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Endocannabinoid biosynthesis and inactivation, from simple to complex

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Cannabinoid receptors, the primary molecular targets of the endocannabinoid system, are activated by specific bioactive lipids termed 'endocannabinoids'. These lipid transmitters are synthesized from cell membrane phospholipids through multiple pathways and are inactivated by enzymatic hydrolysis, and their levels are the major parameter driving the endocannabinoid system activity. An in-depth understanding of their metabolic pathways is essential to unravel the endocannabinoid system's role in physiological and pathological situations and to devise new therapeutic strategies based on the endocannabinoid system. Major advances both in the characterization of anandamide's and 2-arachidonoylglycerol's biosynthesis and inactivation pathways and in the discovery of pharmacological tools used to interfere with their metabolism have been made and are discussed in this review.

Endocannabinoids are endogenous lipid transmitters that act by binding and activating specific G-protein-coupled-receptors termed 'CB$_1$ and CB$_2$ cannabinoid receptors'. To date, N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol are the most thoroughly studied endocannabinoids. Anandamide is a member of the N-acylethanolamine (NAE) family, a large group of bioactive lipids that also includes non-endocannabinoid compounds such as the anorexigenic derivative N-oleoylethanolamine and the anti-inflammatory compound N-palmitoylethanolamine [1]. These lipids are present throughout the body and their levels – more so than those of non-lipid transmitters – are finely regulated by the balance between synthesis and inactivation. There is now strong evidence that dysregulation of the tight control of endocannabinoid levels results in, or is causative of, pathological conditions such as obesity and related metabolic syndrome or neurological disorders, to name a few [2].

Since the beginning of its molecular description in the early 1990s, the endocannabinoid system has been considered an attractive therapeutic target in numerous diseases [3]. Early strategy consisted of targeting cannabinoid receptors using exogenous ligands such as dronabinol or rimonabant. Although efficacious, this approach is hampered by the consequences of receptors persistent activation or blockade and by the lack of tissue specificity of the ligands developed so far. A novel, more subtle, approach is the use of allosteric modulators, thus potentially avoiding some of the aforementioned drawbacks. However, the most promising alternative strategy, now supported by a growing number of studies, is to modulate endogenous ligand levels. Because lipid transmitters are produced on demand (i.e. in a stimulus-dependent fashion), interacting with endocannabinoid metabolism should offer the advantage of modifying their levels and, thus, cannabinoid receptor activation, mainly in tissues where this is needed. Preclinical studies have shown that increasing endocannabinoid levels could find applications for instance in treating anxiety, depression and inflammatory pain. For this strategy to be useful as a therapeutic approach, however, it is crucial to unravel the metabolic pathways involved in endocannabinoid synthesis and inactivation.

Initially, NAEs were thought to be produced by a phospholipase D and hydrolyzed by an amidase called 'fatty acid amide hydrolase', whereas 2-arachidonoylglycerol was believed to be produced by either a phospholipase C (PLC)–diacylglycerol lipase pathway or by a phospholipase A1–PLC pathway and hydrolyzed by a monoacylglycerol lipase. This rather simple picture has now evolved into a more complex array of biochemical pathways, with newly discovered enzymes representing attractive targets to

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enhance or decrease endocannabinoid signaling. Here, I discuss the biochemical pathways leading to endocannabinoid production and inactivation and the most recent pharmacological tools that, by interacting with these enzymes, enable the manipulation of endocannabinoid levels.

Multiple endocannabinoid biosynthetic pathways

NAE biosynthesis

N-acylphosphatidylethanolamines (NAPEs) are considered the general precursors for NAEs, yet the molecular characterization of the enzyme responsible for the activity-dependent transfer of the sn-1 acyl chain from 1,2-diacylglycerophospholipids (e.g. phosphatidylcholine) or 1-acyl lysophospholipids to phosphatidylethanolamine (an N-acyl transferase, NAT) is still awaited (Fig. 1a) [4,5]. An N-acyl transferase able to synthesize NAPEs was recently cloned but differs from NAT – in its relative insensitivity to Ca^{2+} activation, for example – and, therefore, was named 'iNAT' [6]. The second step in the canonical transacylation–phosphodiesterase pathway leading to NAEs is NAPE hydrolysis by a Ca^{2+}-sensitive NAPE-selective phospholipase D (NAPE-PLD) [7]. Its molecular cloning revealed a phosphodiesterase of the metallo-β-lactamase family [8], and the characterization of the purified enzyme confirmed that it is kept constitutively active in its membrane-associated form by membrane components such as phosphatidylethanolamine [8,9]. It is noteworthy that even though NAEs are produced on demand, NAPE-PLD seems to be kept in a constitutively active form.

Although the transacylation–phosphodiesterase pathway is considered the major route for NAE production, generation of NAPE-PLD knockout mice clearly pointed to the presence of additional metabolic pathways responsible for NAE, especially anandamide, synthesis. These mice have wild-type brain levels of anandamide and moderately reduced N-palmitoylethanolamine and N-oleoylethanolamine levels, contrasting with the strong decrease in long-chain saturated NAE levels [10]. Thus, with NAPEs generally accepted as NAE precursors, at least two pathways distinct from NAPE-PLD are now being characterized (Fig. 1a). One pathway has glycerophospho-N-acylethanolamine lipids (GP-NAEs) as key intermediates. The serine hydrolase ABHD4 (α/β-hydrolase 4, Abh4) was put forward as a B-type lipase producing GP-NAEs by double O-deacylation of NAPEs. Accordingly, cells overexpressing ABHD4 have increased levels of both NAPE-lipase and lyso-NAPE-lipase activities, with ABHD4 showing a marked preference for lyso-NAPE substrates over other lysophospholipids [11]. GP-NAEs' role as intermediate in NAE synthesis was confirmed by their detection in mouse brain and by the characterization of glycerophosphodiesterase 1 (GDE1) as a metal-dependent GP-NAE phosphodiesterase [12]. GDE1 is an integral membrane protein highly expressed in CNS tissues and showing highest activity with C16:0, C18:1 and C20:4 substrates. Interestingly, these are typically the substrates least affected by NAPE-PLD invalidation in mouse brain [10].

