The Life Cycle of the Endocannabinoids: Formation and Inactivation

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Contents

Abstract In this chapter, we summarise the current thinking about the nature of endocannabinoids. In describing the life cycle of these agents, we highlight the synthetic and catabolic enzymes suggested to be involved. For each of these, we provide a systematic analysis of information on sequence, subcellular and cellular distribution, as well as physiological and pharmacological substrates, enhancers and inhibitors, together with brief descriptions of the impact of manipulating enzyme levels through genetic mechanisms (dealt with in more detail in the chapter "Genetic Models of the Endocannabinoid System" by Monory and Lutz, this

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volume). In addition, we describe experiments investigating the stimulation of endocannabinoid synthesis and release in intact cell systems.

Keywords 2-arachidonoylglycerol • Anandamide • Diacylglcyerol lipase • Endocannabinoid turnover • Fatty acid amide hydrolase • N-acylphosphatidylethanolamine phospholipase D

Abbreviations

2AG	2-Arachidonoylglycerol
2AG-3P	2-Arachidonoylglycerol-3-phosphate
AEA	Anandamide, *N*-arachidonoylethanolamine
COX	Cyclooxygenase
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
DSI	Depolarization-evoked suppression of inhibition
ECB	Endocannabinoid
EET	Epoxyeicosatrienoic acid
Epac	Exchange protein activated by cyclic AMP
FAAH	Fatty acid amide hydrolase
LOX	Lipoxygenase
LPI	Lysophosphatidylinositol
LPLC	Lysophospholipase C
LPLD	Lysophospholipase D
lysoNAPE	Lyso-*N*-acylphosphatidylethanolamine
MAFP	Methylarachidonylfluorophosphonate
MGL	Monoacylglycerol lipase
NAAA	*N*-Acylethanolamine acid amidase
NAE	*N*-Acylethanolamine
NAPE	*N*-Acylphosphatidylethanolamine
ODA	Oleamide
OEA	*N*-Oleoylethanolamine
PE	Phosphatidylethanolamine
PEA	*N*-Palmitoylethanolamine
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
SEA	N-Stearoylethanolamine
THL	Tetrahydrolipstatin

1 Cannabinoid Signalling in the CNS

The widely accepted phenomenon of synaptic plasticity highlights the fact that the efficiency of synaptic transmission can alter, dependent on the local environment. Two relevant aspects of synaptic plasticity involving cannabinoid receptors are depolarization-evoked suppression of excitation and inhibition (Gerdeman and Lovinger 2003; Diana and Marty 2004). Although dealt with in more detail in the chapter "Endocannabinoid Signaling in Neural Plasticity" by Brad Alger in this volume, in brief, these phenomena are proposed to result from transmitter-mediated post-synaptic depolarization of neurones leading to an elevation of intracellular calcium ions, resulting in the generation of a retrograde messenger which acts on the presynaptic neurone to alter neurotransmitter release. The involvement of the CB_1 cannabinoid receptor has been identified through the use of the relatively selective antagonists, rimonabant (Wilson and Nicoll 2001) and AM251 (Kreitzer and Regehr 2001), as well as animal models with disruption of the gene encoding CB_1 cannabinoid receptors (Varma et al. 2001; Wilson et al. 2001; Kim et al. 2002; Yoshida et al. 2002). The majority of evidence favours the involvement of ester endocannabinoids (ECBs) in mediating these retrograde effects. In contrast, the amide ECBs have been proposed to act in an anterograde fashion, subserving a more conventional neurotransmitter-like role (Egertova et al. 2008).

From these studies and previous investigations (Di Marzo et al. 1994), the hypothesis has emerged that ECBs are made "on demand" as a result of heightened neuronal activity. In this chapter, we will look at synthetic pathways which appear to be consistent with this hypothesis and more recent developments, which suggest alternative strategies for endocannabinoid biosynthesis, potentially of more relevance to the pathophysiological state. In addition, we will examine metabolic pathways which inactivate, or potentially transform, ECBs.

2 What Are Endocannabinoids?

Anandamide (*N*-arachidonoylethanolamine, AEA) is the archetypal ECB described first by Raphael Mechoulam, Roger Pertwee and colleagues in 1992 (Devane et al. 1992). It was identified in the classical fashion by screening solvent extracts of brain in a cannabinoid receptor radioligand binding assay, with subsequent determination of structure by GC-MS and re-synthesis. *N*-Palmitoylethanolamine (PEA) was identified in the same experiments, but was not considered to be an ECB since only one cannabinoid receptor (CB_1) had been identified at the time and PEA had negligible affinity for this. The situation is much more complex now with a variety of putative ECB receptors of the G-protein-coupled, ion channel and nuclear receptor families proposed (see the chapter "Endocannabinoid Receptor Pharmacology" by Mackie and Yao, this volume) along with chemically related agents having affinities for one or more of these. As a note of caution, it is not always clear whether endogenous levels of some of these agents in different tissues are sufficient

to activate cognate receptors allowing them to be labelled as true ECBs (Oka et al. 2003). 2-Arachidonoylglycerol (2AG) has been suggested to be the most biologically important ECB, as it occurs in greater concentrations in tissues, and shows greater efficacy at these targets, than AEA (Sugiura et al. 1997, 1999; Sugiura and Waku 2000, 2002). Although AEA and 2AG are considered the principal ECBs, the range of endogenous agents active at cannabinoid receptors is certainly not limited to these two (Hanus et al. 1993). As a pair, they are closely structurally related in that they are both based on the polyunsaturated fatty acid arachidonate. They are both hydrophobic entities, with partition coefficients (XlogP values, indices of hydrophobicity) of 5.5 and 5.4, respectively. In comparison, conventional neurotransmitters like dopamine, glutamate and GABA have XlogP values of 0.9, −3.3 and −0.7, and are considerably more hydrophilic, partitioning readily into aqueous solutions. This hydrophobicity is also considerably more marked than that of prostaglandin E_2 (2.8) and more similar to leukotriene A_4 (5.0). In comparison to arachidonic acid (6.5), however, the endocannabinoids AEA and 2AG are less hydrophobic. Similarly, the precursor molecules 1-stearoyl-2-arachidonoylglycerol (14.3) and *N*-arachidonoyl-1-stearoyl-2-arachidonoylglycerolphosphoethanolamine (XlogP likely in excess of 20) are likely only to be found dissolved in membranes. 1-Stearoyl-2-arachidonoylglycerol and 1-palmitoyl-2-oleoylglycerol, in particular, partition into enriched domains of membranes (Basanez et al. 1996; Jimenez-Monreal et al. 1998). This hydrophobicity has a marked influence on the life cycle of ECBs. For example, it has been hypothesised that AEA is able to merge into the phospholipid bilayer as an extended conformation with the ethanolamine headgroup protruding, and access the receptor binding site by lateral diffusion without leaving the plane of the membrane (Tian et al. 2005), suggesting a role as an autocrine messenger without the need for a specific release mechanism.

Despite sharing similar structural features, the turnovers of AEA and 2AG follow parallel pathways with little overlap in selectivity. A convenient division of the ECBs is into ester or amide derivatives (see the chapter “Pharmacological Tools in Endocannabinoid Neurobiology” by Mor and Lodola, this volume).

3 Ester Endocannabinoids

Given the levels of 2AG in rodent brain (in our assays of rat brain, between 10 and 30 nmol g^{-1}), and the relative ability of isolated astrocytes and neurones to generate 2AG, it has been suggested that astrocytes are the major source of 2AG in the brain (Walter et al. 2004).

