Chapter 1

Need for Methods to Investigate Endocannabinoid Signaling

Mauro Maccarrone

Abstract

Endocannabinoids (eCBs) are endogenous lipids able to activate cannabinoid receptors, the primary molecular targets of the cannabis (*Cannabis sativa*) active principle Δ^9-tetrahydrocannabinol. During the last 20 years, several *N*-acylethanolamines and acylesters have been shown to act as eCBs, and a complex array of receptors, metabolic enzymes, and transporters (that altogether form the so-called eCB system) has been shown to finely tune their manifold biological activities. It appears now urgent to develop methods and protocols that allow to assay in a specific and quantitative manner the distinct components of the eCB system, and that can properly localize them within the cell. A brief overview of eCBs and of the proteins that bind, transport, and metabolize these lipids is presented here, in order to put in a better perspective the relevance of methodologies that help to disclose molecular details of eCB signaling in health and disease. Proper methodological approaches form also the basis for a more rationale and effective drug design and therapeutic strategy to combat human disorders.

Key words Anandamide, 2-Arachidonoylglycerol, Enzyme assays, Immunochemical assays, Intracellular trafficking, Localization, Metabolic routes, Oxidative pathways, Receptor binding assays, Signal transduction

1 A Modern View of the Endocannabinoid System

Two G protein-coupled receptors, termed type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors, are activated by Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis (*Cannabis sativa*) extracts like hashish and marijuana [1]. Endogenous counterparts of THC [collectively termed "endocannabinoids (eCBs)"], their target receptors, and the enzymes responsible for their synthesis and degradation form an entirely new endogenous signaling system, also known as the "endocannabinoid system (ECS)" [2–4].

The most important eCBs are two arachidonic acid derivatives: *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), shown in Table 1.

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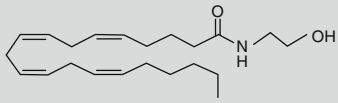
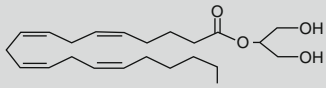
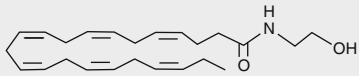
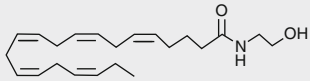
Table 1
Endocannabinoids (eCBs), their molecular targets, and their biosynthetic and catabolic enzymes

Bioactive lipids	Molecular targets	Biosynthetic enzymes	Catabolic/oxidative enzymes
ω – 6 eCBs			
	CB_1 CB_2 TRPV1 PPARα PPARγ GPR55	NAT iNAT NAPE-PLD ABHD4 *Lyso*-PLD GDE1	FAAH NAAA LOXs COX-2 Cyt P_{450}
N-arachidonoylethanolamine (anandamide, AEA)		PTPN22	
	CB_1 CB_2	PLCβ DAGLα	MAGL FAAH
2-Arachidonoylglycerol (2-AG)	TRPV1	DAGLβ	ABHD6
ω – 3 eCBs			
	CB_1 CB_2	Possibly as for other NAEs	Possibly as for other NAEs
N-docosahexaenoylethanolamine (DHEA)	PPARγ		
	CB_1 CB_2	Possibly as for other NAEs	Possibly as for other NAEs
N-eicosapentaenoylethanolamine (EPEA)	PPARγ		

Abbreviations: *ABHD4/6/12* α/β-hydrolase domain 4/6/12, *CB1* type-1 cannabinoid receptors, *CB2* type-2 cannabinoid receptors, *COX-2* cyclooxygenase-2, *Cyt P450* cytochrome P_{450}, *DAGLα/β* diacylglycerol lipase α/β, *FAAH* fatty acid amide hydrolase, *GPR55* orphan G protein-coupled receptor 55, *LOXs* lipoxygenases, *MAGL* monoacylglycerol lipase, *NAAA N*-acylethanolamine-hydrolyzing acid amidase, *NAPE-PLD N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, *NAT N*-acyltransferase, *iNAT* Ca^{2+}-independent *N*-acyltransferase, *PLCβ* phospholipase Cβ, *lyso-PLD lyso*-phospholipase D, *PPARα/γ* peroxisome proliferator-activated receptor α/γ, *PTPN22* protein tyrosine phosphatase, non-receptor type 22, *TRPV1* transient receptor potential vanilloid type 1 channel