Another pathway, to date mainly characterized in macrophage-like RAW264.7 cells, uses phospho-N-arachidonoylethanolamine (pAEA) as a key intermediate in the synthesis of anandamide (Fig. 1a). In the presence of lipopolysaccharide (LPS), these cells have increased anandamide levels but decreased NAPE-PLD

expression. In the same conditions, expression of the phosphatase protein tyrosine phosphatase PTPN22 is increased and pAEA detected. Upon PTPN22 silencing in LPS-activated RAW264.7 cells, anandamide levels are decreased, confirming the role of PTPN22 in LPS-induced enhancement of anandamide levels. Thus, the suggested pathway involves the production of pAEA from N-arachidonoylphosphatidylethanolamine by a PLC, which, in turn, is hydrolyzed into anandamide by a phosphatase, with PTPN22 and SHIP1 being the prime candidates for this activity [13,14]. However, although the conversion of pAEA to anandamide by brain homogenates of PTPN22 and SHIP1 knockout animals is, indeed, reduced significantly (compared with wild-type), only a small reduction in anandamide levels was observed in $PTPN^{-/-}$ mice brain [14]. It is, of course, possible that upon stimulation (e.g. under inflammatory conditions), anandamide levels would further differ between $PTPN^{-/-}$ and wild-type animals. Another question of interest is whether this pathway is more or less selective for anandamide synthesis than the other NAEs; only data relevant to anandamide have been reported.

With the characterization of at least three important pathways through which NAEs are synthesized (Fig. 1a), the question of their selectivity gains even more interest. Current understanding is that depending on the acyl chain (and, thus, the resulting NAE), a given pathway will be preferred over the others (see above) [10]. In the future, it will be important to dissect out the factors – for instance the nature of the acyl chain, the phospholipid membrane composition at the site of synthesis, or the tissue and condition – favoring one pathway over the others for a given NAE. For instance, although both secreted PLA_2 and ABHD4 produce lyso-NAPEs from NAPEs, $sPLA_2$ low expression in the brain suggests a primary role for ABHD4 in CNS lyso-NAE production [11,15]. Another example can be found in the opposite regulation of NAPE-PLD and PTPN22 expression in macrophages upon LPS stimulation, suggesting that, at least in macrophages, PTPN22 rather than NAPE-PLD is responsible for activity-dependent anandamide production [13]. Finally, one has to consider that upon blockade of a pathway (especially in the case of knockout models), an alternative pathway or alternative pathways might compensate for the inactivated one. For example, GDE1-inhibited brain homogenates are still able to convert GP-AEA into anandamide, through pAEA, thus pointing to a link between the ABHD4 pathway and the phosphatase (PTPN22) pathway [14] (Fig. 1a). Our understanding of the intricate relationships between these pathways will benefit from the description of selective pharmacological tools targeting these enzymes.

2-Arachidonoylglycerol biosynthesis

It is well known that 2-arachidonoylglycerol is produced in a stimulus-dependent fashion in both the CNS and peripheral cells [16–18]. Stimuli can be cell depolarization, inducing a strong Ca^{2+} influx; activation of $G_{q/11}$-coupled-receptors, such as metabotropic glutamate receptors; or a combination of both mechanisms leading to increased 2-arachidonoylglycerol production (for a review, see Ref. [19]). This acylglycerol can be synthesized in two steps via generation of 1-acyl-2-arachidonoylglycerol (diacylglycerol, or DAG) from phosphatidylinositol by PLC activity and subsequent hydrolysis of DAG by a diacylglycerol lipase (Fig. 1b) [17,18]. It is thought that this PLCβ–DAGL pathway is involved in 2-arachidonoylglycerol retrograde signaling after activation of $G_{q/}$
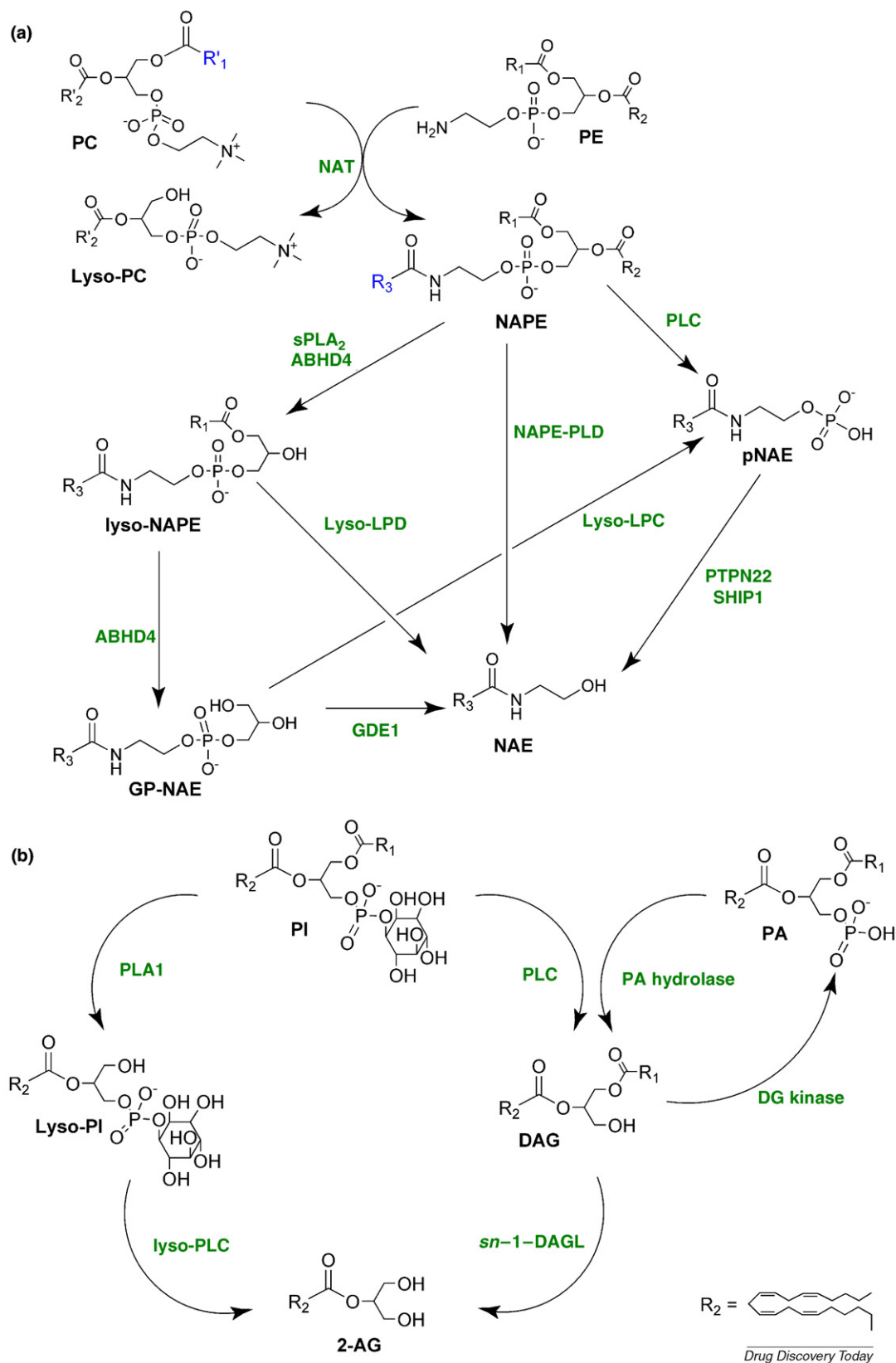
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FIGURE 1