3.1 Synthesis of Ester Endocannabinoids

The “classical” pathway for 2AG synthesis is through the sequential activation of phospholipase C and diacylglycerol lipase (DGL) enzymes (Figs. 1 and 2). The intermediate in 2AG synthesis through this pathway, diacylglycerol (DAG), is more

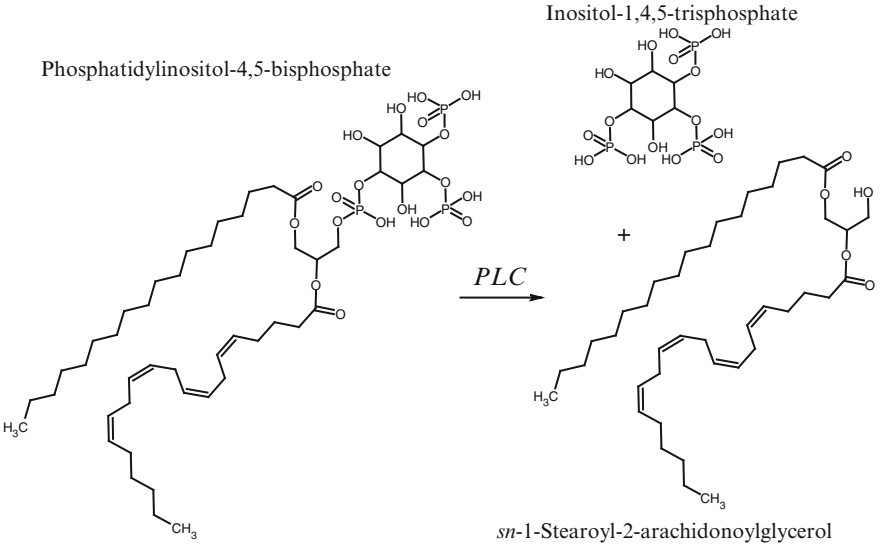

Fig. 1 Reaction scheme for phospholipase C action. Phosphatidylinositol-4,5-bisphosphate, accumulated primarily in plasma membranes, is cleaved to form the water-soluble second messenger inositol 1,4,5-trisphosphate, which causes calcium release from intracellular stores. The co-product, diacylglycerol, shown here as 1-stearoyl-2-arachidonoylglycerol, remains inserted in the plasma membrane and can activate protein kinase C, be recycled to form phospholipids or be hydrolysed to generate monoacylglycerols

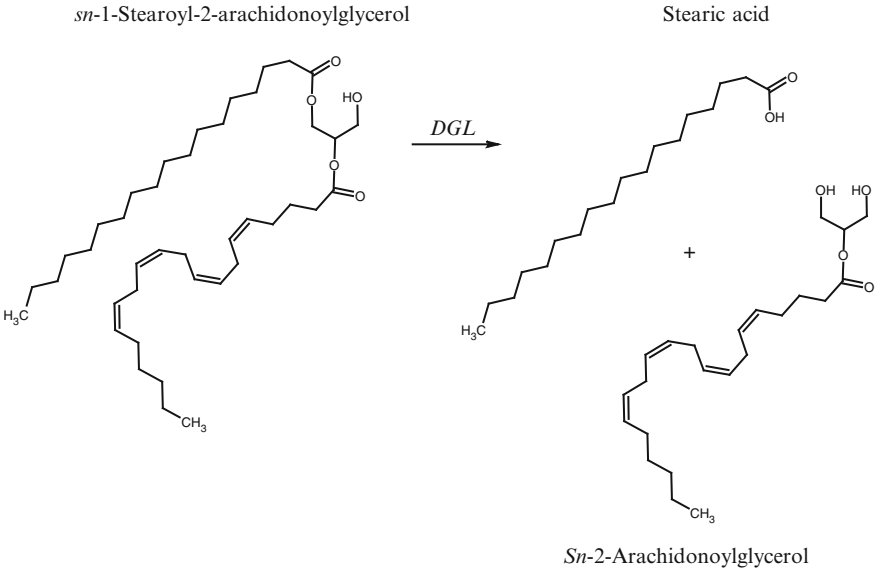

Fig. 2 Reaction scheme for diacylglycerol lipase action. Membrane-associated diacylglycerol, shown here as 1-stearoyl-2-arachidonoylglycerol, is hydrolysed to generate free fatty acid and 2-acylglycerol, both of which are much more water soluble than the parent and may have multiple cellular actions

widely associated as a second messenger in phosphoinositide turnover, activating protein kinase C. Enzymes competing for DAG include DAG kinase (seven isoforms), which is able to generate phosphatidic acid, and DAG acyltransferases (two isoforms prominent in adipose tissue), which generate triacylglycerols.

DGL is a membrane-associated enzyme generated as two separate gene products, DGLα and DGLβ (Table 1). The β isoform (694 aa) is a truncated paralogue of the α isoform (1,004 aa), although both show similar topology, with a short intracellular N-terminus and four transmembrane domains in the first 10% of the molecule. The remainder of the protein encompasses the active site and putative

Table 1 Molecular parameters of ECB-related enzymes

Enzyme	Gene name/Ensembl ID	Size	Species orthologues identity (homology)	Genetic variation
DGLα	*DAGLA*/ ENSG00000134780	1,042 aa/ 115 kDa	h/r 97% (99%) h/m 97% (97%)	Intronless; four non-synonymous SNPs: 595 G/A, 735 G/T, 889 C/T, 945 C/G
DGLβ	*DAGLB*/ ENSG00000164535	672 aa/ 74 kDa 34% (52%) identity to DGLα	h/r 78% (88%) h/m 79% (88%)	Intronless; three non-synonymous SNPs: 456 G/C, 517 G/A, 664 T/C
MGL	*MGLL*/ ENSG00000074416	303 aa/ 33 kDa	h/r 83% (92%) h/m 84% (93%)	Eight exons generating two isoforms of 303 and 273 aa; two non-synonymous SNPs: 202 C/T, 288 A/C
NAPE-PLD	*NAPEPLD*/ ENSG00000161048	393 aa/ 46 kDa	h/r 90% (95%) h/m 89% (94%)	Six exons; four non-synonymous SNPs: 152 A/C, 207 C/G, 380 T/C, 389 C/T
FAAH1	*FAAH*/ ENSG00000117480	579 aa/ 63 kDa	h/r 82% (91%) h/m 84% (91%)	15 exons; four non-synonymous SNPs: 129 C/A, 208 G/A, 370 A/G, 504 G/A
FAAH2	*FAAH2*/ ENSG00000165591	532 aa/ 58 kDa		11 exons; one non-synonymous SNP: 293 G/T
NAAA	*NAAA*/ ENSG00000138744	331 aa/ 36 kDa	h/r 79% (88%) h/m 78% (87%)	11 exons generating three isoforms; three non-synonymous SNPs: 107 G/T, 151 C/G, 334 A/G
COX2	*PTGS2*/ ENSG00000073756	587 aa/ 68 kDa	h/r 84% (91%) h/m 86% (93%)	10 exons; six non-synonymous SNPs: 1 C/T, 228 C/T, 428 G/C, 488 T/C, 511 A/G, 587 C/T

sites for regulation. Analysis of the protein sequence suggests two consensus sequences in the cytoplasmic C-terminus of the DGLα isoform for serine/threonine phosphorylation, one of which (S-727) is a potential target for both protein kinases A and C.

3.1.1 Regulation of Phospholipase C Activity

Although PLC appears capable of hydrolysing a variety of phosphoinositides in vitro, phosphatidylinositol-4,5-bisphosphate (PIP_2) appears to be the physiological substrate (Fig. 1). This substrate and one of the products (DAG) are sufficiently hydrophobic to be retained in the plasma membrane, while the second product of PLC action, inositol-1,4,5-trisphosphate, is much more hydrophilic (XlogP of –7) and so can migrate away from the membrane. Currently, 13 isoforms of PLC have been identified, which are widely distributed in the body (Suh et al. 2008). Within the cell, PLC-β isoforms (β1-β4) are membrane-associated and activated by G-proteins of the Gq family, while PLC-γ isoforms (γ1, γ2) are recruited to membranes by activation by tyrosine kinase-linked receptors of the growth factor family. PLC-δ isoforms (δ1, δ3, δ4) associate with PIP_2 in the plasma membrane and are activated by elevated concentrations of intracellular calcium ions leading to the view that PLC-δ is a calcium amplifier. PLC-ε1 is activated by the low molecular weight G-proteins Ras and Rho, as well as the exchange protein activated by cyclic AMP (Epac). Much less is known about the regulation of the ζ1, η1 and η2 isoforms (Suh et al. 2008). Gene expression of all of these isoforms appears abundant in CNS tissues, with the exception of the ζ1 isoform, which appears to have a crucial role in oocyte fertilisation. Clearly, therefore, the apparent potential for regulation of this route of ECB synthesis is huge.

U73122 is an aminosteroid which has been used to inhibit PLC activity, although its activity has not been assessed against all 13 isoforms. It has been shown to inhibit 2AG synthesis in a macrophage cell line (Berdyshev et al. 2001), 3T3 mouse fibroblasts (Parrish and Nichols 2006) and rat brain synaptosomes (Oka et al. 2007a), as well as inhibiting DSI in the hippocampus (Edwards et al. 2006). However, U73122 has also been shown to interfere with 2AG-evoked regulation of excitability in rat microglial cells (Carrier et al. 2004) or rat hippocampal slices (Hashimotodani et al. 2008). Furthermore, using mice in which genes encoding three of the isoforms of phospholipase C (PLCδ1, PLCδ3 and PLCδ4) were disrupted failed to alter cannabinoid-induced DSI responses (Hashimotodani et al. 2008).

The role of phospholipase C in 2AG generation in the CNS is, therefore, inconclusive.