They belong to the large families of *N*-acylethanolamines and 2-monoacylglycerols, respectively. Besides these *ω* – 6 (*n* – 6) fatty acid compounds, two metabolically important *ω* – 3 (*n* – 3) fatty acid ethanolamines have been discovered: *N*-eicosapentaenoylethanolamine (EPEA) [5] and *N*-docosahexaenoylethanolamine (DHEA) [6], also shown in Table 1. The latter two substances have been proposed as additional CB_1/CB_2 agonists [7], but their pharmacology and biological relevance remain to be clarified.

The actions of eCBs and congeners are controlled through not yet fully characterized cellular mechanisms that include key agents responsible for their biosynthesis, degradation, and oxidation.

Remarkably, during the last few years multiple pathways have been described for the metabolism of AEA (Fig. 1), and of 2-AG (Fig. 2), as detailed in a recent review [8].

Briefly, the main route for AEA biosynthesis consists of two enzymatic reactions. The first is a fatty acyl chain transfer from membrane phospholipids to a phosphatidylethanolamine, resulting in the formation of *N*-acylphosphatidylethanolamine (NAPE), by a yet-unidentified Ca^{2+}-dependent *N*-acyltransferase (NAT) [9], or by a Ca^{2+}-independent counterpart (iNAT) [10]. The second step is catalyzed by a NAPE-specific type D phospholipase (NAPE-PLD) that is the most relevant enzyme among multiple players in AEA formation from NArPE [9, 10], as shown in Fig. 1.