Endocannabinoid biosynthetic pathways. **(a)** So far, all the described NAE synthetic pathways have NAPEs, produced from membrane phospholipids by NAT, as key intermediate. Note that the pNAE pathway was described for anandamide only. **(b)** Starting from membrane PIs, 2-AG is produced via a lyso-PI intermediate or a DAG intermediate. DAG can also be obtained from PA. Abbreviations: 1-Acyl-PC, 1-acyl-phosphatidylcholine; 2-AG, 2-arachidonoylglycerol; ABHD4, α/β-hydrolase 4; DAG,

[11]-type-coupled-receptors. Indeed, the phenomenon is absent in brain preparations from PLCβ knockout mice or after inhibition of these enzymes [20,21]. The molecular cloning of sn1-DAGLα and sn1-DAGLβ was a crucial step in further characterizing 2-arachidonoylglycerol biosynthesis [22]. Cellular activity of these enzymes correlates with 2-arachidonoylglycerol production and, conversely, their inhibition results in decreased endocannabinoid levels. The key intermediate DAG can also be produced from phosphatidic acid by a phosphatidic acid hydrolase, which thus represents an alternative pathway to PLC-driven DAG production [23].

A second pathway leading to 2-arachidonoylglycerol features a 2-arachidonoyl-lysophosphatidylinositol (lyso-PI) intermediate (Fig. 1b). It involves the sequential actions of a phosphatidylinositol-preferring phospholipase A_1, producing the lyso-PI intermediate, and of a lysophosphatidylinositol-selective phospholipase C (lyso-PLC) producing 2-arachidonoylglycerol [24]. Compared with the PLC–DAGL pathway, however, the actual relevance of this 2-arachidonoylglycerol-producing cascade in generating 2-arachidonoylglycerol as an endocannabinoid is less clear. It is important to keep in mind that most of these molecules, including DAG and 2-arachidonoylglycerol, are intermediates in several pathways, the most notable of which is arachidonic acid release. It is likely, therefore, that not all the pathways leading to 2-arachidonoylglycerol are actually involved in physiological cannabinoid signaling.

As mentioned above, 2-arachidonoylglycerol production is stimulated by cell depolarization or $G_{q/11}$-coupled-receptor activation. It seems, however, that differing pathways are responsible for 2-arachidonoylglycerol synthesis, depending on the stimuli. Indeed, 2-arachidonoylglycerol-mediated retrograde suppression of synaptic transmission after $G_{q/11}$-coupled-receptor activation is absent in PLCβ knockout mouse neurons, whereas 2-arachidonoylglycerol-mediated depolarization-induced suppression of inhibition (DSI) or excitation (DSE) is still present in both PLCβ knockout and PLCδ knockout mouse neurons [20,25]. However, stimuli-induced 2-arachidonoylglycerol production in mouse forebrain is affected by $G_{\alpha q}/G_{\alpha 11}$ protein invalidation, whereas basal production is unaffected by the mutation [26]. Although additional studies are needed to further dissect the pathways activated by a given stimuli, these differences already suggest that using specific inhibitors, one could finely tune 2-arachidonoylglycerol signaling.

Multiple degradation pathways

Endocannabinoids are produced on demand and released in the extracellular environment, where they can bind and activate CB_1 and CB_2 cannabinoid receptors. However, their hydrolysis – resulting in the termination of endocannabinoid signaling – is an intracellular event. Although endocannabinoids, lipophilic in nature, can freely cross cell membranes, evidence suggests the existence of mechanisms facilitating endocannabinoid internalization. One such mechanism could be an endocannabinoid transporter still awaiting molecular characterization (for a review, see

Ref. [27]). Lipophilicity is also expected to hinder endocannabinoid cytosolic trafficking toward their hydrolysis site (e.g. the endoplasmic reticulum for anandamide). Accordingly, fatty acid binding proteins 5 and 7 were recently identified as intracellular carriers for anandamide, increasing its rate of hydrolysis upon overexpression [28].

