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MINI REVIEW

The endocannabinoid system: a general view and latest additions

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After the discovery, in the early 1990s, of specific G-protein-coupled receptors for marijuana's psychoactive principle Δ^9-tetrahydrocannabinol, the cannabinoid receptors, and of their endogenous agonists, the endocannabinoids, a decade of investigations has greatly enlarged our understanding of this altogether new signalling system. Yet, while the finding of the endocannabinoids resulted in a new effort to reveal the mechanisms regulating their levels in the brain and peripheral organs under physiological and pathological conditions, more endogenous substances with a similar action, and more molecular targets for the previously discovered endogenous ligands, anandamide and 2-arachidonoylglycerol, or for some of their metabolites, were being proposed. As the scenario becomes subsequently more complicated, and the experimental tasks to be accomplished correspondingly more numerous, we briefly review in this article the latest 'additions' to the endocannabinoid system together with earlier breakthroughs that have contributed to our present knowledge of the biochemistry and pharmacology of the endocannabinoids.
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Abbreviations: AEA, *N*-arachidonoyl-ethanolamine (anandamide); 2-AG, 2-arachidonoyl-glycerol; 2-AGE, 2-arachidonyl-glyceryl ether (noladin); AMT, anandamide membrane transporter; DAG, diacylglycerol; FAAH, fatty acid amide hydrolase; GPCRs, G-protein-coupled receptors; MAGLs, monoacylglycerol lipase; NADA, *N*-arachidonoyl-dopamine; NAPE, *N*-acyl-phosphatidylethanolamines; NAPE-PLD, NAPE-selective PLD; NArPE, *N*-arachidonoyl-phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphoinositides; PI-PLC, PI-selective phospholipase C; PLD, phospholipase D; *sn*-1 DAGL, *sn*-1-selective DAG lipase; THC, (-)-Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid type 1 receptor; virhodamine, *O*-arachidonoyl-ethanolamine

Introduction

The discovery in the early 1990s of specific membrane receptors of marijuana's psychoactive component (-)-Δ^9-tetrahydrocannabinol (THC) opened the way to the revelation of a whole endogenous signaling system now known as the *endocannabinoid system*. Apart from the cannabinoid CB_1 and CB_2 receptors (Pertwee, 1997), this system comprises also their endogenous ligands (the *endocannabinoids*) and the proteins for their synthesis and inactivation, as well as other molecular targets for the endocannabinoids. However, as new findings on the regulation of the levels and action of the endocannabinoids, and new data on their possible physiological and pathological role, are reported every day in the literature, it is easy to understand that the story of the endocannabinoid system is far from set. For example, while until the end of the 20th century only two endocannabinoids, anandamide (*N*-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG) had been discovered (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995), in just a couple of years, three more candidates to the role of cannabinoid receptor agonists have been proposed: 2-arachidonyl-glyceryl ether (noladin, 2-AGE), *O*-arachidonoyl-ethanolamine (virhodamine) and *N*-arachidonoyl-dopamine (NADA) (Bisogno *et al*., 2000; Huang *et al*., 2002; Porter *et al*., 2002). These findings not only suggest that the endocannabinoid family is larger than initially thought but also enlarge our view on the possible molecular mechanisms for the biosynthesis, action and inactivation of these lipid mediators. This brief article aims at giving a picture as much updated as possible on the 'old' and 'new' components of the endocannabinoid system, while highlighting the latest and most important findings in this field.

Regulation of the endocannabinoid signal – formation

Any molecule acting as an endogenous mediator of physiological and pathological responses needs mechanisms for its biosynthesis and, following its action on specific molecular targets, for its rapid inactivation. Based on original observations carried out with AEA (Di Marzo *et al*., 1994), it was postulated that this endocannabinoid is not stored in 'resting' cells but is, instead, synthesized and released 'on demand' following physiological and pathological stimuli, such as neuronal depolarization and bacterial lipopolysaccharides, possibly depending on the Ca^{2+}-dependent remodelling of phospholipid precursors (Di Marzo *et al*., 1998b). Although this mechanism holds true also for 2-AG, which apart from being an endocannabinoid is also an intermediate in (phospho)

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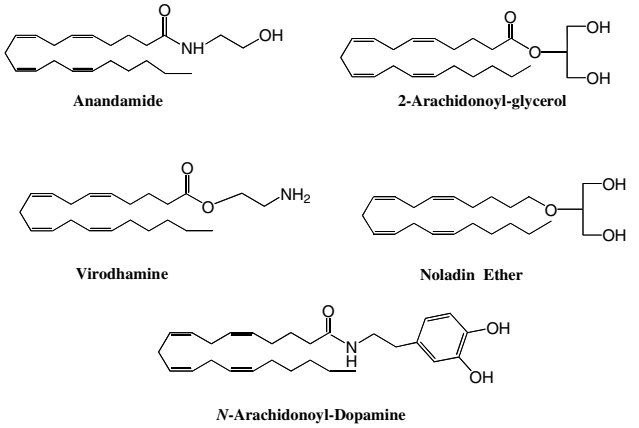