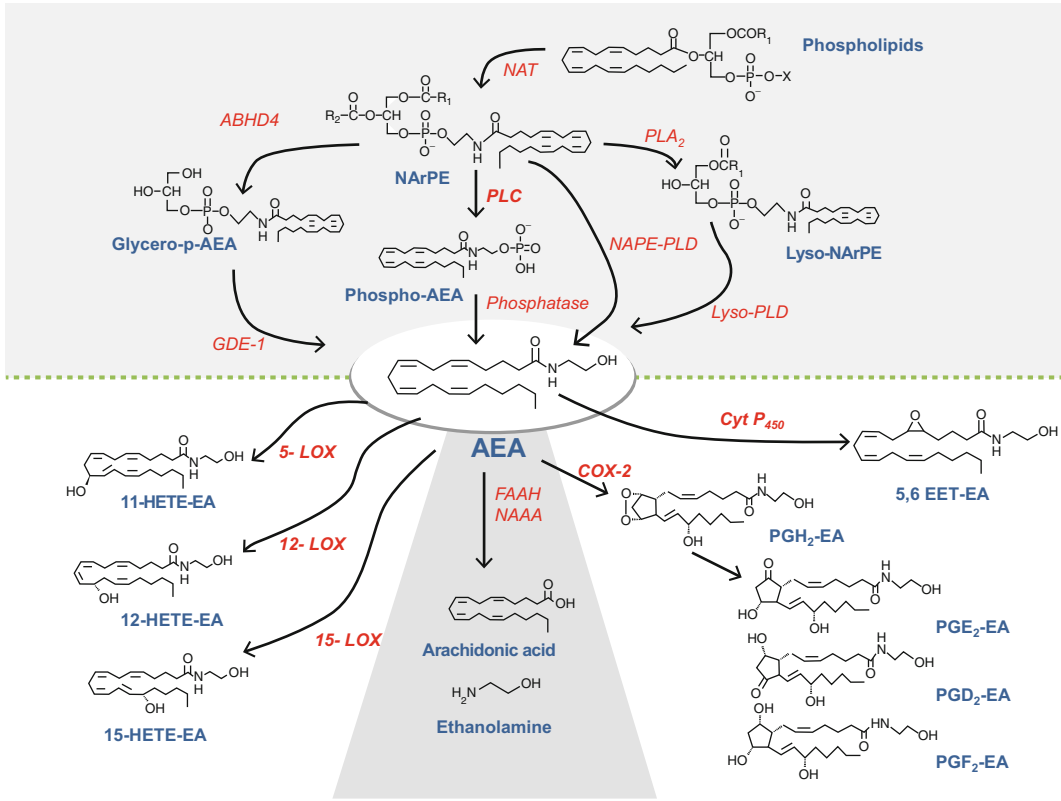


Fig. 1 Alternative biosynthetic, degradative, and oxidative pathways of AEA and congeners. *AA* arachidonic acid, *ABHD4* α/β-hydrolase domain 4, *pAEA* phospho-AEA, *COX-2* cyclooxygenase-2, *Cyt P450* cytochrome P_{450}, *EET-EA* epoxyeicosatrienoyl ethanolamides, *EtNH2* ethanolamine, *FAAH* fatty acid amide hydrolase, *GP-AEA* glycerophospho-AEA, *GDE1* glycerophosphodiester phosphodiesterase 1, *12-HAEA* 12-hydroxyanandamide, *12-LOX* 12-lipoxygenase, *NAAA N*-acylethanolamine-hydrolyzing acid amidase, *NAPE-PLD N*-acyl-phosphatidyl ethanolamine-hydrolyzing phospholipase D, *NAT N*-acyltransferase, *iNAT* Ca^{2+}-independent *N*-acyltransferase, *lyso-NArPE lyso-N*-arachidonoylphosphatidylethanolamine, *NArPE N*-arachidonoylphosphatidylethanolamine, *pNArPE N*-arachidonoylethanolamine plasmalogen, *PLA2* phospholipase A_2, *PLC* phospholipase C, *lyso-PLD lyso*-phospholipase D, *PMF2α* prostamides F2α, *PTPN22* protein tyrosine phosphatase, non-receptor type 22