Besides the hydrolytic enzymes described below, endocannabinoids can be enzymatically transformed by other enzymes (cyclooxygenase 2 is one example) into pharmacologically active chemical entities (e.g. prostamides) [29] (Fig. 2). Although of interest because they represent pathways to additional bioactive lipids, to date only the hydrolytic enzymes reviewed below are considered to be responsible for terminating endocannabinoid signaling.

NAE hydrolysis

Although differing in the nature of their acyl moiety, NAEs are characterized by a common ethanolamide moiety and, thus, are hydrolyzed by a common group of amidases (Fig. 2). Fatty acid amide hydrolase (FAAH), cloned in 1996 and now extensively characterized, has received a great deal of attention because its pharmacological or genetic invalidation results in strongly enhanced NAE levels, both in the CNS and in the periphery [30]. FAAH is a membrane-bound enzyme member of the amidase signature family of enzymes characterized by a Ser-Ser-Lys catalytic triad. Crystal structures of FAAH have been reported and showed the presence of an acyl-chain-binding channel accommodating NAEs' acyl moiety and a cytoplasmic port enabling the ethanolamine leaving group to reach the cytoplasm [31,32]. FAAH has an alkaline optimal pH and preferentially hydrolyzes anandamide over other NAEs, such as N-oleoylethanolamine or N-palmitoylethanolamine. These differences in substrate hydrolysis are not apparent in $FAAH^{-/-}$ mouse brain tissue, however, since the three lipid levels were affected similarly by enzyme deletion [33]. Although this is suggestive of a FAAH primary role in controlling NAE levels, two additional amidases hydrolyzing anandamide and related compounds have been described.

A second amidase signature enzyme, FAAH-2, was found in human, but not rodent, tissues [34]. Although it shares the Ser-Ser-Lys catalytic triad with FAAH, the two enzymes have only limited sequence homology (∼20%). They also differ in their subcellular localization: FAAH-2 is localized in cytosolic lipid droplets and not in the endoplasmic reticulum as is FAAH [35]. Although FAAH-2 is less efficacious than FAAH at hydrolyzing NAEs [34,35], its high expression in peripheral tissues – including liver, where anandamide is known to have crucial metabolic roles – suggests that FAAH-2 might have a rescue role in hydrolyzing NAEs upon FAAH inactivation. A third NAE-hydrolyzing enzyme, N-acylethanolamine-hydrolyzing acid amidase (NAAA), is highly expressed in immune cells, specifically in macrophages, and localized into the lysosomes, where it is activated by autoproteolytic cleavage [36–38]. Accordingly, and in contrast to FAAH, NAAA is most active at acidic pH [39]. Another distinguishing feature is the nature of the catalytic nucleophile, which is a Cys residue in NAAA, compared with a Ser

diacylglycerol; DG kinase, diacylglycerol kinase; GDE1, glycerophosphodiesterase 1; GP-NAE, glycerophospho-NAE; lyso-NAPE, lysophosphatidyl-NAPE; lyso-PC, lysophosphatidylcholine; lyso-PI, lysophosphatidylinositol; lyso-PLC, lysophosphatidylinositol-selective phospholipase C; NAE, N-acylethanolamine; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, N-acylphosphatidylethanolamine-selective phospholipase D; NAT, N-acyltransferase; PA, phosphatidic acid; PA hydrolase, phosphatidic acid hydrolase; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA1, phospholipase A1; PLC, phospholipase C; pNAE, phospho-NAE; PTPN22, phosphatase protein tyrosine phosphatase 22; sPLA2, secreted phospholipase A2; SHIP1, SH2 domain-containing inositol phosphatase.

residue in FAAHs [38,40]. Given that the preferred substrate of NAAA is N-palmitoylethanolamine (the levels of which are increased during inflammation) and that NAAA is highly expressed in macrophages, NAAA is to be considered an interesting target in tackling inflammatory states. This is also relevant in the CNS where microglia is known to mediate neuroinflammation and to respond to N-palmitoylethanolamine [41]. Along this line, an additional N-palmitoylethanolamine-hydrolyzing activity distinct from NAAA and FAAHs was detected in a microglial cell line [42].