3.1.2 Regulation of DGL Activity

DGL (Table 1) hydrolyses DAG to generate monoacylglycerol and free fatty acid (Fig. 2) with some selectivity for the *sn*-1 position (Bisogno et al. 2003). The

substrate specificity is not well understood, but a dually monounsaturated DAG appeared better hydrolysed than a mixed monounsaturated/saturated or monounsaturated/polyunsaturated DAG (Bisogno et al. 2003). DGL action, therefore, takes a predominantly membrane-associated substrate and generates two products, both of which are much more able to migrate away from the membrane. The recombinant enzymes are activated by calcium at supra-physiological concentrations of 100 μM or above, albeit to levels less than those evoked by glutathione (Bisogno et al. 2003). Whether these modulations are replicated with the enzyme in situ awaits further investigation.

Tetrahydrolipstatin (THL, also known as orlistat), an agent used to target pancreatic lipase in the treatment of obesity, was also found to inhibit the recombinant enzymes with IC_{50} values of 60–100 nM (Bisogno et al. 2003), although the activity in bovine aorta was more sensitive by an order of magnitude (Lee et al. 1995). THL is ineffective at 25 μM against MGL or FAAH activities, but does show inhibition of NAPE-PLD (IC_{50} of 10 μM) and triacylglycerol lipase (IC_{50} of 10 μM) (Szabo et al. 2006). Intriguingly, it also shows some occupancy of cannabinoid receptors (CB_1 IC_{50} of 4 μM vs. CB_2 IC_{50}>25 μM) (Szabo et al. 2006). RHC80267 shows low potency inhibition of DGL in platelets with an IC_{50} of 1–4 μM, with some selectivity vs. other enzymes expressed (no inhibition at 100 μM against phospholipase C or phospholipase A_2 activities (Sutherland and Amin 1982) or MGL (Rindlisbacher et al. 1987)). A recent investigation of 'activity-based protein profiling' of mouse brain using fluorophosphonate probes indicated that these two agents interfered with multiple serine hydrolases (Hoover et al. 2008), including FAAH and ABHD12 (see below). Intriguingly, the two isoforms of DGL were not identified using this methodology, suggesting either low abundance in this tissue, or reduced activity against the fluorophosphonate substrate. Despite this apparent lack of selectivity, it was noted that very few enzyme activities were inhibited by both THL and RHC80267, leading the authors to suggest the use of both agents to identify the role of DGL in biological processes.

These inhibitors have been used to identify the essential role of DGL in 2AG accumulation in the action of the Ca^{2+} ionophores ionomycin in neuroblastoma cells (Bisogno et al. 1999; Szabo et al. 2006) and A23187 in RTMGL1 rat microglial cells (Carrier et al. 2004) as well as ATP in astrocytes (Walter et al. 2004).

Currently, there are no published reports of genetic interference with DGL expression.

3.1.3 Alternative Pathways of DAG and 2AG Synthesis

Although the best established route of 2AG biosynthesis described above involves a two-step process utilising sequential activities of PLC (Fig. 1) and DGL (Fig. 2) activities, at least three further routes are possible using phosphatidylinositol, phosphatidylcholine or phosphatidylserine as starting points (Fig. 3).

A Ca^{2+}-independent phospholipase A_1 (PLA_1) activity in rat brain hydrolyses phosphatidylinositol to generate LPI, lysophosphatidylinositol (Kobayashi et al.

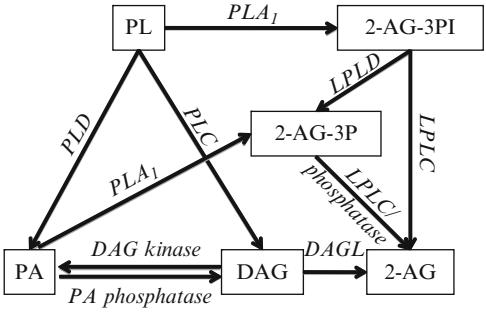

Fig. 3 Alternative routes of 2AG formation. Aside from the canonical route of 2AG formation through PLC/DGL action, diacylglycerol can also be formed via phosphatidic acid generated by PLD action. Additionally, PLA_1 activity can generate a lysophospholipid, which may be used to generate 2AG directly through a LPLC activity, or indirectly through a LPLD/LPLC sequence. Phospholipase A_1 hydrolysis of phosphatidic acid can also allow generation of 2AG, through the intermediate 2AG-3-phosphate, which can then be hydrolysed by a phosphatase/ lysophospholipase C activity

1996), which has recently been suggested to be the endogenous ligand for a cannabinoid-related receptor, GPR55 (Oka et al. 2007b, 2009). A PLA_1 activity able to hydrolyse phosphatidylinositol in cytosolic and microsomal fractions of rat brain has been described, which was less active than a PLC-like phosphodiesterase activity (Hirasawa et al. 1981). Later reports described a PLA_1 activity found in the soluble fraction of brains, which exhibited some selectivity for phosphatidylinositol over other phospholipid substrates (Ueda et al. 1993a, b).

In molecular terms, three isoforms of PLA_1 have been identified (Aoki et al. 2007). PS-PLA_1 (also known as PLA1A, ENSG00000144837) is a soluble enzyme released by activated platelets (Sato et al. 1997), which hydrolyses phosphatidylserine to produce lysophosphatidylserine and a fatty acid. Two further, membrane-associated PLA_1 activities have been identified (mPA-PLA1α, LIPH or PLA1B, ENSG00000163898 and mPA-PLA1β, LIPI or PLA1C, ENSG00000188992), which appear to hydrolyse preferentially phosphatidic acid, giving rise to lysophosphatidic acid and a fatty acid (Hiramatsu et al. 2003). Other lipase activities, such as hepatic lipase (LIPC, ENSG00000166035) and endothelial lipase (LIPG, ENSG00000101670), have also been reported to exhibit phospholipase A_1 activity when presented with phosphatidylcholine as a substrate (Gillett et al. 1993; Jaye et al. 1999).

Following PLA_1 degradation of phospholipid, a lysophospholipase C (LPLC) activity of rat brain, with some selectivity for LPI, is able to generate 2AG (Tsutsumi et al. 1994). This enzyme, although not precisely identified at the molecular level, appears to be an integral membrane protein (Tsutsumi et al. 1995).

Two further alternative routes of 2AG synthesis, independent of the phosphatidylinositol/PLC pathway, involve phospholipase D (PLD) activity, which favours

phosphatidylcholine as a substrate, generating phosphatidic acid. In mouse N18TG2 neuroblastoma cells stimulated by the calcium ionophore ionomycin, this appears to be the major synthetic route (Bisogno et al. 1999), with sequential formation of phosphatidic acid, DAG and then 2AG. The conversion of phosphatidic acid to DAG is catalysed by phosphatidic acid phosphatases or lipid phosphate phosphatases (Brindley 2004).

The phosphatidic acid phosphatase can be inhibited by high concentrations (100 μM) of the β-adrenoceptor antagonist propranolol, which has allowed identification of the involvement of this enzyme in 2AG biosynthesis in cultured neuroblastoma (Bisogno et al. 1999) and microglial (Carrier et al. 2004) cells. It has, however, not been widely applied to investigate mechanisms of ECB biosynthesis, presumably because of "non-specific" effects, for example, directly interfering with electrophysiological recordings (Hashimotodani et al. 2008) due to its local anaesthetic-like action.

A further alternative pathway for 2AG synthesis involves the generation of 2-arachidonoylglycerol-3-phosphate (2AG-3P), a lysophosphatidic acid (Nakane et al. 2002). This may theoretically be generated from phosphatidic acid by phospholipase A_1 or from lysophospholipids by lysophospholipase D (LPLD). A phosphatidic acid-hydrolysing PLA_1 activity was identified in porcine platelet membranes (Inoue and Okuyama 1984), and subsequently in rat liver (Kucera et al. 1988) and bovine brain (Higgs and Glomset 1994), leading to cloning of the enzyme from bovine testis (DDHD1, ENSG00000100523) (Higgs et al. 1998).

To date, a single isoform of LPLD has been identified at the molecular level. This is autotaxin (ENPP2, ENSG00000136960), a membrane-associated enzyme initially characterised as an ecto-nucleotide pyrophosphatase/phosphodiesterase. The primary physiological role of this enzyme, however, is thought to be the regulation of levels of lysophosphatidic acid, which it produces from lysophosphatidylcholine (Goding et al. 2003). It remains to be determined whether this entity is able to regulate ECB production, however; the fact that it contains extracellular enzymatic activity allows some speculation about a particular signalling role.

