 was the first synthetic compound developed to counteract AEA accumulation into cells with IC_{50} values in the 1–10 μM range of concentrations (Beltramo et al., 1997). Unfortunately, this compound is not selective. It can also inhibit FAAH, presumably by acting as an alternative substrate (Fegley et al., 2004), and activate vanilloid TRPV1 receptors (Jarrahian et al., 2000; Zygmunt et al., 2000; De Petrocellis et al., 2000; Ross

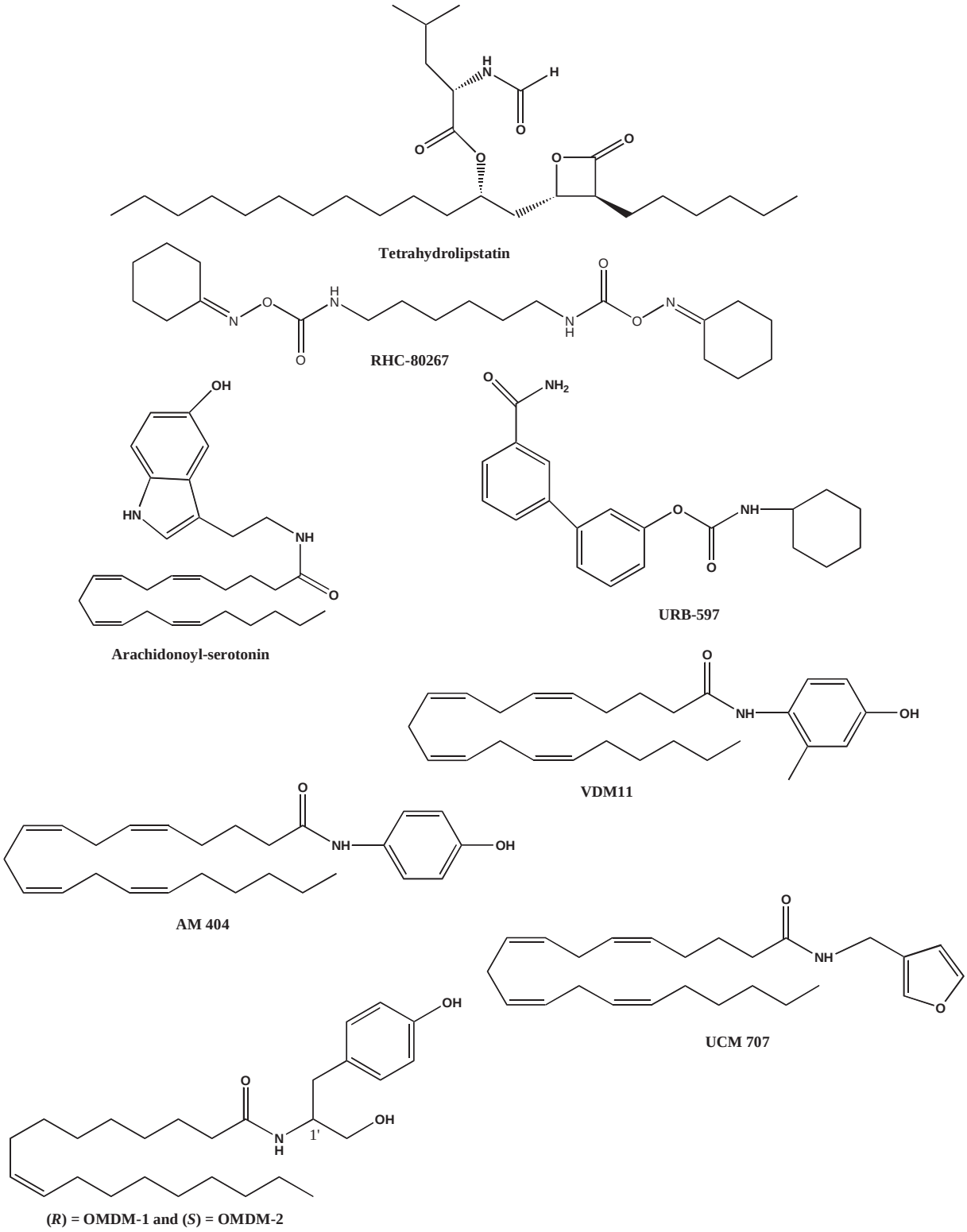


Fig. 5. Chemical structures of same inhibitors of endocannabinoid biosynthesis and inactivation.