Figure 1 Established and newly proposed endocannabinoids. Chemical structures of the five endogenous cannabinoid ligands identified so far.

glyceride metabolism, no conclusive data on the biosynthetic mechanisms of noladin, NADA and virodhamine have been reported so far (Figure 1). In general, pharmacological and electrophysiological data have shown that both Ca^{2+} influx and activation of some metabotropic receptors, either co-operatively or independently, can induce the formation of nonchemically identified endocannabinoids acting as retrograde synaptic signals (for a review, see Wilson & Nicoll, 2002). After their biosynthesis, AEA and 2-AG are immediately released into the extracellular milieu *via* a mechanism still unknown, which data from our and other laboratories suggests as being dependent on the same putative membrane transporter proposed to underlie endocannabinoid cellular uptake (see below). Extracellular endocannabinoids then act mostly, and with varying selectivity, on cannabinoid receptors, possibly including subtypes other than CB_1 and CB_2. Endocannabinoids such as AEA and/or NADA may also act on intracellular sites of ion channels, such as those of vanilloid TRPV1 receptors and T-type Ca^{2+} channels. The termination of the action of the endocannabinoids on extracellular targets is achieved through their reuptake by cells, a process that can be facilitated by yet-to-be-characterized membrane transporters, usually followed by one or more enzymatic reactions.

Biosynthesis of AEA and its congeners

The metabolic pathways of the class of lipids to which AEA belongs, the *N*-acylethanolamines, have been investigated since the 1960s. Work performed by H Schmid's group in the 1970s and 1980s (for reviews, see Schmid *et al*., 1996; 2002) had shown that these compounds are biosynthesized *via* a phospholipid-dependent pathway (Figure 2), that is the enzymatic hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamines (NAPEs). The enzyme catalyzing this reaction is a phospholipase D (PLD) selective for NAPEs (NAPE-PLD) with low affinity for other membrane phospholipids. NAPEs, in turn, are produced through the acyl transfer from the *sn*-1 position of phospholipids to the *N*-position of phosphatidylethanolamine (PE), catalyzed by a Ca^{2+}-dependent *trans*-acylase. Neither the PLD nor the *trans*-acylase appeared to exhibit any selectivity for a particular fatty acid moiety. Indeed, this route was shown to underlie also AEA biosynthesis in central neurons after depolarization (Di Marzo *et al*., 1994), and subsequent studies (Di Marzo *et al*., 1996; Sugiura *et al*., 1996; Cadas *et al*., 1997) confirmed the occurrence of *N*-arachidonoyl-phosphatidylethanolamine (NArPE), the NAPE precursor of AEA, in murine brain, testes and leukocytes, and showed that cell-free preparations could convert NArPE into AEA and biosynthesize NArPE from phospholipid precursors (Figure 2). The enzymes catalyzing these reactions were only partially characterized, and, in particular, the NAPE-PLD was shown later to lack the *trans*-phosphatidylating activity typical of other PLD enzymes (Petersen & Hansen, 1999), to be dependent on Ca^{2+} for optimal activity (Ueda *et al*., 2001) and to be stimulated by polyamines (Liu *et al*., 2002). The precursor/product relationship between NArPE and AEA, at least in the CNS, was later confirmed, among other things, by the findings of a similar distribution of the two compounds in nine different brain areas (Bisogno *et al*., 1999a), and of levels of NArPE and AEA that increase correspondingly in rat brain during the course of its development (Berrendero *et al*., 1999).

Biosynthesis of 2-AG

The levels of 2-AG in unstimulated tissues and cells are usually much higher than those of AEA, and are sufficient in principle to permanently activate both cannabinoid receptor subtypes (Sugiura *et al*., 1995; Stella *et al*., 1997). However, these amounts are probably overestimated due to artefactual 2-AG production, for example following rat decapitation (Sugiura *et al*., 2001). At any rate, the 2-AG found in cells and tissues is probably not uniquely used to stimulate cannabinoid receptors, as this compound is at the crossroads of several metabolic pathways, and is an important precursor and/or degradation product of phospho-, di- and tri-glycerides, as well as of arachidonic acid. It is likely that a particular pool of 2-AG is produced *via* a special pathway only for the purpose of functioning as endocannabinoid. Of the several possible biosynthetic pathways already known for 2-AG formation (for a review, see Sugiura *et al*., 2002), those routes leading to the synthesis of an endocannabinoid should be Ca^{2+} dependent since the *de novo* formation of 2-AG, like for AEA, is induced in neurons by membrane depolarization (Bisogno *et al*., 1997b; Stella *et al*., 1997). Several stimuli have been shown to lead to the formation of 2-AG in intact neuronal and non-neuronal cells (Sugiura *et al*., 2002), but only seldom the pathways for 2-AG biosynthesis have been investigated (Stella *et al*., 1997; Bisogno *et al*., 1999b; Berdyshev *et al*., 2001). In most cases (Figure 2), 2-AG is produced from the hydrolysis of 2-arachidonate-containing diacylglycerols (DAGs) catalyzed by an *sn*-1-selective DAG lipase (*sn*-1 DAGL). DAGs serving as 2-AG precursors can be produced in turn from the hydrolysis of 2-arachidonate-containing phosphatidic acid (PA), catalyzed by a PA phosphohydrolase, or of phosphoinositides (PI), catalyzed by PI-selective phospholipase C (PI-PLC). Two *sn*-1 DAGL isozymes have been just cloned and enzymatically characterized (Bisogno *et al*., 2003). They are mostly found in the plasma membrane, are stimulated by Ca^{2+}, appear to possess a catalytic triad typical of serine hydrolases, and catalyze in intact cells the Ca^{2+}-dependent formation of 2-AG with endocannabinoid function. Furthermore, the two enzymes exhibit a pattern of expression fitting with the proposed role of 2-AG either as a mediator of neurite growth, during brain development (Berrendero *et al*., 1999; Williams *et al*., 2003), or