Although the enzymatic pathway involved in 2AG-3P synthesis has not been unequivocally defined, levels in rat brain of 2AG-3P (530 pmol g^{-1}) were lower than those of 2AG (37,000 pmol g^{-1} (Artmann et al. 2008)), suggesting either lower rates of 2AG-3P synthesis or higher rates of 2AG-3P dephosphorylation. The rapid conversion of 2AG-3P to 2AG (70% in 2 min) by rat brain homogenate (Nakane et al. 2002) suggests that this may be a feasible route for 2AG synthesis in vivo.

3.2 Hydrolysis of Ester Endocannabinoids

FAAH appears to be the primary enzyme involved in amide ECB hydrolysis (see Sect. 4.3 below). Initial characterization of cell-free preparations from cells expressing recombinant FAAH (Goparaju et al. 1998) or endogenously expressing FAAH

(Di Marzo et al. 1998) suggested that 2AG might also be hydrolysed through this route. However, tissues from mice with disruption of the gene encoding FAAH show an unchanged ability to hydrolyse 2AG, suggesting FAAH plays only a minor role in turnover of ester ECBs (Lichtman et al. 2002). The identification of monoacylglycerol lipase (MGL) as a serine hydrolase enzyme capable of hydrolysing ester ECBs in vitro drew attention to an alternative route of ECL turnover (Dinh et al. 2002). Although this enzyme was shown to have a central role in lipid turnover over 30 years ago (Tornqvist and Belfrage 1976), it appears to have an additional important role in the regulation of ester ECBs. It is generally thought to be a cytosolic enzyme, with the primary sequence consistent with a lack of predicted transmembrane domains. Experimentally, however, both soluble and membrane-associated activities are observed (Dinh et al. 2002; Saario et al. 2004; Vandevoorde et al. 2005), with some pharmacological evidence to indicate minor differences between the two (Vandevoorde et al. 2005; Duncan et al. 2008).

Primary sequence analysis indicated the possibility for phosphorylation of MGL and, presumably, regulation of activity by protein kinases, in particular calcium/calmodulin kinase II and cyclic AMP- and cyclic GMP-dependent protein kinases (Dinh et al. 2002). Although this has not been investigated directly, this suggests the possibility that 2AG hydrolysis can be regulated by intracellular levels of calcium and cyclic nucleotides. Immunostaining analysis suggested predominant expression of MGL in nerve fibres and cell bodies of brain regions rich in CB_1 cannabinoid receptors (Dinh et al. 2002).

The substrate specificity of MGL showed hydrolytic activity towards 2AG, but not AEA (Dinh et al. 2002), but with little specificity between acylglycerols (Ghafouri et al. 2004; Vandevoorde et al. 2005).

The available MGL inhibitors described to date have little selectivity. Fluorophosphonate analogues, such as methylarachidonylfluorophosphonate (MAFP), are potent inhibitors of MGL activity in the nanomolar range (Saario et al. 2004; Duncan et al. 2008). However, they exhibit similar activity at FAAH (De Petrocellis et al. 1997), and are thus unhelpful in defining a role of MGL in intact tissues. URB754, on the other hand, appeared at first to have selectivity for MGL over FAAH (Makara et al. 2005). Subsequently, a retraction was published indicating that a contaminant of the preparation was found to be responsible (Makara et al. 2007). URB602 was initially described as a non-competitive selective inhibitor of MGL activity (Hohmann et al. 2005). However, this compound has been reported not to show selectivity over FAAH (Vandevoorde et al. 2007; Duncan et al. 2008). OMDM169, a recently reported analogue of the DAGL inhibitor THL, shows submicromolar potency at MGL activity and enhances levels of 2AG, but not AEA, in ionomycin-stimulated N18TG2 neuroblastoma cells, but still is only tenfold selective over FAAH (Bisogno et al. 2009). JZL184, a carbamate analogue, on the other hand, appears to be almost 1,000-fold selective for MGL over FAAH (Long et al. 2009). Intraperitoneal administration of this agent caused an elevation of 2AG, but not AEA, in KCl-perfused microdialysate of mouse nucleus accumbens.

Although a knockout mouse with the gene encoding MGL has not yet been described, siRNA silencing of the enzyme in HeLa human cervical carcinoma cells

causes an elevation of cellular 2AG levels equivalent to those obtained in the presence of MAFP (Dinh et al. 2004).

A functional, activity-based protein profiling approach to studying the enzymes in rat brain responsible for 2AG hydrolysis indicated MGL accounted for the vast majority of activity. Two further, poorly characterised enzymes, abhd6 and abhd12, were identified as contributing up to 15% of 2AG hydrolysis, but evidence for their physiological significance is currently lacking.

4 Amide Endocannabinoids

The canonical pathway of AEA formation in neural tissues is thought to be via a two-step reaction – a transacylase-phosphodiesterase pathway. The intermediate involved is a low abundance phospholipid, which acts as a precursor for *N*-acylethanolamides (NAEs), including AEA. It is generally considered that the formation of this precursor, rather than its metabolism, is the rate-determining step in AEA synthesis.

4.1 Synthesis of NAPEs

N-Acylphosphatidylethanolamines (NAPEs) were described in plants about 40 years ago (Dawson et al. 1969), and observed to be mobilised during seed germination and to accumulate during stress. More recently, they were identified as precursors of the ethanolamide ECBs (Di Marzo et al. 1994). They are synthesised through the action of an acyltransferase (E.C. 2.3.1.-), which catalyses the lysophospholipase A_1-style hydrolysis of a fatty acid from the sn-1 position of phosphatidylcholine and transfers it to the amine of phosphatidylethanolamine (PE) (Fig. 4). In mammalian systems, this activity was initially identified in dog heart and reported to be calcium-dependent (Natarajan et al. 1982; Reddy et al. 1983). In mouse cerebral cortical neurones, NAPE formation was also enhanced substantially in the presence of the calcium ionophore A23187 (Hansen et al. 1995). Intriguingly, although the adenylyl cyclase activator forskolin and the Gs-coupled receptor agonist vasoactive intestinal polypeptide both failed to enhance NAPE accumulation in cultured neurones, they potentiated the stimulatory effects of the calcium ionophore ionomycin (Cadas et al. 1996a). The protein kinase inhibitor, H89, was able to prevent this potentiation, indicating a role for phosphorylation of a key enzyme in this process. In a comparison of cell types, NAPE synthesis appeared restricted to cultured neurons rather than astrocytes (Cadas et al. 1996a). In cultured neurons, the use of exogenous PLD activities indicated that approximately half of cellular NAPEs were available for hydrolysis, indicating a likely accumulation in the plasma membrane (Cadas et al. 1996b). A molecular correlate for this calcium-dependent transferase has yet to be identified.

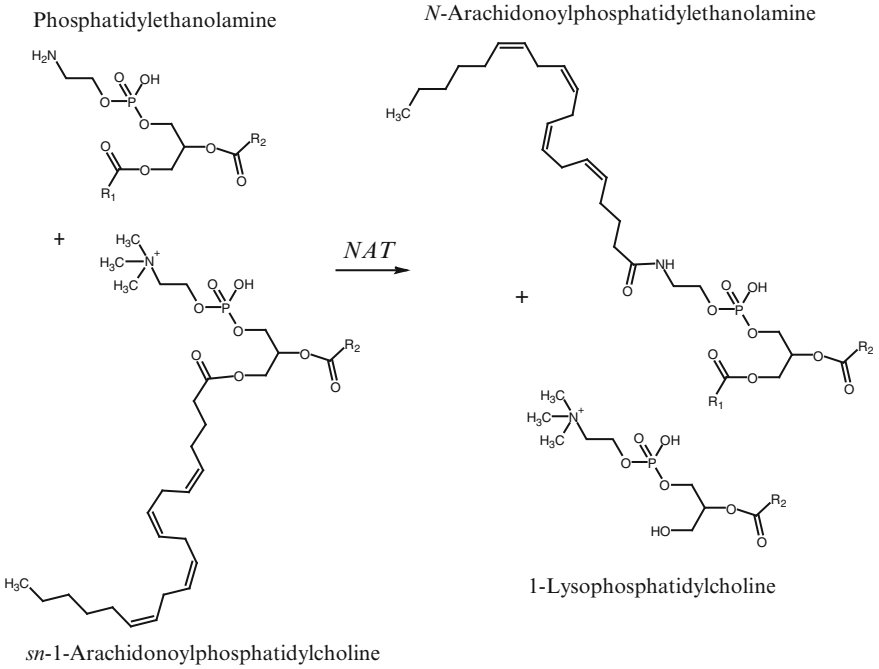

Fig. 4 Reaction scheme for *N*-acyltransferase action. The two phospholipids, phosphatidylethanolamine and phosphatidylcholine are co-substrates for *N*-acyltransferase activity, where the *sn*-1 fatty acid (depicted here as arachidonic acid) from the phosphatidylcholine is transferred to the amine of the phosphatidylethanolamine

In contrast, a Ca^{2+}-independent PE *N*-acyltransferase has recently been described (Jin et al. 2007). This predominantly cytosolic activity appears to be identical to a protein termed rat lecithin-retinol acyltransferase-like protein 1 (RLP-1, ENSG00000168004). Given that this enzyme is highly expressed in testis and pancreas, with much lower levels expressed in brain, it seems unlikely that it contributes significantly to ECB precursor formation in neural tissues.