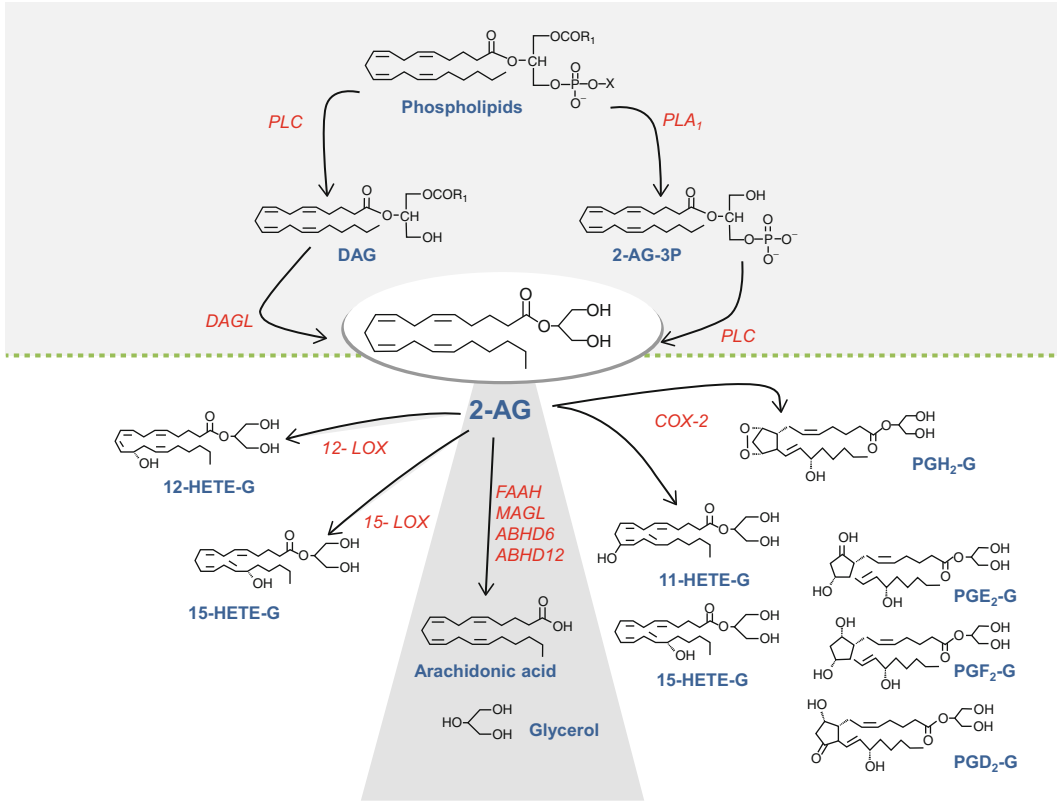


Fig. 2 Alternative biosynthetic, degradative, and oxidative pathways of 2-AG and congeners. *AA* arachidonic acid, *2-AG-3P* 2-arachidonoylglycerol-3-phosphate, *COX-2* cyclooxygenase-2, *DAG* diacylglycerol, *DAGL* diacylglycerol lipase, *12-HETE-G* 12-hydroxy-arachidonoyl-glycerol, *ABHD6/12* α/β-hydrolase domain 6/12, *12-LOX* 12-lipoxygenase, *MAGL* monoacylglycerol lipase, *PLC* phospholipase C, *PLCβ* phospholipase Cβ, *PGE2-G* prostaglandin glycerol E_2-G

As for the biosynthesis of 2-AG and congeners, the best known biosynthetic pathway requires the combined action of phospholipase C (PLC) and diacylglycerol lipase (DAGL that is present in two forms, α and β) [11], but alternative pathways of 2-AG biosynthesis are also known, as shown in Fig. 2.

Degradation of eCBs and congeners can start with their transmembrane uptake, a process that remains highly debated because true "eCBs membrane transporters (EMT)" have not yet been cloned; however, EMT activity and pharmacological inhibition have been repeatedly described [12]. Once inside the cell, eCBs are hydrolyzed to terminate signal transduction. The main catabolic enzyme of AEA is fatty acid amide hydrolase (FAAH) [13], a widely distributed intracellular membrane-bound serine hydrolase [14]. An additional lysosomal cysteine hydrolase termed

N-acylethanolamine-hydrolyzing acid amidase (NAAA) is also known [15], and cleaves AEA and congeners under acidic conditions (Fig. 1). As for 2-AG and congeners, monoacylglycerol lipase (MAGL) is the main responsible for their degradation, along with two additional serine hydrolases, known as α/β-hydrolase domain 6 and 12 (ABHD6 and ABHD12) [16].

Moreover, the oxidative metabolism of eCBs has (patho)physiological relevance, because it leads to the production of new biologically active metabolites [17]. In particular, AEA and 2-AG are metabolized by lipoxygenases (LOXs) [18] and by cyclooxygenase-2 (COX-2) [17–19], and additionally AEA can be oxygenated also by cytochrome P450 [20], as shown in Figs. 1 and 2.

Incidentally, it should be stressed that an emergent issue is how eCBs can reach their distinct sites of action within the cell (e.g., membrane or nuclear receptors, or metabolic enzymes) at the right time and at the right concentration, in order to trigger the appropriate response to a stimulus [21]. In this context, the existence of intracellular storage organelles (adiposomes or lipid droplets) [22], as well as of constitutive intracellular transporters (AEA intracellular transporters, AITs), has been reported for AEA [2]. A functional role for these AITs in eCB signaling has been recently documented [23], providing a proof of concept that indeed they can drive eCBs towards distinct transduction pathways. This is particularly striking in the central nervous system, where at each synapse distinct ECS elements in different neuronal and non-neuronal cells contribute to proper neurotransmission [24, 25]. The same complexity in other organs of our body (e.g., cardiovascular, digestive, musculoskeletal, immune, and reproductive systems) has been the subject of a comprehensive review [26].

Finally, strong pharmacological and biochemical evidence has demonstrated that eCBs are able to interact also with non-CB_1/non-CB_2 receptors, further increasing the complexity of the ECS and of the signaling pathways trigged thereof (Fig. 3). In particular, the best known of these targets is the transient receptor potential vanilloid type 1 (TRPV1) channel, which is activated by both AEA [27] and 2-AG [28]. Other potential receptors activated by eCBs are peroxisome proliferator-activated receptor (PPAR) α and γ [29], and the orphan G protein-coupled receptor GPR55 [30].