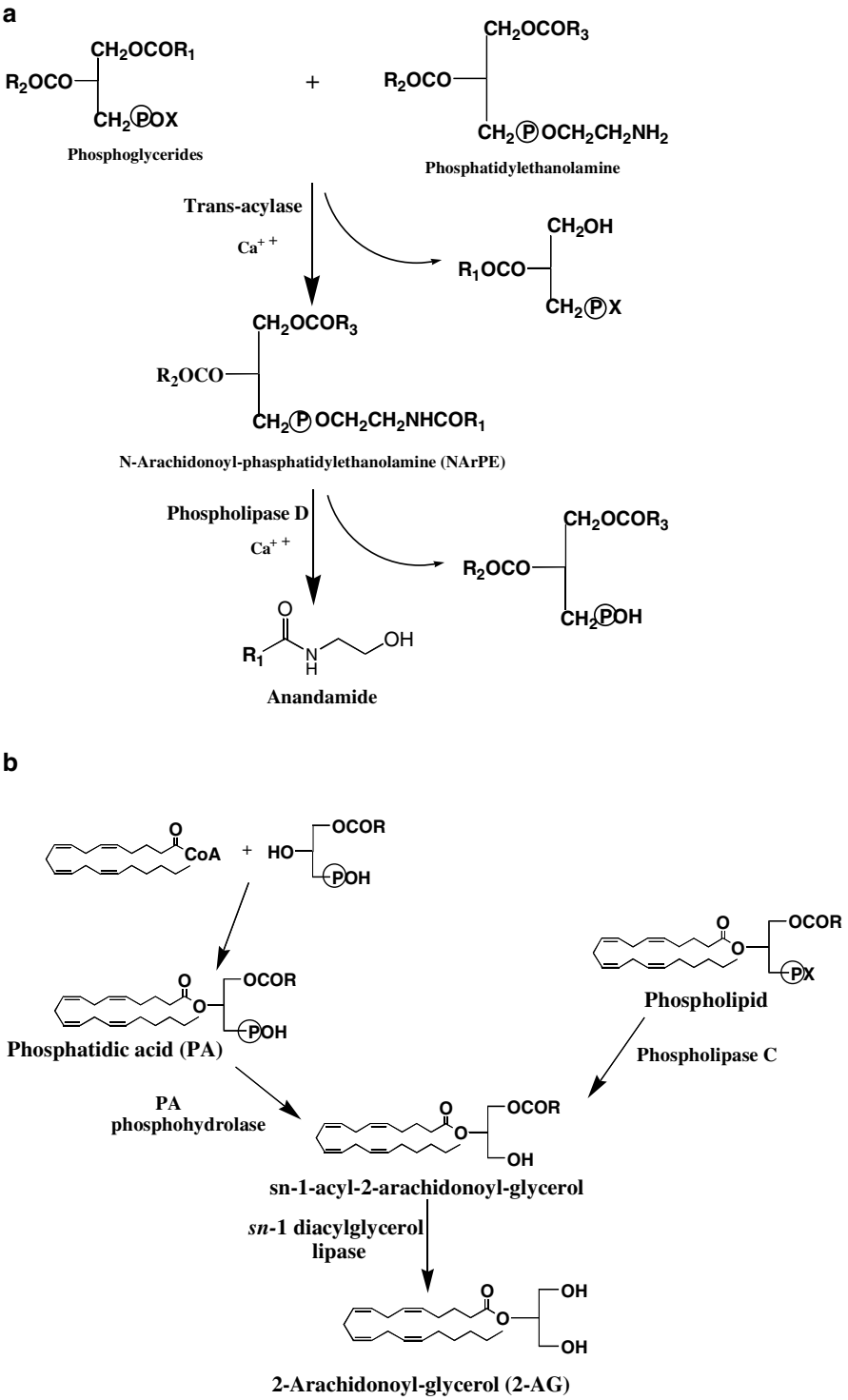

Figure 2 Proposed biosynthetic pathways of (a) anandamide and (b) 2-AG. P denotes a phosphate group, R_1 the acyl chain in arachidonic acid, R, R_2 and R_3 acyl chains of other fatty acids, and X the base in phosphoglycerides. In the case of 2-AG, the phosholipids mostly found to act as precursors for the *sn*-1-acyl-2-arachidonoyl-glycerol are phosphoinositides.