The presence of at least three NAE hydrolases, with only partially overlapping tissue expression, raises the question of their respective roles in regulating NAE levels. Whereas it is generally accepted that FAAH is the principal contributor to anandamide hydrolysis in the CNS, the role of FAAH-2 and NAAA is still an open question. From a therapeutic perspective, as FAAH inhibition increases anandamide, N-palmitoylethanolamine and N-oleoylethanolamine levels, it remains to be seen whether selected NAE levels can be independently regulated depending on the inhibited enzyme.
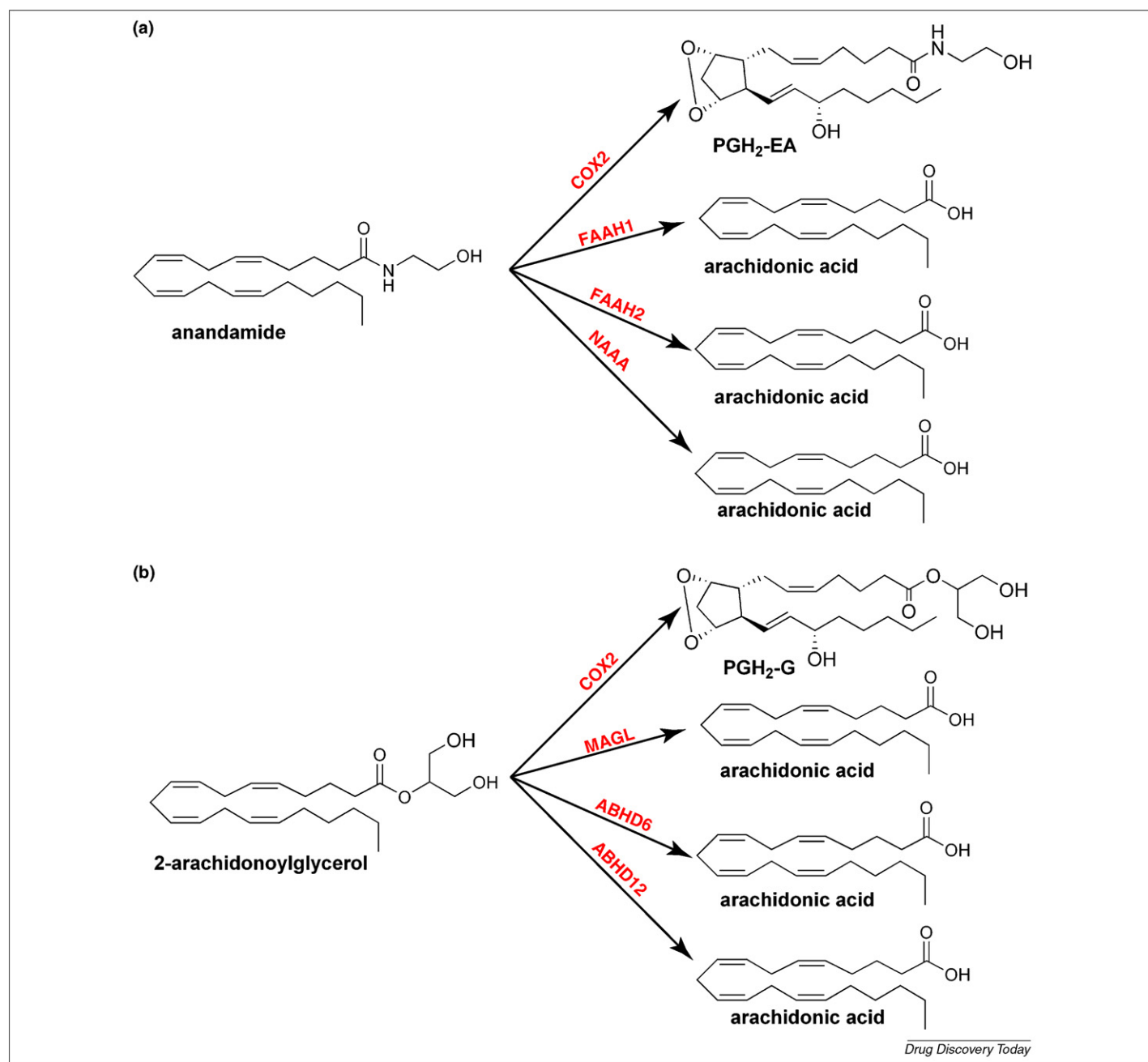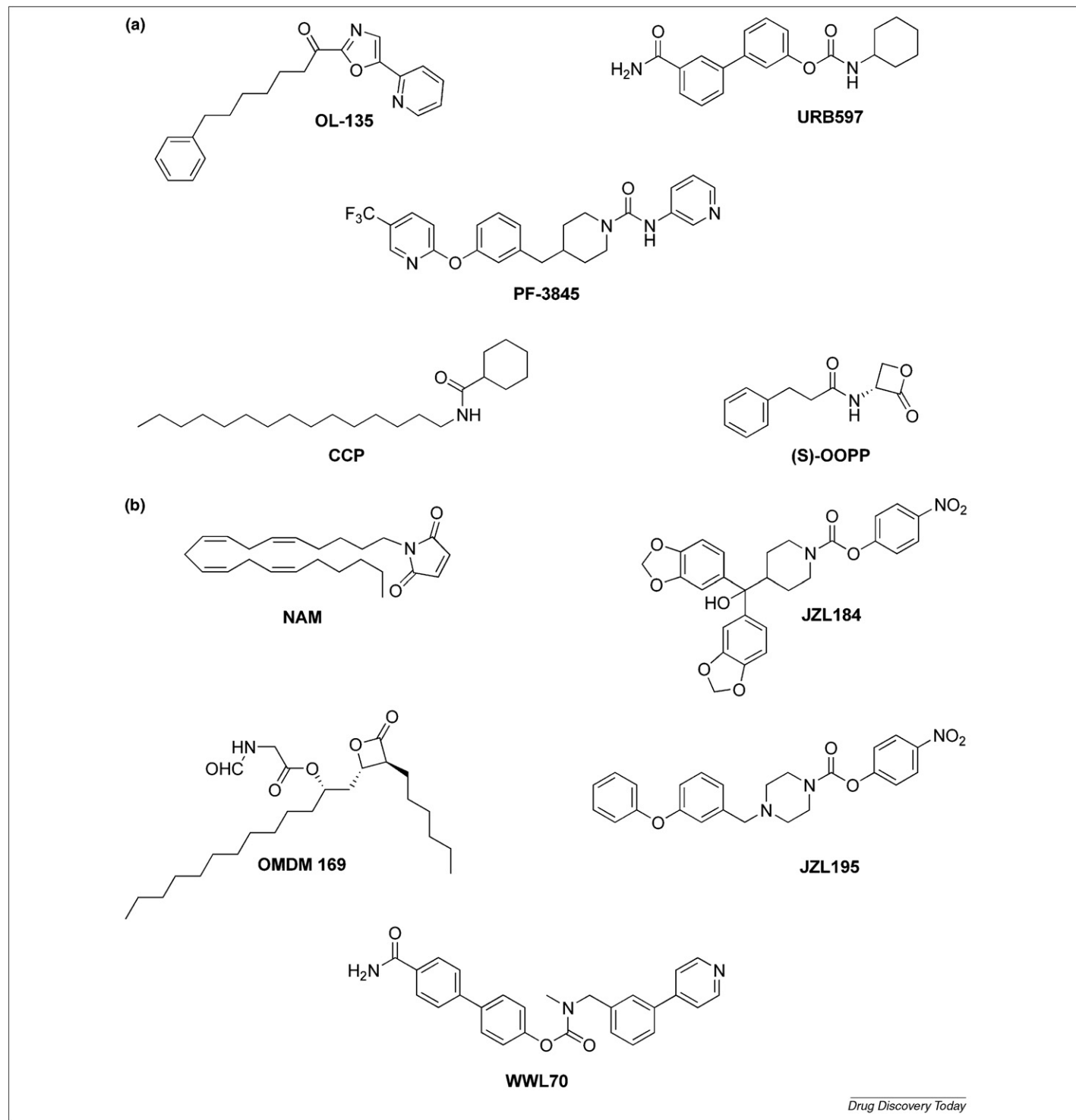