et al., 2001). Recently, other synthetic compounds more selective for the AMT, with no or little effects with other proteins of the endogenous cannabinoid system, have been identified. UCM-707, the furyl-derivative of arachidonic acid, inhibits the AEA membrane transporter in human lymphoma U937 cells with an IC50 of 0.8 μM (Lopez-Rodriguez et al., 2003a,b). This compound is selective for AEA uptake vs. TRPV1 receptors and FAAH. However, this compound is less active in other cell types (Ruiz-Llorente et al., 2004; Fowler et al., 2004). VDM11 and VDM13 inhibit

AMT with the same potency as AM404 and do not activate vanilloid TRPV1 receptors (De Petrocellis et al., 2000, 2001a). Finally OMDM-1 and OMDM-2 are the first selective inhibitors of AEA cellular uptake to be developed from a fatty acid different from arachidonic acid, i.e. oleic acid (Ortar et al., 2003). Compared to AM404 and VDM-11, both OMDM-1 and OMDM-2 are more stable to enzymatic hydrolysis in rat brain homogenates, although the enzymatic stability of UCM707 has not been investigated yet (de Lago et al., 2002). While the existence of the AMT is still controversial, strong evidence of the use of anandamide uptake inhibitors as possible drugs to alleviate symptoms of diseases in animal models have been reported. VDM11 was shown to ameliorate spasticity in a mouse model of multiple sclerosis, the chronic relapsing experimental allergic encephalomyelitis in mice, to inhibit intestinal hyperactivity and diarrhea induced in mice by cholera toxin, to ameliorate movement in a rat model of Parkinson's disease, and to reduce hyperactivity in glutamatergic neurons in this model (Baker et al., 2001; Pinto et al., 2002; Mascolo et al., 2002; Izzo et al., 2003; Gubellini et al., 2002). UCM707 was shown to reduce acute excitotoxicity in central nervous system neurons (Marsicano et al., 2003). Finally, OMDM-1 and OMDM-2 are also suitable for in vivo use, as they have been used to inhibit spasticity in an animal model of multiple sclerosis, the chronic relapsing experimental allergic encephalomyelitis in mice (de Lago et al., 2004).

Several inhibitors of FAAH have been described so far (Bisogno et al., 2002; Deutsch et al., 2002). Most of these compounds, such as the trifluoro methyl ketones and methyl arachidonoyl fluoro phosphonate (MAFP), are not selective for FAAH, they bind to CB_1 and inhibit phospholipase A_2 (PLA_2) (Deutsch et al., 1997a; De Petrocellis et al., 1997). Arachidonoyl diazomethyl ketone is a mixed inhibitor but it also inhibits 5-lipooxygenase (De Petrocellis et al., 1997). The acyl sulfonyl fluorides, such as AM374, are very potent FAAH inhibitors with low affinity for CB_1 receptors, although these compounds have never been tested on PLA_2 (Deutsch et al., 1997b). A series of MAFP analogs, one of which, O-1624, being quite selective for FAAH vs. CB_1 receptors, have been reported, but again they were not tested on PLA_2 (Martin et al., 2000). The only FAAH inhibitor with IC_{50} values in the low μM range tested against CB_1 and CB_2 receptors and PLA_2 enzymes and found to be inactive is the *N*-arachidonoyl-5-hydroxytryptamin (Bisogno et al., 1998). A new class of potent FAAH inhibitors, the alkylcarbamic acid aryl esters, have been characterized, one of which, URB597, exhibited selectivity for FAAH (IC_{50} values in the nM range) towards CB_1 and CB_2 receptors, AMT and MAGL, although they were not tested against PLA_2 (Tarzia et al., 2003). These compounds appear to inhibit anxiety by enhancing brain AEA levels (Kathuria et al., 2003). Finally, alpha-keto-heterocycles were recently developed using a proteomics approach, and one of these compounds was extremely selective for FAAH and was found to exert interesting analgesic activities (Lichtman et al., 2004).