as retrograde signal mediating depolarization-induced suppression of neurotransmission and heterosynaptic plasticity, in the adult brain (for reviews, see Wilson & Nicoll, 2002; Chevaleyre & Castillo, 2003). In fact, the enzymes are located on axons during development and postsynaptically in adult neurons (Bisogno *et al*., 2003). Inhibitors of these DAGLs, and hence, most likely, of 2-AG biosynthesis, can now be developed and they might prove useful pharmacological tools to dissect the role of 2-AG from that of other endocannabinoids in many physiological and pathological conditions.

Biosynthesis of noladin, virodhamine and NADA

Still very little is known on the biosynthesis of the three most recently proposed endocannabinoid, 2-AGE, virodhamine and NADA. Regarding the former compound, it must be said that

its finding in mammalian tissues (Hanus *et al*., 2001; Fezza *et al*., 2002) raised some controversy, since: (1) the only acyl ethers found to occur in animals previously were 2-acyl ethers (e.g. alkenyl ethers such as platelet-activating factor and plasmalogens); and (2) the enzymes catalyzing the formation of these compounds from fatty acyl alcohols are known to have *sn*-1 specificity and to recognize short-medium chain, saturated fatty acids. Indeed, data against the existence of 2-AGE in the rat brain have been recently published (Oka *et al*., 2003), although it must remembered that the occurrence of AEA, now a well-established fact, had also been doubted immediately after its discovery (Kempe *et al*., 1996). Some as yet unknown biosynthetic pathway, different from that leading to plasmalogens, may exist for this compound. We have found that neuroblastoma N18TG2 intact cells are not capable of converting arachidonate-containing phospholipids into 2-AGE when stimulated with ionomycin, that is under conditions where high levels of 2-AG are produced (Fezza *et al*., 2002). This might suggest a nonphospholipid-mediated, or a Ca^{2+}-independent pathway for the formation of this putative endocannabinoid in neurons.

No data have been published, instead, regarding the biosynthesis of virodhamine, which seems to accompany AEA in all tissues analyzed (Porter *et al*., 2002). The reported nonenzymatic transformation of *N*-acylethanolamines into the corresponding *O*-acyl esters, and *vice versa* (Markey *et al*., 2000), in the presence of bases or acids, might suggest that virodhamine, which is an antagonist at cannabinoid CB_1 receptors and a partial agonist at CB_2 receptors, and anandamide, which, conversely, is an antagonist at cannabinoid CB_2 receptors and a partial agonist at CB_1 receptors, might be converted into each other, with a subsequent switch from CB_1- to CB_2-mediated responses, and *vice versa*.

Original evidence for the formation of NADA from arachidonic acid and dopamine or tyrosine (Huang *et al*., 2002) suggested biosynthetic pathways common to those of either the recently discovered arachidonoyl amino acids (Huang *et al*., 2001) or of dopamine. Preliminary data suggest that NADA is produced uniquely following the reaction between dopamine and arachidonoylCoA (F Fezza, MJ Walker & V Di Marzo, unpublished observations).

Regulation of the endocannabinoid signal – inactivation

Cellular reuptake

The first step in the inactivation *endocannabinoids* consists of their being rapidly ($t_{1/2} \leq 5$ min) cleared away from their extracellular molecular targets. Being lipophilic in nature, endocannabinoids can diffuse through the plasma membrane if their amount in the extracellular milieu is higher than their intracellular concentration. However, in order to be rapid, this process needs to be driven by controllable and selective mechanisms, such as either a membrane transporter protein, or an intracellular enzymatic process capable of rapidly reducing the intracellular concentration of the two compounds, or both (Figure 3). Indeed, AEA and 2-AG are taken up by cells *via* selective, saturable, temperature-dependent and Na^+-independent 'facilitated transport' mechanisms, known as the anandamide membrane transporter(s) (AMT) (Di Marzo *et al*., 1994; Beltramo *et al*., 1997; Bisogno *et al*., 1997a; Hillard *et al*., 1997; Maccarrone *et al*., 1998; 2000a, b; see also Fowler & Jacobsson *et al*., 2002 for a review). If one or more proteins is (are) responsible for these mechanisms, however, it (they) has (have) not yet been isolated nor cloned. Overall, the collected data suggest that the same mechanism mediates the cellular uptake of all endocannabinoids (Fezza *et al*., 2002; Huang *et al*., 2002; Wilson & Nicoll, 2002), although none of the selective