FIGURE 2

Schematic representation of the endocannabinoid inactivating pathways. **(a)** Anandamide endocannabinoid signaling is inactivated by FAAH1, FAAH2 or NAAA-mediated hydrolysis into arachidonic acid or by COX2 oxidation into PGH$_2$-EA. **(b)** 2-Arachidonoylglycerol signaling is terminated by MAGL, ABHD6 or ABHD12-mediated hydrolysis or by COX2 oxidation into PGH$_2$-G. Note that arachidonic acid, PGH$_2$-EA and PGH$_2$-G can be further transformed into other bioactive lipids, such as prostaglandins and endocannabinoid-derived prostaglandins, respectively. Abbreviations: ABHD6 and 12, α/β-hydrolase 6 and 12; COX2, cyclooxygenase 2; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAAA, N-acylethanolamine-hydrolyzing acid amidase; PGH$_2$-EA, PGH$_2$-ethanolamide; PGH$_2$-G, PGH$_2$-glyceryl ester.

2-Arachidonoylglycerol hydrolysis

It is now firmly established that the serine hydrolase monoacyl-glycerol lipase (MAGL) is the main contributor to brain 2-arachidonoylglycerol hydrolysis [43–45]. Because of its activity against 2-arachidonoylglycerol and its presynaptic expression, MAGL has long been considered the pivotal enzyme controlling the duration of 2-arachidonoylglycerol-mediated retrograde signaling [46,47]. However, this was only confirmed recently using a potent and selective MAGL inhibitor (JZL184, Fig. 3) [48,49]. Similarly, novel evidence demonstrated that 2-arachidonoylglycerol increased levels upon MAGL inhibition induce CB_1-receptor-dependent behavioral effects [50,51]. Beyond the CNS, MAGL inhibition

FIGURE 3

Endocannabinoid hydrolysis inhibitors. **(a)** URB597, OL-135 and PF-3845 are selective FAAH inhibitors that were shown to increase cellular or *in vivo* NAE levels. CCP and (S)-OOPP are NAAA inhibitors, the latter increased PEA levels in immune cells both *in vitro* and *in vivo*. **(b)** NAM, JZL184 and OMDM169 are MAGL inhibitors. JZL195 is a selective 'dual' MAGL–FAAH inhibitor increasing anandamide and 2-arachidonoylglycerol levels *in vivo*. WWL70 is an inhibitor of ABHD6. *Abbreviations*: ABHD6, α/β-hydrolase 6; FAAH, fatty acid amide hydrolase; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; NAEs, *N*-acylethanolamines.

resulted in substantially increased monoacylglycerol levels in several peripheral tissues, including liver and adipose tissue [52]. Thus, MAGL also regulates the levels of monoacylglycerols, such as palmitoylglycerol and oleoylglycerol, that have not been implicated in endocannabinoid signaling.

Besides regulating 2-arachidonoylglycerol brain levels, MAGL activity affects brain arachidonic acid levels. Using organophosphorous inhibitors, Nomura and coworkers showed that MAGL inhibition results in decreased fatty acid concentrations. Because these inhibitors do not inhibit cPLA2, their effect on arachidonic acid tissue content was suggested to be mediated by MAGL [53,54]. MAGL involvement was later confirmed using the selective inhibitor JZL184 (Fig. 3) [50,52]. Nevertheless, the physiological relevance of MAGL in controlling arachidonic acid metabolism – and, thus, a wealth of downstream mediators – remains to be established.

From a drug discovery perspective, an interesting MAGL feature is the possibility to target either the catalytic Ser residue or one of the Cys residues located in the vicinity of the active site. This hypothesis was suggested using molecular modeling of MAGL and later confirmed by mass spectrometric and mutational studies that identified several Cys residues as probable targets of several MAGL inhibitors (e.g. N-arachidonoylmaleimide, disulfiram and isothiazolinones) [44,55–57]. The presence of three Cys residues close to the active site was unequivocally demonstrated by the recently reported human MAGL crystal structures. Indeed, two groups independently solved and reported the tridimensional structure of the enzyme that presents itself as the predicted α/β-hydrolase [58,59]. Overall, the two structures do not show marked differences. One characteristic feature of MAGL structure is the presence of a lid made of two loops surrounding the α4 helix. This helix has a high content of apolar and lipophilic residues pointing outside the enzyme, suggesting that it enables MAGL to anchor itself to cell membranes. This hydrophobic helix, therefore, could explain why MAGL is found in both cytoplasmic and membrane fractions.

A residual 2-arachidonoylglycerol hydrolysis upon MAGL inhibition or immunodepletion, as well as 2-arachidonoylglycerol hydrolysis in cells not expressing MAGL, suggested the existence of additional enzymes controlling the endocannabinoid levels in vivo [43,44,60]. FAAH could be one of these enzymes because it efficiently hydrolyzes 2-arachidonoylglycerol in vitro, and URB597 was shown in some studies to increase 2-arachidonoylglycerol tissue levels [61,62]. Note, however, that in numerous studies, FAAH inhibition (e.g. using URB597 [63] or PF-3845 [64]) or genetic invalidation [63] had no effect on 2-arachidonoylglycerol levels. This suggests either a role for FAAH in specific conditions and/or tissues only or a lack of specificity for some of the inhibitors used in these studies.