Very recently, NAPEs have been described to have functions beyond acting simply as precursors for ECBs. Reportedly, NAPEs are synthesised in the gut, prompted by fat ingestion, and released into the circulation where they appear to have a hormonal function. Administration of exogenous NAPE led to reduced food intake which was independent of CB_1 receptors (Gillum et al. 2008).

4.2 Synthesis of Amide Endocannabinoids

As the (perhaps inappropriately considered) archetypal ECB, AEA synthesis has received the most attention. In the chemistry lab, AEA can be synthesised as a

simple condensation product of arachidonic acid and ethanolamine, but in vivo, generation of the ethanolamide ECBs is thought to occur mainly as a result of hydrolysis of a minor membrane phospholipid, *N*-arachidonoylphosphatidylethanolamine (Di Marzo et al. 1994). This is a substrate for a phospholipase D-type activity (NAPE-PLD, ENSG00000161048, Table 1) which can produce a wide range of endogenous fatty acid ethanolamides, including AEA (Okamoto et al. 2004).

4.2.1 Pharmacological and Biochemical Manipulation of NAPE-PLD Activity

An early report of crude preparations of rat heart homogenates identified a membrane-associated NAPE-PLD activity (Fig. 5) capable of hydrolysing NAPEs to generate phosphatidic acid and diacylglycerols (Schmid et al. 1983). With the inhibition of phosphatidic acid phosphatase activity, the production of diacylglycerol was inhibited indicating that the latter was produced in a two-step process. In addition to the phosphatidic acid, NAEs were produced apparently in equimolar quantities, and in the absence of synthesis of *N*-acylethanolamine phosphates (indicating the lack of involvement of PLC). Similar levels of lyso-*N*-acylphosphatidylethanolamine (lysoNAPE) to phosphatidic acid were observed, indicating

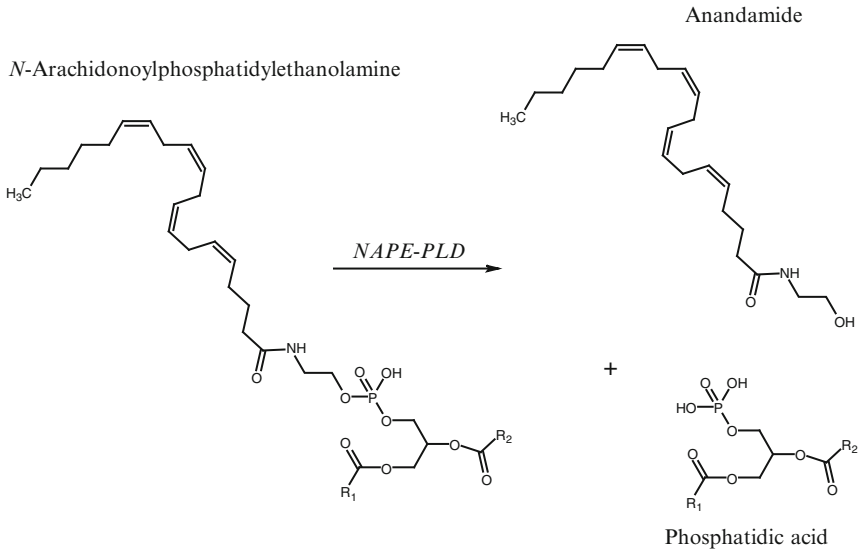

Fig. 5 Reaction scheme for NAPE-PLD action. The action of a selective phospholipase D activity allows cleavage of *N*-arachidonoylphosphatidylethanolamine to generate anandamide and phosphatidic acid. The latter is highly hydrophobic (dipalmitoylphosphatidic acid has an XlogP value of 12.9) and so stays associated with the membrane, while the anandamide can more readily move into the aqueous milieu

activity of phospholipase A_1 and/or A_2 in this preparation. In this crude preparation, supplementation with calcium or magnesium ions at concentrations up to 5 mM was without effect (Schmid et al. 1983). The same crude preparations were also able to hydrolyse LNAPE and an ether analogue of NAPE with activities only slightly less than those with NAPE itself, although it is uncertain whether these activities reside in the NAPE-PLD activity or are present in parallel enzymes (Schmid et al. 1983).

NAPE-PLD activity from rat brain microsomes was observed to generate AEA at a slightly lower rate compared to other shorter chain, more saturated NAEs (Sugiura et al. 1996). In the presence of calcium ions, the generation of these latter shorter chain, more saturated NAEs appeared enhanced, while AEA production was unchanged, although the mechanism for this selective action has not been elucidated. A further stimulus for NAPE-PLD activity is the presence of polyamines. Spermine, spermidine and putrescine were able to replace calcium ions or detergent (see below) as enhancers of NAPE-PLD activity at concentrations within the physiological range (Liu et al. 2002), although whether polyamine levels are a physiological influence on AEA levels has not been identified.

Intriguingly, addition of the non-ionic detergent Triton X-100 (up to 0.2%) led to a doubling of NAPE-PLD activity, while the same concentrations of an alternative non-ionic detergent, Tween 20, inhibited activity to 4% of the level in control preparations (Schmid et al. 1983). Ionic detergents, such as SDS or taurodeoxycholate, also inhibited NAPE-PLD activity in these preparations. It may be that these influences are more physical than biochemical, with the possibility that Triton X-100 allows a particular conformation of enzyme:substrate interaction to occur, which the other detergents are unable to facilitate. Recently, it was noted that solubilisation of NAPE-PLD from the membrane by detergents revealed a greater sensitivity to divalent cations, including calcium (Wang et al. 2008a), leading to the suggestion that a membrane component was able to substitute for calcium. A heat-stable membrane fraction was able to enhance enzyme activity, which was later suggested to be the phospholipid PE. It was surmised that membrane components, including PE, were able to maintain activity of NAPE-PLD in a tonically active state, implying that formation of the NAPE precursor was the rate-determining state in amide ECB synthesis (Wang et al. 2008a).

Although this is dealt with by Monory and Lutz in the chapter "Genetic Models of the Endocannabinoid System" in this volume, it is pertinent to consider briefly the impact of genetic manipulation of NAPE-PLD activity. Cloning of the gene encoding this enzyme allowed identification of a primary sequence distinct from classical phospholipase D activities, with characteristics of a metallo-β-lactamase family (Okamoto et al. 2004), including the obligate incorporation of a zinc atom (Wang et al. 2006). Subsequently, it was observed that disruption of the gene encoding NAPE-PLD leads to increased levels of many forms of the precursor NAPE and decreased levels of the cognate NAEs (Leung et al. 2006). In particular, OEA, PEA and SEA levels were reduced, while their cognate precursors were enhanced. In comparison, AEA and DHEA, as well as their precursors, were unaltered. This has been taken as evidence for alternative pathways for synthesis of NAEs, particularly AEA (see below).

Using viral over-expression of NAPE-PLD activity in HeLa cells, it was observed that cellular levels of OEA and PEA were increased, without altering AEA levels (Fu et al. 2008). This suggests either that AEA synthesis can be selectively driven by synthesis of the precursor NAPE, or that AEA synthesis does not involve NAPE-PLD activity.

4.2.2 Alternative Pathways of Amide ECB Generation

In NAPE-PLD knockout mice, lower brain levels of saturated *N*-acylethanolamines were detected but concentrations of polyunsaturated NAEs, including AEA, were essentially unchanged (Leung et al. 2006), indicating the existence of more than one synthetic pathway (Fig. 6). Indeed, a further three routes for AEA synthesis have been proposed, although their roles in the physiological generation of AEA in neural preparations is unclear.