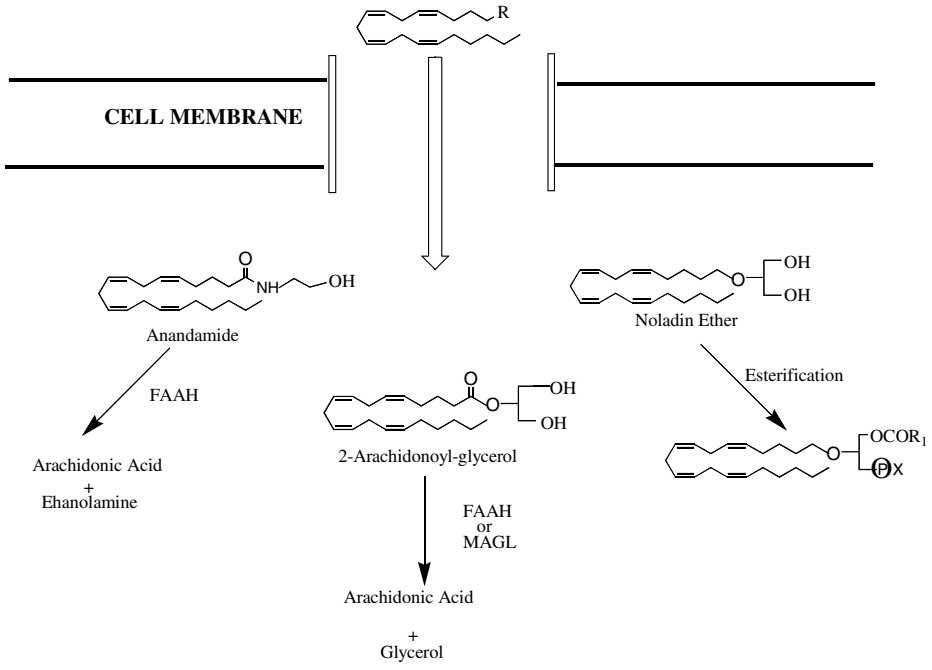

Figure 3 Inactivation (cellular reuptake and intracellular metabolism) of anandamide and 2-arachidonoylglycerol (2-AG). P denotes a phosphate group, R the head group of the endocannabinoid, R_3 the acyl chain of other fatty acids and X the base in phosphoglycerides. FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase.

inhibitors of the putative endocannabinoid transporter developed so far (De Petrocellis *et al*., 2000; Lopez-Rodriguez *et al*., 2001; Ortar *et al*., 2003) has been tested yet on virodhamine, 2-AGE and NADA. However, it must be emphasized that until molecular evidence for the AMT is found, its existence will have to be doubted *bona fide*. Some authors have suggested that intracellular degradation of AEA by the microsomial enzyme mostly responsible for its hydrolysis, the 'fatty acid amide hydrolase' (FAAH, see below), can be sufficient alone to drive the facilitated diffusion of this compound from the extracellular milieu into the extracellular space (Glaser *et al*., 2003). Nevertheless, several observations still strongly, albeit indirectly, support the existence of the AMT (for a more detailed review, see Hillard & Jarrahian, 2003): (1) several cell types can be found that can rapidly take up AEA from the extracellular medium even though they do not express FAAH; (2) several compounds have been developed that are capable of inhibiting AEA cellular uptake without inhibiting AEA enzymatic hydrolysis *via* FAAH (De Petrocellis *et al*., 2000; Di Marzo *et al*., 2001b, c; Lopez-Rodriguez *et al*., 2001; Ortar *et al*., 2003); FAAH inhibitors enhance, and anandamide uptake inhibitors inhibit, anandamide accumulation in cells (Kathuria *et al*., 2003); (3) substances that inhibit the AMT *enhance* the effect of AEA that are exerted at extracellular sites (i.e. at CB_1 receptors) and *inhibit* those that are exerted at intracellular targets (i.e. VR1 receptors, see De Petrocellis *et al*., 2001) – if these compounds were simply acting by inhibiting FAAH, they should enhance AEA effects in both cases; (4) noladin and NADA, two endocannabinoids that are either resistant or refractory to enzymatic hydrolysis, respectively, are still taken up by cells in a temperature-dependent way, and their uptake is inhibited competitively by AEA (Fezza *et al*., 2002; Huang *et al*., 2002) and (5) lipopolysaccharide inhibits FAAH expression without affecting AEA cellular uptake (Maccarrone *et al*., 2001); conversely, nitric oxide, peroxynitrite and superoxide anions stimulate AEA cellular reuptake (Maccarrone *et al*., 2000a), while acute or chronic ethanol inhibit this process (Basavarajappa *et al*., 2003), without affecting FAAH activity. These data suggest that, although intracellular hydrolysis does greatly influence the rate of AEA-facilitated diffusion, the uptake process might still be mediated by a mechanism distinct from the one catalyzing AEA hydrolysis.