Using a functional proteomic approach, two additional enzymes, ABHD6 and ABHD12, were identified as 2-arachidonoylglycerol hydrolases [45]. These novel 2-arachidonoylglycerol α/β hydrolases make up for most of the non-MAGL-dependent 2-arachidonoylglycerol hydrolysis found in mouse brain. Because MAGL, ABHD6 and ABHD12 display different subcellular – and probably cellular – localizations, it is believed that they might control independent pools of 2-arachidonoylglycerol and, thus, signaling events. Because arachidonic acid is also a bioactive lipid at the crossroad of several signaling pathways, the presence of three 2-arachidonoylglycerol-hydrolyzing enzymes might also be relevant for endocannabinoid-unrelated processes.

Inhibitors of endocannabinoid biosynthesis and hydrolysis

Numerous endocannabinoid metabolism inhibitors have been described over the years. However, only a limited number have been proven efficacious in modulating endocannabinoid levels in
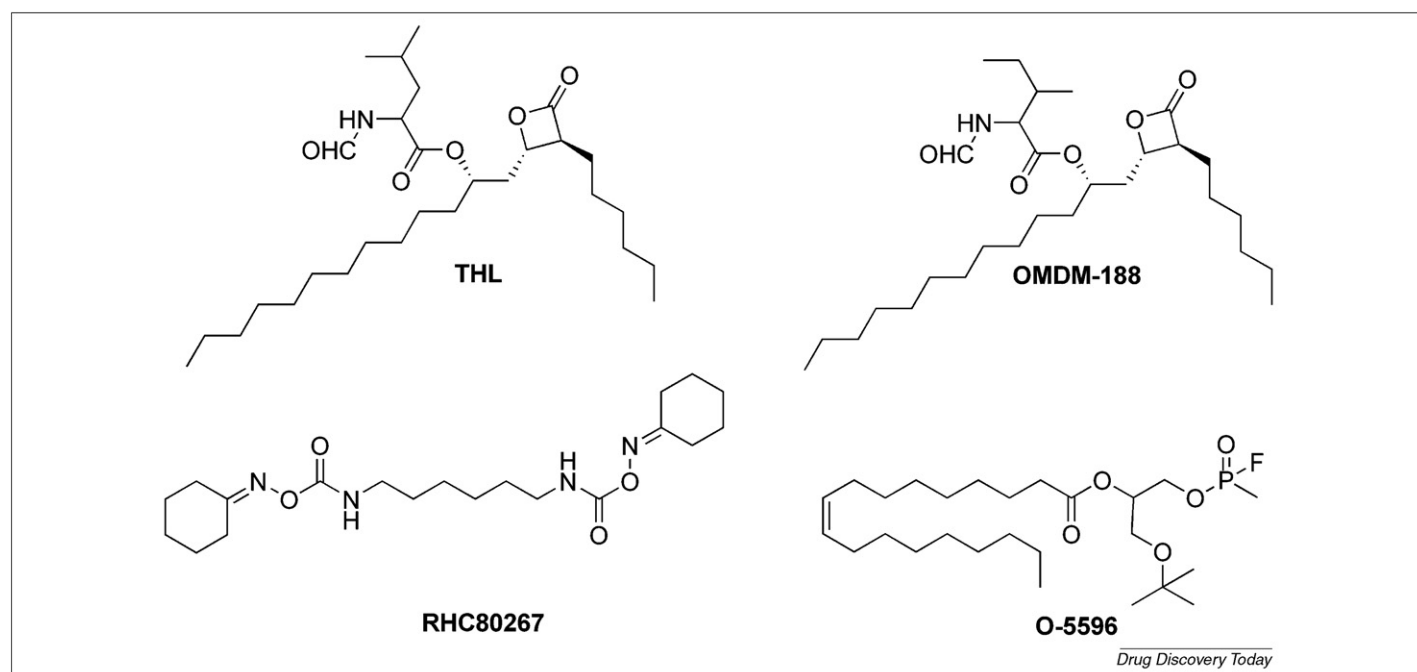

FIGURE 4

2-Arachidonoylglycerol biosynthesis inhibitors. Chemical structures of diacylglycerol lipase (DAGL) inhibitors reported to increase 2-arachidonoylglycerol levels.

cell culture or *in vivo*. With the development of proteome-wide activity assays and endocannabinoid quantification techniques, potent, selective and efficacious inhibitors are becoming available for several enzymes.

Endocannabinoid biosynthesis inhibitors

The few endocannabinoid synthesis inhibitors reported to date target 2-arachidonoylglycerol biosynthesis [65]. Compounds such as tetrahydrolipstatin and RHC80267 (Fig. 4) have been extensively used to inhibit DAGL-mediated 2-arachidonoylglycerol production in neuronal cell cultures. Refined tools are desirable, however, because their selectivity has been put into question [66]. Second-generation inhibitors such as the fluorophosphonate-based O-5596 and the tetrahydrolipstatin analog OMDM-188 might represent interesting starting points for more selective drugs [67,68]. Such inhibitors should be useful tools because one can speculate that reducing endocannabinoid production in tissues in which it is pathologically overactive might be an interesting alternative to cannabinoid receptor antagonists. Although very attractive, this approach still represents a challenge because NAEs and 2-arachidonoylglycerol are produced through a multitude of synthetic pathways and because they share these pathways with numerous other bioactive lipids. Thus, inhibition of an enzyme might not be sufficient to actually decrease their production, whereas, conversely, inhibiting an enzyme involved in 2-arachidonoylglycerol or NAE synthesis could result in unexpended changes in cell membrane composition or bioactive lipid production. These elements demonstrate how useful tools enabling to selectively target each individual enzyme would be for our understanding of the endocannabinoid system.