9. Other molecular targets for the endocannabinoids

Although great progress has been made towards the understanding of the biochemical and molecular mechanisms that underlie to the actions of the endocannabinoids, several findings suggest that those compounds may act also on non-cannabinoid receptor targets. First of all, pharmacological and biochemical data suggest the existence of non-CB_1 non-CB_2 receptors activated in vitro by physiologically relevant concentrations of AEA (Di Marzo et al., 2002b; Pertwee, 2004). The first example of such data was reported by Sagan et al. in mouse astrocytes. The authors reported that AEA and the cannabinoid receptor agonist WIN 55212-2 inhibit cyclic AMP formation through G-protein-coupled receptors distinct from CB_1 and CB_2 cannabinoid receptors in cultured astrocytes from the striatum of mouse embryos (Sagan et al., 1999). Furthermore, AEA and WIN 55212-2 are still functionally active in the GTP-γ-S-binding assays carried out in CB_1 knockout mouse brain membranes, apparently by acting on a G-protein-coupled receptor with a distribution different from that of the cannabinoid CB_1 receptors (Di Marzo et al., 2000; Breivogel et al., 2001; Monory et al., 2002). A novel, non-CB_1 cannabinoid receptor has been proposed to mediate the inhibitory effect of WIN 55212-2 on glutamate release in hippocampal pyramidal cells, and this putative receptor might bind also some vanilloid receptor ligands (Hajos et al., 2001; Di Marzo et al., 2001, 2002a,b,c; Brooks et al., 2002). Finally, the vascular endothelium contains a novel cannabinoid receptor. In fact, AEA causes endothelium-dependent vasorelaxation reverted by the cannabinoid receptor antagonist SR141716A but this effect is still persistent in arteries from CB_1 or CB_1/CB_2 knockout mice (Jarai et al., 1999). The non-psychoactive cannabinoid, abnormal-cannabidiol, (ABN-CBD), is a selective agonist of this receptor, whereas a synthetic analog of ABN-CBD, O-1981, is a selective antagonist (Offertaler et al., 2003). ABN-CBD causes endothelium-dependent vasorelaxation and stimulates phosphorylation of p42/p44 MAPK and protein kinase B/AkT (Begg et al., 2003). This ABN-CBD sensitive receptor has been proposed to be involved also in microglial cell migration (Walter et al., 2003). Very recently, it was shown that ABN-CBD increases human umbilical vein endothelial cell migration by activating this receptor (Mo et al., 2004), which appears to be activated also by NADA (O'Sullivan et al., 2004).

Interactions of AEA with ion channels involved in Ca^{2+} and K^+ homeostasis, such as TASK-1 K^+ (Maingret et al., 2001) channels and T-type Ca^{2+} channels (Chemin et al., 2001), which are both inhibited by the endocannabinoid, have been also reported. Furthermore, AEA and NADA

activate vanilloid TRPV1 receptors (Zygmunt et al., 1999; Huang et al., 2002), the molecular target of the pungent component of hot red peppers, capsaicin (Caterina et al., 1997). The interaction between AEA and vanilloid receptors was originally controversial because the concentrations of AEA necessary to induce the typical TRPV1 responses appeared to be higher than those required for CB_1 activation (Szolcsanyi, 2000). Recent data indicate, however, that AEA potency at vanilloid receptors is influenced by several factors, and might be sensibly enhanced under particular conditions. These factors include different assay conditions and, in particular, the ability of AEA to reach the intracellular binding site of TRPV1 receptors. Because of the "short life" of endocannabinoids, it is likely that the "endovanilloid" activity of AEA may be increased by retarding its physiological inactivation, possibly through the modulation of the molecular mechanisms that decrease intracellular AEA concentration or improve its interaction with TRPV1. In fact, activation of AMT, inhibition of FAAH and co-treatment with AEA congeners, independently of FAAH, significantly increase the activity of extracellular AEA at vanilloid receptors (De Petrocellis et al., 2001a,b; Smart et al., 2002). Furthermore, since CB_1 stimulation may have opposite effects to those caused by TRPV1 stimulation, the presence of CB_1 antagonists may enhance the apparent potency of AEA at TRPV1, and this is particularly important in tissues were the two receptors are co-expressed (Ahluwalia et al., 2003). Finally, new evidence indicates that the potency of AEA and NADA at TRPV1 receptors can be increased by intracellular events activate during pathological conditions such as PKC- and PKA-mediated phosphorylation of TRPV1 (Premkumar and Ahern, 2000; Premkumar et al., 2004; De Petrocellis et al., 2001c). A typical example of how AEA can activate TRPV1 during pathological conditions is represented by the finding that the levels of AEA increase in inflamed ileum of rats treated with toxin A, and that subsequently AEA mediates the inflammatory effects of toxin A through a TRPV1-dependent mechanism (McVey et al., 2003).

In conclusion, considerable progress has been made in this research field although multidisciplinary expertise will be necessary to fully understand the action and the function of the endocannabinoid system. In particular it will be necessary: to assess the physiological role of virodhamine, NADA and 2-AGE and find their biosynthetic pathways; to develop selective inhibitors of endocannabinoids biosynthesis; to clone the AMT, if such a protein does exist, and the novel receptors proposed for AEA. It is possible to foresee that the complete knowledge of the role of the endocannabinoid system in physiological and pathological conditions will allow the development of new drugs whose mechanism of action might be based on the modulation of this system. This might lead to innovative therapeutically strategies to counteract the symptoms or the progress of central and peripheral diseases.

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