Endocannabinoid metabolism – hydrolysis

FAAH, an enzyme originally purified and cloned from rat liver microsomes (Cravatt *et al*., 1996), catalyzes the hydrolysis of both AEA (as well as other long-chain fatty acid amides) and 2-AG to arachidonic acid and ethanolamine or glycerol, respectively. Since the enzyme also catalyzes the hydrolysis of arachidonoyl methyl ester, it is possible that virodhamine is also one of its substrates. This possibility, however, has not been tested yet. The structural and kinetic properties of FAAH have been widely reviewed in the literature (for a recent review, see Cravatt & Lichtman, 2003) and several more or less selective FAAH inhibitors have also been developed (for a review, see Deutsch *et al*., 2002). FAAH has an alkaline optimal pH and is found in microsomial membranes. Its hydrophobic domain is important for the formation of active oligomers, whereas its localization on intracellular membranes might be regulated by an SH-3 consensus, proline-rich sequence also necessary for enzymatic activity. The catalytic amino acid of FAAH has been identified as Ser241, and two other residues of the amidase consensus sequence, Ser217 and Cys249, participate in the enzymatic activity. Recently, the X-ray structure of FAAH crystals in complex with its substrate was elucidated (Bracey *et al*., 2002) and suggested that the enzyme might be able to associate with the plasma membrane. The promoter region on the FAAH gene has been identified (Puffenbarger *et al*., 2001; Waleh *et al*., 2002), and seems to be upregulated by progesterone and leptin (Maccarrone *et al*., 2003a, b), and downregulated by estrogens and glucocorticoids (Waleh *et al*., 2002).

FAAH 'knockout' mice have been developed that are more responsive to exogenously administered AEA (Cravatt *et al*., 2001), and whose brains contain 15-fold higher levels of brain AEA than wild-type mice. The phenotype of these mice is also characterized by lower sensitivity to some painful stimuli, which suggests that inhibition of FAAH might lead to the development of novel analgesics. 2-AG levels were not increased in FAAH 'knockout' mice (Lichtman *et al*., 2002), in agreement with previous reports on the existence of additional hydrolases for 2-AG degradation in porcine brain and rat circulating platelets and macrophages, as well as in mouse J774 macrophages (Di Marzo *et al*., 1999; Goparaju *et al*., 1999). This(ese) enzyme(s), known as monoacylglycerol lipase(s) (MAGLs), is(are) found in both membrane and cytosolic fractions and also recognize(s) other unsaturated monoacylglycerols, such as, for example, mono-oleoyl-glycerol, which indeed acts as a competitive inhibitor for 2-AG inactivation by intact cells (Ben-Shabat *et al*., 1998; Di Marzo *et al*., 1998a). A MAGL with enzymatic properties and subcellular distribution very similar to this(ese) roughly characterized enzyme(s) has been cloned (Karlsson *et al*., 1997; Dinh *et al*., 2002). As previously found for FAAH (Egertova *et al*., 1998), rat MAGL is expressed in brain regions with high cannabinoid CB_1 receptor density, such as the hippocampus, but, unlike FAAH, it occurs in presynaptic neurons and is likely to be coexpressed with CB_1 receptors (Dinh *et al*., 2002). This finding supports the role of rat MAGL in the degradation of that pool of 2-AG that acts as a retrograde endocannabinoid synaptic signal. No selective inhibitor has been developed yet for this enzyme.

Endocannabinoid metabolism – esterification, oxidation and other reactions

Unlike AEA, 2-AG can be esterified into (phospho)glycerides directly, that is prior to its hydrolysis by FAAH or MAGL, *via* phosphorylation and/or acylation of its two free hydroxyl groups (for a review, see Sugiura *et al*., 2002). This pathway was suggested to occur in mouse N18TG2 neuroblastoma and rat basophilic leukemia (RBL-2H3) cells and in mouse J774 macrophages (Di Marzo *et al*., 1998a, b; 1999). Direct esterification provides a mechanisms for the inactivation of also noladin (Fezza *et al*., 2002), whose ether bond cannot be hydrolyzed enzymatically.

Enzymatic oxidation of the arachidonoyl moiety of endocannabinoids is catalyzed, at least in cell free homogenates and in some intact cells *in vitro*, by enzymes of the arachidonate cascade, that is lipoxygenases, cyclooxygenase-2 and cytochrome p450 oxidases (for a review, see Kozak & Marnett, 2002a). Some of these reactions might be important for those