Studies by Natarajan et al. (1984) provided in vitro evidence for multi-step enzymatic activities capable of producing NAEs from NAPEs. This involved the hydrolysis of one or both acyl chains from NAPEs followed by cleavage of the phosphodiester bond of the resulting lysoNAPE or glycerophospho (GP)-NAE, respectively. A secreted PLA_2 has been shown to catalyse the deacylation of

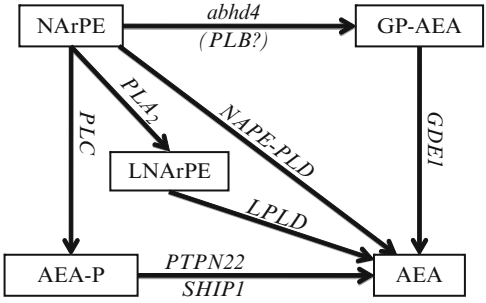

Fig. 6 Alternative routes of AEA formation. Aside from the canonical pathway of NAPE hydrolysis to form AEA, through NAPE-PLD action, phospholipase C action can generate anandamide-phosphate, which can subsequently be hydrolysed by at least two phosphatases, PTPN22 and SHIP1, to generate AEA. Phospholipase A_2 hydrolyses NAPE to generate a lysoNAPE, which can then be hydrolysed by a lysophospholipase D activity to generate AEA. A third route, utilising a phospholipase B-like action of abhd4 generates glycerophospho-AEA. A membrane-associated glycerophosphodiesterase, GDE1, is able to then generate AEA

NAPE to yield lysoNAPE in vitro (Sun et al. 2004), although this enzyme was primarily expressed in the gut, with little expression in the brain. This suggests the existence of additional enzymes responsible for the calcium-independent NAPE hydrolase activity detected in NAPE-PLD knockout mouse brain.

The lysoNAPE evolved following a PLA_2-mediated hydrolysis of NAPE can itself be hydrolysed by a LPLD activity which produces NAEs and lysophosphatidic acid (LPA). This activity was, however, found to be enriched in brain and testis (Sun et al. 2004). Given the profound biological actions of LPA, it is interesting to speculate on the dual functions of products of this enzyme.

More recently, Simon and Cravatt (2006) identified a novel enzyme αβ-hydrolase 4 (abhd4, ENSG00000100439) as a lysophospholipase/phospholipase B that selectively hydrolyzes NAPEs and lysoNAPEs to yield GP-NAE. This enzyme is indeed present in the brain and probably represents the NAPE-PLD-independent route for NAE biosynthesis observed in both NAPE-PLD-knockout and wild-type mice. Currently, very little is known about the distribution of abhd4 between neuronal and glial populations, as well as its subcellular location. The enzyme shows little selectivity between acyl groups, generating PEA at an equal rate to AEA, and is inhibited by fluorophosphonates, with 5 μM MAFP proving an effective inhibitor (Simon and Cravatt 2006). As yet, no selective inhibitors have been described. However, since NAPE-PLD is insensitive to MAFP up to 100 μM (Petersen and Hansen 1999), it is possible that this agent allows some discrimination of the two routes of AEA synthesis.

GDE1 (ENSG00000006007) is an integral membrane protein which has been identified as a glycerophosphodiesterase (Zheng et al. 2000). Initial characterization indicated an interaction with RGS16, a regulator of G-protein signalling, implying the possibility that enzyme activity might be modulated by cell-surface receptors. Indeed, in a recombinant system, the enzyme was able to hydrolyse glycerophosphoinositol preferentially (compared to glycerophosphocholine) and this activity was enhanced by isoprenaline and reduced by phenylephrine (Zheng et al. 2003). Recently, the substrate profile of this enzyme was extended to include GP-NAE, including a glycerophospho derivative of AEA (Simon and Cravatt 2008). Since the enzyme activity is stimulated by magnesium ions and inhibited by calcium ions, chelation of these allowed accumulation of several GP-NAEs in a rat brain membrane fraction, including saturated, mono-unsaturated and polyunsaturated fatty acid derivatives. Analysis of multiple recombinant glycerophosphodiesterase activities suggested identity with GDE1. Taken together, these data suggest a role for abhd4 and GDE1 in calcium-independent generation of AEA (and other NAEs) in neural tissue.

Another route that has been identified is the PLC hydrolysis of NAPE and the consequent production of acylethanolamine-*O*-phosphates, which may subsequently be hydrolysed by the phosphatases, PTPN22 or SHIP1 (Liu et al. 2008). Intriguingly, the PLC route was suggested to react more rapidly ($<$10 min) than the PLB route ($\geq$1 h). Whether the PLC activity which is able to hydrolysis NAPE is a member of the conventional phosphoinositide-specific PLC activities described earlier is as yet unknown.

The physiogical role of PTPN22 and SHIP1 in ECB turnover in neural tissues is, as yet, almost completely unexplored.

4.3 Hydrolysis of Amide Endocannabinoids

The best characterised and investigated pathway of ECB turnover is the hydrolysis of amide ECBs. This is partly because of the relative ease of assay and synthesis of substrates, but also because inhibitors of ECB hydrolysis show some promise as therapeutic agents.

4.3.1 FAAH1 Activity

FAAH was cloned from rat tissues on the basis of identifying the enzyme responsible for hydrolysis of oleamide, an ECB-related fatty acid amide (Cravatt et al. 1996). Expression of human and rat enzymes in recombinant systems indicated intracellular location of both enzymes, although the two appeared to have distinct patterns. The rat enzyme appeared to associate with Golgi and ER membranes, predominantly in perinuclear regions, while the human enzyme appeared more associated with cytoskeletal elements (Cravatt et al. 1996). In neural tissues, FAAH-like immunoreactivity is associated primarily with neurons, in a pattern extensively (although not completely) complementary to the expression of CB_1 cannabinoid receptors (Egertová et al. 1998; Tsou et al. 1998).

Hydrolysis rates of AEA were greater than those of oleamide, OEA and PEA in mouse brain and liver, but were diminished by ~99% in both tissues in mice in which the *faah* gene was disrupted (Lichtman et al. 2002) indicating the predominant role for FAAH in the hydrolysis of AEA, at least in "normal" neural tissues. It is, therefore, easy to understand the focus on development of FAAH inhibitors as therapeutic alternatives to receptor agonists. Intriguingly, there is a possibility that endogenous inhibitors of FAAH are able to regulate ECB turnover. Thus, *N*-arachidonoyl amino acids, such as *N*-arachidonoylglycine and *N*-arachidonoylalanine show species-dependent inhibition of FAAH activity (Grazia Cascio et al. 2004), although whether these are physiological regulators of ECB hydrolysis is unknown. Of potentially more direct influence is the observation that FAAH has a wide substrate profile, such that many endogenous fatty acid amides, including OEA and PEA, but not limited to NAEs, are also substrates for the enzyme and are present in quantities up to 100 times those of AEA. This observation led to the hypothesis that these compounds act as "entourage" compounds. That is, although they have no direct activity at CB_1 or CB_2 cannabinoid receptors, they are able to slow the hydrolysis of AEA through competition for FAAH activity sufficiently so that they can indirectly influence cannabinoid activity. The issue is complicated further by studies of the putative ECB-like receptor GPR119, which suggested that it was activated by OEA, PEA and SEA (Overton et al. 2006). Whether there is a

convergence between GPR119 and conventional cannabinoid receptors awaits further investigation.

Synthetic inhibitors of FAAH abound and can be divided into two broad groups. One group is based around mimicking endogenous ligands, while the second is structurally unrelated compounds. Although α-keto ethyl esters and trifluoromethyl-ketone analogues of AEA were effective FAAH inhibitors, the overlap in pharmacophore meant that activity at CB_1 receptors and other eicosanoid-metabolising enzymes reduced their applicability (Koutek et al. 1994). Assessment of a number of carbamate analogues identified an irreversible inhibitor with nanomolar potency, URB597 (Kathuria et al. 2003). Although this compound has some "off-target" activity (Zhang et al. 2007), including activation of TRPA1 channels (Niforatos et al. 2007), the profile of its action in vitro and in vivo is consistent with a predominant action to elevate NAEs. Although systemic administration of URB597 has been demonstrated to increase levels of AEA, OEA and PEA in rat CNS tissues (Gobbi et al. 2005; Russo et al. 2007). Moise et al. (2008) reported that it elevated brain levels of OEA and PEA but not AEA in the hamster brain, indicating the possibility of species-selective effects of the inhibitor on multiple enzyme activities.

The observation that some, but not all, non-steroidal anti-inflammatory drugs, previously thought to exert their therapeutic effects through inhibition of cyclooxygenase activity, were also able to inhibit FAAH activity at relevant concentrations (Fowler et al. 1997) raised the possibility that some of the therapeutic effects of these agents might be mediated through cannabinoid receptors.