In Table 1 old and new members of the ECS are listed together. Unsurprisingly, ECS has been shown to regulate different physiological processes in the central nervous system [2–4] and at the periphery [26], thereby suggesting that its signaling may foster the development of pathway-selective drugs for therapeutic benefit [2–4].

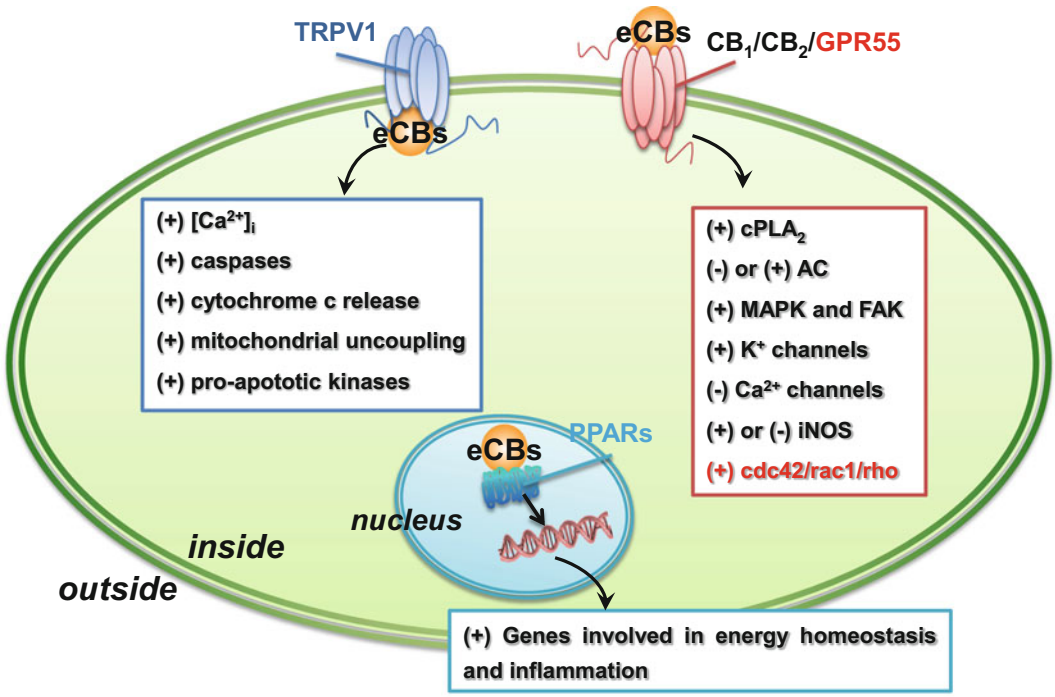


Fig. 3 Signal transduction pathways triggered by endocannabinoids through their main target receptors. *AC* adenylyl cyclase, *CB1* type-1 cannabinoid receptor, *CB2* type-2 cannabinoid receptor, *cPLA2* cytosolic phospholipase A_2, *eCBs* endocannabinoids, *FAK* focal adhesion kinase, *GPR55* orphan G protein-coupled receptor 55, *MAPK* mitogen-activated protein kinase, *iNOS* inducible nitric oxide synthase, *PPARs* peroxisome proliferator-activated receptors, *TRPV1* transient receptor potential vanilloid type 1 channel

2 Conclusions

Taken together, it appears all the more important to develop methods and protocols that allow to properly assay activity and location of the different ECS elements, possibly with specifications that make the same method fully effective in different cells, tissues, and organisms. For most ECS elements reliable methods are indeed available, and will be presented in this theme issue on "Endocannabinoid signaling: Methods and protocols" by those who developed and/or improved them over the last few years. Such a book is a manual that puts together all current methodologies to investigate eCB signaling in a timely manner. Thus, I believe that it will help chemists, drug designers, biochemists, molecular biologists, cell biologists, pharmacologists, and (electro)physiologists to successfully navigate with appropriate tools the *mare magnum* of eCB research.

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