endocannabinoids, like NADA and noladin, which are stable or refractory to enzymatic hydrolysis. Cyclooxygenase-2, and subsequent action of the various prostaglandin synthases, transforms AEA and 2-AG into the corresponding prostaglandin ethanolamides (or prostamides) and prostaglandin glyceryl esters, which, in some assays, exhibit high potency even though they have no activity on either cannabinoid or prostanoid receptors (Woodward *et al*., 2001; Ross *et al*., 2002). These metabolites are rather stable to further metabolism, except for prostamide E_2 that undergoes slow dehydration/isomerization to prostamide B_2 (Kozak *et al*., 2001). Prostaglandin E_2 glyceryl ester is rapidly hydrolyzed in rat, but not in human, plasma (Kozak *et al*., 2001). Regarding lipoxygenase products of AEA and 2-AG, these are formed with both 12- and 15-, but not so easily with 5-, lipoxygenases (Kozak & Marnett, 2002a; van der Stelt *et al*., 2002). In the case of AEA, only the 12-hydroxy-derivative still binds to cannabinoid receptors (Edgemond *et al*., 1998; van der Stelt *et al*., 2002). Undefined lipoxygenase products of AEA have been suggested to act *via* vanilloid TRPV1 receptors (see below) (Craib *et al*., 2001), although there is no direct evidence for the interaction of hydroxy-anandamides or leukotriene-ethanolamides with these receptors. The 15-(*S*)-hydroxy-derivative of 2-AG was recently shown to be formed in intact cells, and to activate the peroxisome proliferation activator receptor-α (Kozak *et al*., 2002b). In general, it can be hypothesized that oxidation of AEA and 2-AG, while leading to the partial or complete inactivation of their 'cannabinergic' signal, might produce in some cases compounds active on other molecular targets, thus potentially representing a switch from a type of signal to another.

Finally, the cathecolamine moiety of NADA is likely to be subject to both enzyme-catalyzed and nonenzymatic oxidation. Methylation of the 3-hydroxy-group of NADA by catecholamine *O*-methyl transferase has indeed been observed (Huang *et al*., 2002). The reaction product is significantly less active on TRPV1 receptors (Huang *et al*., 2002), but its activity at CB_1 receptors has not been investigated.

Endocannabinoid mechanism of action – old and new

By definition, endocannabinoids are supposed to act primarily at cannabinoid CB_1 and/or CB_2 receptors, and they do so with varying efficacy, depending also on the type of intracellular event that one measures (for a review, see Mukhopadhyay *et al*., 2002). AEA, NADA and 2-AGE are functionally more selective for CB_1, virodhamine appears to prefer CB_2 and 2-AG is equipotent at both receptor subtypes. Functional stimulation of CB_1/CB_2 by endocannabinoids triggers, *via* activation of $G_{i/o}$ proteins, those intracellular signalling events that are normally coupled to these two G-protein-coupled receptors (GPCRs) (Pertwee, 1997), that is: (i) inhibition of stimulus-induced adenylate cyclase, and subsequent impairment of cAMP/protein kinase A-mediated short- and long-term effects; (ii) stimulation of mitogen-activated protein kinase signalling; (iii) in the case of CB_1 receptors, inhibition of voltage-gated, P, Q and N-type Ca^{2+} channels, and stimulation of inwardly rectifying G-protein-coupled K^+ channels and (iv) again only in the case of CB_1, stimulation of phosphatidylinositol 3-kinase and of intracellular Ca^{2+} mobilization, seemingly through activation of PLC-γ, by the $\beta\gamma$ subunits of $G_{i/o}$ proteins (for a review, see Guzman *et al*., 2002).

There is now mounting evidence, based so far only on pharmacological and biochemical data, for the existence of non-CB_1, non-CB_2 GPCRs for endocannabinoids, and for AEA in particular (for a comprehensive review, see Di Marzo *et al*., 2002b). Of these possibly novel sites of action, two are activated by physiologically relevant concentrations of AEA. The first one was proposed to be present in murine astrocytes and found to respond also to the noncannabinoid agonist of CB_1 and CB_2 receptors, WIN55.212-2 (Sagan *et al*., 1999). This, or a very similar, receptor appears to inhibit adenylyl cyclase and, by blocking gap-junctions, Ca^{2+} signalling in astrocytes. A GPCR with a distribution different from CB_1 receptors and sensitive to both AEA and WIN55.212-2, but not to other cannabinoid receptor agonists, was indeed described in several brain areas of CB_1 knockout mice (Breivogel *et al*., 2001). This receptor might be similar to the site of action suggested to mediate the inhibitory effect of WIN-55,212-2 on glutamate release in the mouse hippocampus (Hajos & Freund, 2002), and to be sensitive to amides of fatty acids with vanillyl moieties, such as arvanil and its analogues (Di Marzo *et al*., 2001b,c; 2002c), and to capsaicin (Hajos and Freund, 2002). Finally, a different GPCR for AEA and the nonpsychtropic cannabinoid, abnormal-cannabidiol, has been described in vascular endothelial cells. This putative receptor mediates the local vasodilatory, but not the systemic hypotensive, effects of AEA, and is blocked by both cannabidiol and a synthetic analogue O-1981 (Járai *et al*., 1999; Offertaler *et al*., 2003). It appears to be coupled to guanylyl cyclase or to p42/44 mitogen-activated protein kinase and protein kinase B/Akt.