4.3.2 FAAH2 Activity

A second isoform of FAAH, FAAH2 (ENSG00000165591), has a limited species distribution in mammals, being found in man, other primates, elephants and rabbits, but not mice, rats, pigs, dogs, sheep or cows (Wei et al. 2006). Although the subcellular distribution of this isoform has not been precisely identified, it was predicted to be membrane-associated with the active site oriented towards the luminal side of the membrane. Whether FAAH2 regulates ECB levels in the extracellular medium or in subcellular organelles is, as yet, unknown. Although FAAH2 appears to hydrolyse the conventional fatty acid ethanolamine ECB-like compounds, the activity against AEA, OEA and PEA is greatly reduced, while ODA hydrolysis is similar to that evoked by FAAH1 (Wei et al. 2006). Unlike FAAH1, FAAH2 appears not to be expressed in brain or small intestine, but in contrast to FAAH1, shows low expression in heart, muscle and ovary (Wei et al. 2006). Both isoforms show high expression in kidney, liver, lung and prostate. In comparison with FAAH1, there appear to be no inhibitors of FAAH2 with greater than 100-fold selectivity (Wei et al. 2006), although both URB597 and OL135 show more than tenfold selectivity. In contrast, JNJ1661010 appears to be 100-fold selective for FAAH1 (Karbarz et al. 2009). It appears unlikely, therefore, that FAAH2 is a major regulator of AEA levels in human neural tissues.

4.3.3 NAAA Activity

N-Acylethanolamine acid amidase (NAAA, ENSG00000138744) activity is a lysosomal enzyme, with an acid pH optimum, and structural similarity to acid ceramidase. The mature enzyme is glycosylated and requires proteolysis for activation (Wang et al. 2008b). It appears to hydrolyse preferentially PEA compared to AEA in cell-free systems (Ueda et al. 1999). In intact cells, however, NAAA appeared capable of hydrolysing a variety of fatty acid ethanolamides, including AEA (Sun et al. 2005), but not 2AG (Tsuboi et al. 2005). The enzyme is expressed to relatively high levels in lung, spleen and large intestine, but in contrast to FAAH activity, is less well expressed in liver, testis and brain (Tsuboi et al. 2005). Under normal circumstances, therefore, it appears unlikely to contribute significantly to AEA turnover in neural tissues.

In counterpoint to FAAH activities, NAAA is not inhibited by MAFP concentrations up to 10^{-5} M (Ueda et al. 1999). The enzyme is also insensitive to URB597, but can be inhibited by a retroamide, *N*-cyclohexylcarbonylpentadecylamine, in the micromolar range (Tsuboi et al. 2004). As yet, genetic disruption of this enzyme has not been reported.

5 Other Routes of ECB Transformation

Other than FAAH, NAAA and MGL, the most prominent route of ECB inactivation appears to be through oxidative metabolism. Intriguingly, there is the possibility that this is not simply an inactivation, but rather a transformation to metabolites, which may themselves be active, albeit not only at canonical cannabinoid receptors.

Aside from oxidative metabolism, a further form of transformation of NAEs was identified using tissue from $FAAH^{-/-}$ mice (Mulder and Cravatt 2006). *O*-Phosphorylcholine derivatives of NAEs (PC-NAEs) were identified in the brain and/or spinal cord of $FAAH^{-/-}$ mice, but not wild-type mice. Intriguingly, although AEA levels were elevated in $FAAH^{-/-}$ mice, there were no detectable levels of PC-AEA, although PC-PEA and PC-OEA were detectable. Whether these metabolites are generated in other species or under pathological conditions or indeed whether they have biological activity in their own right is unknown. The mechanism of PC-NAE formation is also unknown; however, an enzyme activity has been identified which is capable of hydrolysing PC-NAEs to generate *O*-phosphorylcholine and NAE (Mulder and Cravatt 2006). This is ENPP6 (ENSG00000164303), a membrane-associated member of the nucleotide pyrophosphate/phosphatase family (Sakagami et al. 2005), which is expressed highly in human (although not mouse) brain. ENPP6 exhibits LPLC activity with some selectivity for lysophosphatidylcholine over any other lysophospholipid, including LPI (Sakagami et al. 2005). Although PC-NAEs were poor substrates for FAAH activity, they were efficiently hydrolysed by recombinant ENPP6 (Mulder and Cravatt 2006).

5.1 Oxidative Metabolism of ECBs

5.1.1 Cyclooxygenase Activity

Cyclooxygenase (COX) activities are membrane-bound enzymes responsible for the production of prostanoids (prostaglandins and thromboxanes) from arachidonic acid. Of the two isoforms, COX-1 is generally held to be constitutively expressed and responsible for the "house-keeping" roles of prostanoids, while COX-2 is generally inducible, although it is constitutively expressed in some tissues (e.g. spinal cord) and thought to be responsible for the inflammatory, pyrexic and hyperalgesic prostanoids. Given that the two major ECBs, AEA and 2AG, are arachidonate derivatives, it is, in retrospect, not too surprising that COX metabolises ECBs to produce prostanoid-like molecules. Intriguingly, AEA and 2AG appear to be poor substrates for COX-1, but are readily metabolised by COX-2 (Yu et al. 1997; Kozak et al. 2000). Perhaps more intriguing is the observation that ECBs, through the CB_1 receptor, are able to induce COX-2 expression in the cerebral microvasculature (Chen et al. 2005), indicating the possibility of diversion of ECBs through alternative metabolic routes following repeated administration.

The products of COX-2 oxidative metabolism of ECBs are biologically active (Sang et al. 2006, 2007; Hu et al. 2008) and so COX-2 metabolism represents transformation of ECBs rather than inactivation. The prostanoid ethanolamides and glyceryl esters appear not to be active at conventional cannabinoid or prostanoid receptors, however, but rather through separate targets, as yet undefined at the molecular level (Fowler 2007; Woodward et al. 2008). The major route of prostaglandin inactivation, via 15-hydroxyprostaglandin dehydrogenase, appears to be less effective for oxidation of COX-2 metabolites of ECBs (Kozak et al. 2001). In parallel, FAAH or MGL hydrolysis of the COX-2 metabolites of AEA or 2AG, respectively, was reduced in comparison to the untransformed parent ECB (Vila et al. 2007). It appears, therefore, that whilst the ECBs themselves are transient species, COX-2 metabolism is able to generate derivatives which are far more long-lasting.

Numerous cellular and tissue preparations have been shown to be able to metabolise administered ECBs through the COX-2 pathway (Kim and Alger 2004; Patsos et al. 2005; Ahn et al. 2007; Ho and Randall 2007; Rockwell et al. 2008; Jhaveri et al. 2008; Bajo et al. 2009); however, definitive evidence for COX-2 metabolism of endogenous ECBs is currently lacking. Intriguingly, however, the observation that typical antibody-based assays for prostanoids fails to distinguish prostaglandins from prostamides suggests that there is more to be elucidated from the COX-2 metabolism of ECBs (Glass et al. 2005). The picture is further obscured by the observation that many, but not all, non-steroidal anti-inflammatory drugs, previously thought to target COX activities selectively, are also able to inhibit FAAH activity at pharmacologically relevant concentrations (Fowler et al. 1997, 1999, 2003). The possibility exists, therefore, that clinical efficacy of some of these

agents may be due to a combination of preventing the accumulation of inflammatory prostanoids, as well as promoting the accumulation of anti-inflammatory ECBs (Jhaveri et al. 2008).

5.1.2 Lipoxygenase Activity

Mammalian lipoxygenases (LOXs) are bound to membranes inside the cell, including the nuclear membrane, and generate hydroperoxides of unsaturated fatty acids (typically of the 1Z,5Z pentadiene structure) by introducing molecular oxygen at the points of unsaturation. For arachidonate, 5-, 12- and 15-hydroperoxidation generates hydroperoxyeicosatetraenoic acids (HPETEs). 5-LOX metabolism of arachidonate, primarily in white blood cells, generates leukotrienes, while sequential oxidative metabolism of arachidonate by 5- and 15-LOXs generates lipoxins. Although a sequence of metabolic steps allows AEA to be metabolised to 12-oxygenated species in splenocytes, this appears to be due to FAAH-mediated hydrolysis of AEA generating arachidonate, thereafter metabolised by 12-LOX (Bobrov et al. 2000). Both 12- and 15-LOX, but not 5-LOX, appear capable of metabolising AEA and 2AG (Hampson et al. 1995; Ueda et al. 1995; Edgemond et al. 1998; Moody et al. 2001; Kozak et al. 2002). Indeed, incubation of AEA with plant-derived 5-LOX generates a 12-hydroperoxide derivative (Van Zadelhoff et al. 1998).

In the brain, the majority of LOX activity appears to reside in the pineal gland (Nishiyama et al. 1993; Hada et al. 1994), through which enzyme activity a product consistent with 12-hydroxyAEA was identified (Hampson et al. 1995). Rat brain homogenates are able to generate both 12-hydroxyAEA and 15-hydroxyAEA (Veldhuis et al. 2003). Human platelets are able to convert AEA to 12(S)-hydroxyAEA, while both 12(S)-hydroxyAEA and 15(S)-hydroxyAEA appeared following incubation of AEA with human polymorphonuclear lymphocytes (Edgemond et al. 1998).