Endocannabinoid hydrolysis inhibitors
NAE hydrolysis inhibitors

The spectrum of FAAH inhibitors is large and comprises numerous potent and selective compounds (for a review, see Ref. [30]). The most widely used is the carbamate derivative URB597, which inactivates FAAH by carbamylation of the Ser nucleophile (Fig. 3). Proteome-wide screening of serine hydrolases has enabled the development of highly selective compounds, such as the α-ketoheterocycle derivative OL-135 and the urea derivative PF-3845 [30,64]. All three compounds enhance anandamide levels after *in vivo* administration and, thus, are useful tools to dissect anandamide and FAAH-dependent pathways. *In vitro*, however, both OL-135 and URB597 are actually more potent at inhibiting FAAH-2 [34]. Contrasting with the plethora of FAAH inhibitors, only two NAAA inhibitors, *N*-cyclohexanecarbonylpentadecylamine and (S)-OOPP, have been reported [40,69]. (S)-OOPP increases *N*-palmitoylethanolamine levels, without affecting anandamide levels, both in stimulated cells and in mouse tissues [40].

2-Arachidonoylglycerol hydrolysis inhibitors

Considerable progress is being made toward the characterization of potent and selective 2-arachidonoylglycerol hydrolysis inhibitors. For example, the MAGL competitive inhibitor OMDM169 (Fig. 3) increases 2-arachidonoylglycerol, but not anandamide, levels in ionomycin-stimulated N18TG2 cells and increases 2-arachidonoylglycerol levels at the site of formalin-induced paw inflammation. However, because of its structural similarity with tetrahydrolipsta-

tin, OMDM169 also inhibits pancreatic lipase and DAGL-α [67]. Contrasting with more classical approaches, the activity of the carbamate-based derivative JZL184 toward serine hydrolases was assessed in a proteome-wide assay, revealing a highly potent and selective MAGL inhibitor. Administration of JZL184 increases 2-arachidonoylglycerol, but not anandamide, levels in the CNS and peripheral tissues and results in strong cannabinoid-related effects [50–52]. Note that JZL184 is one order of magnitude less potent toward rat MAGL than mouse and human orthologs. Although JZL184 also inhibits two lung serine hydrolases (carboxylesterases ES1 and TGH2), its selectivity profile remains excellent [52]. Because JZL184 is able to inhibit MAGL without affecting ABHD6 or ABHD12 activity, it is a powerful tool to investigate MAGL's role in regulating 2-arachidonoylglycerol levels without the confounding effect of the additional 2-arachidonoylglycerol-hydrolyzing activities. *N*-Arachidonoylmaleimide (NAM) is able to inhibit MAGL activity *in vitro* and shows some selectivity compared with FAAH [44,45]. When administered *in vivo*, NAM raises 2-arachidonoylglycerol levels but does not show any cannabinoid effect *per se*. However, co-administration of NAM with 2-arachidonoylglycerol results in hypothermia, inhibition of locomotion, antinociception and catalepsy, showing that it can efficiently protect 2-arachidonoylglycerol from degradation [70]. As for FAAH, the recent report of MACL crystal structure will certainly be of great help in the design of inhibitors based on original templates. Finally, novel inhibitors that target ABHD6 and ABHD12 are being developed. ABHD6 is dose dependently inhibited by the carbamate derivative WWL-70, whereas ABHD12 is inhibited by the DAGL inhibitor tetrahydrolipstatin [45,71]. These, and second-generation inhibitors, will be crucial in delineating the respective roles of MAGL, ABHD6 and ABHD12 in 2-arachidonoylglycerol hydrolysis *in vivo*.

Tools designed to assess the activity and selectivity of these inhibitors toward their respective target both *in vitro* (e.g. high-throughput assays using purified and/or recombinant enzymes) and *in vivo* (e.g. proteomic approaches combined with click chemistry) are available [72,73]. These mediators are now easily quantified from a host of tissues, although generally *ex vivo*. Further interesting insights could be gained from quantifying 2-arachidonoylglycerol and NAE levels 'in situ'. Indeed, although extracellular endocannabinoids might not be solely responsible for the observed endocannabinoid effects, generalization of techniques such as microdialysis in combination with the use of the novel selective inhibitors could further improve our understanding of the endocannabinoid system. Thus, an integrative approach combining data from both tissue and extracellular endocannabinoid quantification, in conjunction with a reading of *in vivo* enzyme activity, should ultimately give us a sense of the actual *in vivo* regulation of endocannabinoid metabolism. Because both the biosynthetic and degradation pathways are likely to vary depending on cell type, measuring AEA or 2-arachidonoylglycerol levels in whole tissues could well mask the effect of the deletion or inhibition of a given pathway, especially if the pathway is only relevant to a limited portion of the cells present in that tissue. This could be solved, in part, using cell lines and primary cell cultures.

Concluding remarks

Endocannabinoids are produced in an activity-dependent manner and interact with the CB_1 and CB_2 cannabinoid receptors, and

their signaling is quenched by enzymatic inactivation. As discussed, because endocannabinoids are either acylamides (anandamide) or acylesters (2-arachidonoylglycerol), their metabolic pathways differ. Considerable progress in our understanding of these pathways has been made possible in the past few years through a combination of genetic models, proteomic approaches and selective inhibitors. A long way from the initial picture of endocannabinoid metabolism, we now have a glimpse of a much more complex network of enzymes controlling endocannabinoid levels and, thus, endocannabinoid system activity. Far from being negative, this complexity opens new and exciting possibilities. For example, the ability to selectively control anandamide and 2-arachidonoylglycerol CNS levels enabled their respective, and sometime synergistic, roles in several behavioral processes to be determined [51].

Nonetheless much remains to be done such as to obtain selective inhibitors of the newly identified biosynthetic enzymes. These inhibitors, as well as genetic models, are eagerly awaited to investigate the precise role of each enzyme in regulating NAE and 2-arachidonoylglycerol production. For instance, we still have to understand which enzyme (if only one enzyme), is responsible for anandamide production in the CNS. Another question is whether it will be possible to regulate the levels of selected NAEs without affecting the others. As for 2-arachidonoylglycerol, the precise role of ABHD6 and ABHD12 in controlling its levels and function needs to be clarified.

The endocannabinoid system is characterized by a multiplicity of targets, mediators and enzymes regulating its activity. The recent, and future, progress made in its comprehension will certainly offer new and exciting therapeutic perspectives.

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