Some plasma membrane channels involved in Ca^{2+} and K^+ homeostasis also appear to be targeted directly by submicromolar concentrations of AEA (Di Marzo *et al*., 2002b). TASK-1 K^+ channel (Maingret *et al*., 2001) and T-type Ca^{2+} channels (Chemin *et al*., 2001) are blocked by AEA, whereas vanilloid type 1 (VR1, also known as TRPV1) receptors, the sites of action of the pungent component of 'hot' red peppers, capsaicin (Szallasi & Blumberg, 1999), are activated by this endocannabinoid (Zygmunt *et al*., 1999; Smart *et al*., 2000). In heterologous expression systems, the potency of AEA to induce typical TRPV1-mediated effects (e.g. cation currents, Ca^{2+}-influx and cell depolarization) is 5–20-fold lower than its average potency at CB_1 receptors. However, recent data (for a review, see Di Marzo *et al*., 2001a; 2002a) indicate that the potency of AEA at TRPV1 can be increased up to 10-fold by factors occurring during pathological states, and the list of TRPV1-mediated pharmacological effects of this endocannabinoid is becoming increasingly longer. Importantly, a recent study showed that increased levels of endocannabinoids acting at TRPV1 cause ileitis in toxin A-treated rats (McVey *et al*., 2003). Furthermore, blockade of CB_1 receptors unmasks TRPV1-mediated effects of AEA exerted at low concentrations (Ahluwalia *et al*., 2003). These latter findings support the view that, at least under certain conditions, TRPV1 and CB_1 receptors may be considered as ionotropic and metabotropic receptors for the same class of endogenous fatty acid amides, including so far AEA and NADA (Di Marzo *et al*., 2002a, b). This latter compound activates TRPV1 in isolated cells with potency and efficacy higher than AEA and only slightly lower than capsaicin (Huang *et al*., 2002). Importantly, the

stimulatory effect of AEA on TRPV1 receptors (De Petrocellis *et al*., 2001), as well as the inhibitory action on T-type Ca^{2+} channels (Chemin *et al*., 2001), are exerted by binding to sites on the cytosolic side of these proteins. This observation raises the intriguing possibility that AEA, when biosynthesized by cells that express TRPV1 (such as sensory and central neurons, keratinocytes and epithelial cells), activates this protein *prior* to its release from cells and its action on other targets on the same or nearby neurons. On the other hand, when extracellular AEA activates sequentially CB_1 and VR1 receptors in the same cell, this leads to an enhancement of its VR1-mediated responses (Hermann *et al*., 2003).

The endocannabinoid system: a lot done, and yet much more to do

In consideration of the fact that the cloning of the first cannabinoid receptor was only reported in 1990, and the first endocannabinoid discovered only 2 years later, considerable progress has been made, in little more than a decade, towards the understanding of the regulation of the 'cannabinergic' signal. The endocannabinoid system appears to be conserved at least in all vertebrate phyla, and to be present, possibly with some major differences in the structure of receptors and in their function, also in invertebrates (Salzet & Stefano, 2002; McPartland & Glass, 2003), thus implying its participation in vital functions. Also from the pharmacological and pharmaceutical points of view, great breakthroughs in this field have been achieved, last but not least the fact that drugs or preparations targeting the cannabinoid receptors might soon be on the market. Sativex®, an extract of *Cannabis* strains with a known concentration of THC and cannabidiol, in the form of a sublingual spray, useful in the treatment of symptoms of multiple sclerosis, and rimonabant® (SR141716A), a selective CB_1 antagonist that might be marketed in the future as an antiobesity drug, are the two examples of a possible entirely new generation of products to be developed from the endocannabinoid system. New therapeutic drugs might be obtained in the future also from selective inhibitors of endocannabinoid biosynthesis and inactivation, but first several other milestones will have to be reached. In particular, it will be necessary:

- to clone the key enzymes catalyzing AEA biosynthesis and to understand their regulation at the molecular level;
- to find biosynthetic pathways for virodhamine, NADA and 2-AGE, and to clarify their regulation;
- to establish transgenic mice lacking functional genes for endocannabinoid biosynthesis, and to study their phenotype;
- to develop selective and potent inhibitors of endocannabinoid biosynthesis and of 2-AG degradation that can be used *in vivo*;
- to clone the novel receptors proposed for AEA and to establish their actual role in endocannabinoid biology;
- to understand conclusively the relationships between the cannabinoid receptor- and noncannabinoid receptor (e.g. TRPV1)-mediated actions of AEA (and NADA);
- to carry on identifying, first in animal models and then possibly in the clinic, those pathological conditions that can be caused, at least in part, by a malfunctioning endocannabinoid system, or whose onset, progress and/or symptoms are controlled tonically by the endocannabinoids.

The joint multidisciplinary effort of many scientists will be necessary to fulfill these task in the shortest time possible, in order to understand fully the physiological and pathological function of the endocannabinoid system, and to assess finally if further endocannabinoid-based medicines with advantages over other drugs are to be developed in the future.

Note added in proof

Another breakthrough in endocannabinoid research has recently been achieved with the cloning of the NAPE-PLD for AEA biosynthesis (Okamoto *et al*., J. Biol. Chem., in press). The enzyme is a member of the zinc metallo-hydrolase family.

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