As with COX activity, LOX metabolism represents transformation, rather than inactivation, of ECBs, since LOX products are active at CB_1 and CB_2 receptors (Edgemond et al. 1998), as well as TRPV1 (Craib et al. 2001) and PPAR (Kozak et al. 2002) receptors.

5.1.3 Cytochrome P450s and Epoxygenase Activity

Arachidonic acid is subject to an additional form of oxidative metabolism, in which 5,6-epoxyeicosatrienoic acid (EET), 8,9-EET, 11,12-EET or 14,15-EET may be formed. These epoxides are thought to be important signalling molecules in the vascular system and are metabolised by epoxide hydrolases to form diols. Human liver microsomes, containing multiple cytochrome P450 activities, were found to catalyse epoxide formation at all four unsaturations of AEA (Snider et al. 2007). One isoform of cytochrome P450, 4X1, generates 14,15-EET ethanolamide from

AEA (Stark et al. 2008), while a second, 2D6, is not only able to generate all four epoxides from AEA, but also oxidises them further to generate diol analogues of AEA (Snider et al. 2008).

One isoform of cytochrome P450, 4F2, oxidises the ω-carbon of the fatty acid chain to produce *N*-20-hydroxyarachidonoylethanolamine.

The potential for oxidation at the terminal alcohol of AEA has recently been demonstrated, with alcohol dehydrogenase metabolism generating *N*-arachidonoylglycine (Aneetha et al. 2009), which may prove to be the endogenous ligand for the putative ECB-like receptor GPR18 (Kohno et al. 2006).

6 Stimulation of ECB Synthesis and Release

It is widely thought that AEA and 2AG (which are very lipophilic compounds) are produced from their precursor membrane phosphoglycerides via Ca^{2+}-sensitive biosynthetic pathways, activated on demand, rather than being pre-synthesised and stored in secretory vesicles awaiting exocytosis, as is the case for many neurotransmitters. Hence, it is likely that ECB agents act largely as local (autocrine/paracrine) mediators rather than conventional hormones. ECBs can, however, be detected in the plasma, although their tissues of origin are not clear, and a longer range hormonal action should not be completely disregarded. AEA and 2AG are regarded as retrograde mediators in the brain where post-synaptic depolarisation leads to the elevation of intracellular Ca^{2+} from intracellular stores, entry through receptor/voltage-operated Ca^{2+} channels (or both). This is assumed to stimulate Ca^{2+}-sensitive enzymes such as NAPE-PLD catalysing the biosynthesis of AEA and, particularly, 2AG. The released ECB mediators retrogradely traverse the synapse to activate presynaptic CB_1 receptors resulting in inhibition of voltage-activated calcium channels, activation of K^+ channels and inhibition of neurotransmitter release.

However, in spite of the massive research effort expended on the ECBs in recent years, there have been remarkably few direct studies of stimulated ECB synthesis and release, particularly in native cells and tissues. The chemical nature of the ECBs probably explains the difficulty in measuring extracellular concentrations, their high lipophilicity suggesting that they are unlikely to exist alone in the aqueous extracellular medium for any length of time. However, elegant electrophysiological studies by Brad Alger (see the chapter "Endocannabinoid Signaling in Neural Plasticity" by Alger, this volume) have unambiguously shown that depolarisation and agonist-mediated Ca^{2+} mobilisation stimulates release of ECBs, indicated by depolarisation-induced suppression of inhibition and excitation in the CA1 region of the hippocampus (Kim et al. 2002). More direct studies (Bisogno et al. 1997) showed that stimulation of mouse neuroblastoma cells with the Ca^{2+} ionophore, ionomycin, caused the synthesis, release and subsequent degradation of 2AG. Stella and Piomelli (2001) also demonstrated Ca^{2+} mobilising receptor-mediated enhancement of 2AG, OEA and PEA, but not AEA, in rat cortical

neurones. These authors (Stella et al. 1997) had previously reported that high frequency stimulation of hippocampal slices had increased the synthesis of 2AG but not AEA.

Despite the weight of evidence favouring the Ca^{2+}-mediated formation of 2AG but less so of AEA, Di Marzo's group have proposed that AEA acts as a kind of intracellular "volume switch" for Ca^{2+}; van der Stelt et al. (2005) reported that in dorsal root ganglion (DRG) cells, purinoceptor or muscarinic cholinergic receptor activation leads to AEA synthesis which acts on TRPV1 channels, gating extracellular Ca^{2+} allowing more AEA synthesis, thus providing a feed-forward mechanism. This potentially vicious cycle is suggested to be interrupted by released AEA acting on extracellular facing CB_1 receptors and inhibiting TRPV1 function, as demonstrated by Millns et al. (2001). In more recent studies, Vellani et al. (2008) again suggested a central role for the TRPV1 channel in the control of DRG and, by extension, sensory nerve activity. They showed that, in addition to activation by AEA, TRPV1 channels were activated and/or sensitised by stimulating protein kinases A or C leading to enhanced AEA but not 2AG or PEA levels. This indicates that, in addition to Ca^{2+} mobilisation, the generation of other second messengers following receptor activation has the potential to modulate ECB synthesis and release.

In our own studies of ECB synthesis and release in rat cerebral cortical slices in vitro, we have found little evidence for Ca^{2+}-mobilising stimuli elevating the

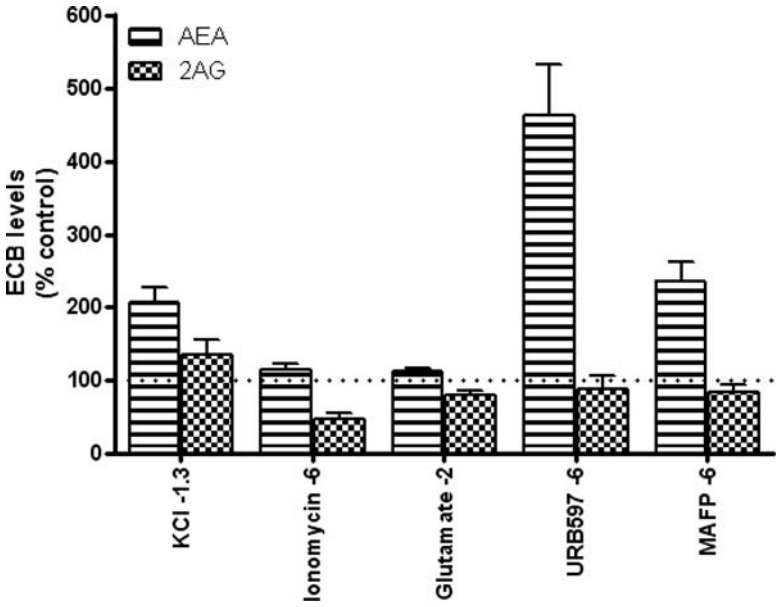

Fig. 7 Accumulation of ECBs in rat brain cerebral cortex in vitro. Brain slices were incubated for 30 min in the absence and presence of ligands and/or calcium-free Krebs' ringer solution before extraction and quantification of ECBs (Sarmad et al. 2008)

levels of ECBs but it is notable that the FAAH inhibitor URB597 (see above) causes a robust increase in tissue levels and accompanying release, suggesting that, in this preparation, there is a high on-going turnover of ECBs independent of calcium ions (Fig. 7).

There have been a few attempts to monitor in vivo ECB release using microdialysis coupled with LC/MS analysis. Béquet et al. (2007) reported that, in the rat hypothalamus, local depolarisation following high K^+ or glutamate perfusion enhanced AEA and 2AG release independently of Ca^{2+}. Their experiments supported a release-modulating role for CB_1 receptors in that the antagonist rimonabant enhanced, while the CB agonist WIN55212-2 reduced, AEA release, although, intriguingly, the same treatments induced opposite changes in 2AG. The mechanisms underlying the control of release clearly require further investigation. At the present time, even basic questions such as whether the release process is an active, energy-dependent mechanism or simply a passive flow down concentration gradients remain unanswered.

7 Conclusion

Despite a massive research effort over the last two decades, there is still a plethora of questions to be addressed concerning the ECB system. There has been a probably unwarranted concentration on AEA, given its archetypal status, and the challenge now is to clarify the roles of the many related fatty acids and their interactions, not only with CB_1 and CB_2 receptors but with the ever-growing family of G-protein-coupled, nuclear and ion channel receptors responsive to ECBs. The immense complexity of the synthetic and metabolic pathways followed by the ECBs provides a great challenge but also an opportunity for the development of selective therapeutic agents to tackle some of the diseases involving the ECB system which are described in later chapters in this volume.

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