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Endogenous Cannabinoid Signaling

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INTRODUCTION

There are two principal lines of evidence supporting the existence of an endogenous cannabinoid system. First, it is clear that cannabimimetic drugs exert their effects by binding with high affinity to selective membrane receptors (1–3). Second, substances with cannabimimetic properties, but with chemical structures that greatly differ from those found in plants, have been discovered in animal tissues (4–7). These indications are relevant, but they are open nevertheless to several possible objections. For example, although endogenous cannabimimetic compounds exist, it is conceivable that they may never engage cannabinoid receptors under physiological conditions. Indeed, if these receptors were constitutively active in the absence of agonist, as suggested by studies with heterologous expression models (8), there may be no need for an intrinsic cannabinoid "system," intended as a group of interconnected cells that produce and respond to endogenous cannabinoids.

These and similar concerns highlight the many gaps left unfilled in our understanding of intrinsic cannabinoid modulation. It is now generally agreed that substances with cannabimimetic properties are released during neuronal activity, and that these substances are inactivated by a set of mechanisms parallel to, but distinct from, those utilized for the elimination of established neurotransmitters. But where in brain and peripheral tissues do these reactions take place? And under what circumstances? Are there discrete cannabinergic pathways comparable to, say, dopaminergic ones? Or do endogenous cannabinoids act as local mediators? What behavioral needs regulate endogenous cannabinoid release? And what physiological adaptations are served by such release? To address these questions critically, we need first to recognize certain biochemical and physiological peculiarities of the cannabinoid signaling system which distinguish it from classical neurotransmitters. The nature of these peculiarities is the key theme that will be developed in the present review.

ENDOGENOUS CANNABINOIDS

A distinctive structural feature of the two endogenous cannabimimetic substances identified thus far, arachidonylethanolamide (anandamide) and 2-arachidonylglycerol (2-AG), is that both are chemical derivatives of the polyunsaturated fatty acid, arachidonic acid (Fig. 1). As such, these compounds show a certain degree of structural resemblance with the eicosanoids, an ubiquitous class of bioactive lipids produced by the enzymatic oxygenation of nonesterified arachidonic acid (for review, see 9, 10). Despite these chemical affinities, anandamide and 2-AG are clearly set apart from the eicosanoids by their different biosynthetic routes, which do not appear to involve release and oxidative metabolism of arachidonate. Anandamide and 2-AG are thought to be produced instead through stimulus-dependent cleavage of two distinct phospholipid precursors present in the membranes of neurons, immunocytes and other cells.

PATHWAYS OF FORMATION

Anandamide

Early reports of anandamide biosynthesis through energy-independent condensation of nonesterified arachidonate with ethanolamine have been subsequently attributed to a reversal of the anandamide amidohydrolase reaction, which participates in anandamide degradation (11,12) (discussed in 13, 14), or to the artifactual formation of compounds with some chromatographic properties of anandamide (15) (discussed in 16). Since anandamide amidohydrolase requires high concentrations of arachidonate and ethanolamine when acting in reverse, much higher than those normally found in cells (17,18), this enzyme is unlikely to play a physiological role in anandamide formation (for a possible exception in uterine tissue, see 19).

Another model of anandamide is illustrated schematically in Fig. 2. According to this model, anan-
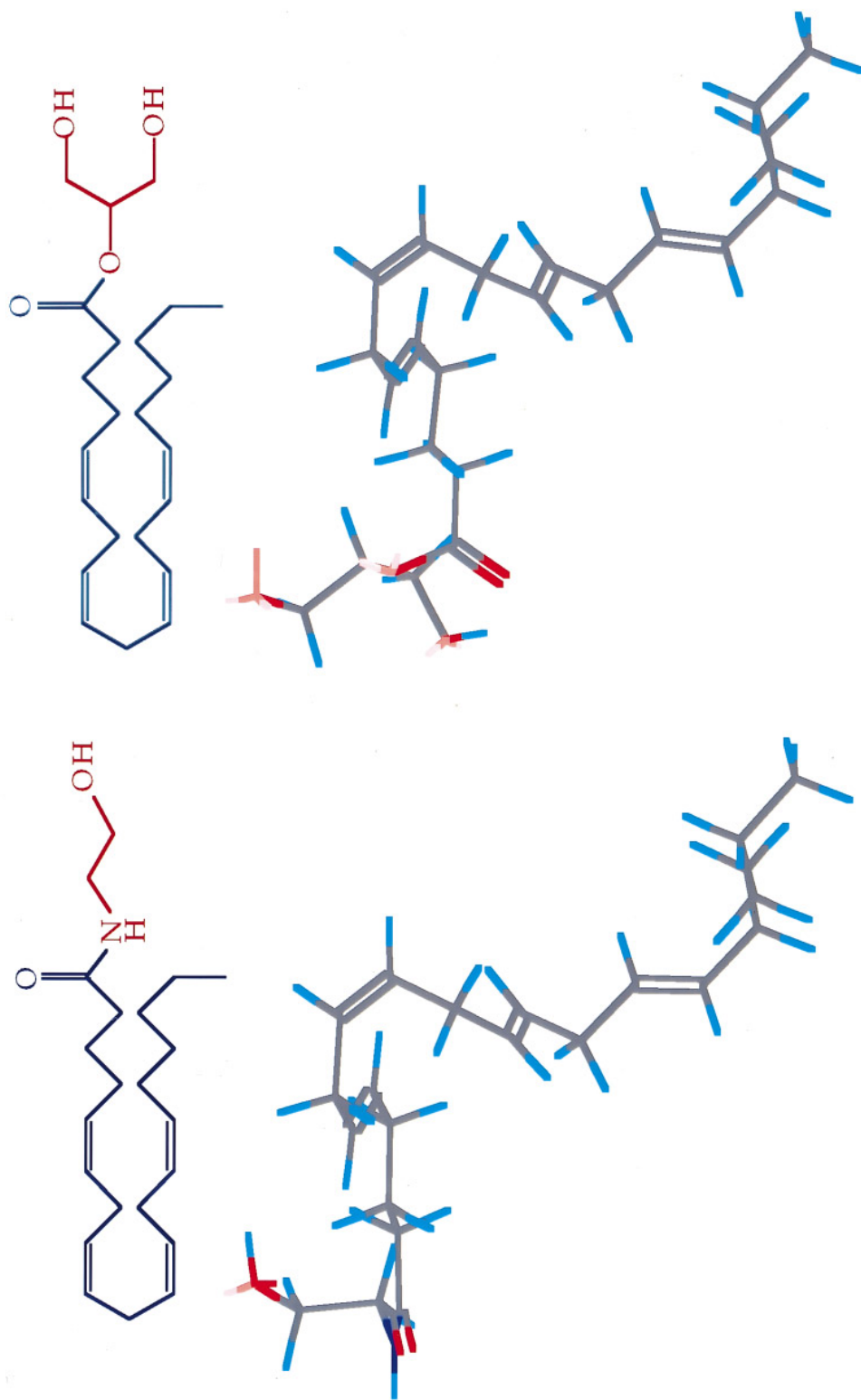
2-Arachidonylglycerol

Anandamide

FIG. 1. Structures and possible low-energy conformations of anandamide (arachidonylethanolamide) and 2-arachidonylglycerol. Local energy minima for each compound were computed by utilizing the Allinger's MM2 force field, as implemented in the Chem3D program (CambridgeSoft). In the top panel, the arachidonic acid moiety is depicted in red; ethanolamine and glycerol are depicted in blue. In the bottom panel, carbon atoms are shown in brown, hydrogen is shown in blue, oxygen is shown in red, and nitrogen is shown in purple.
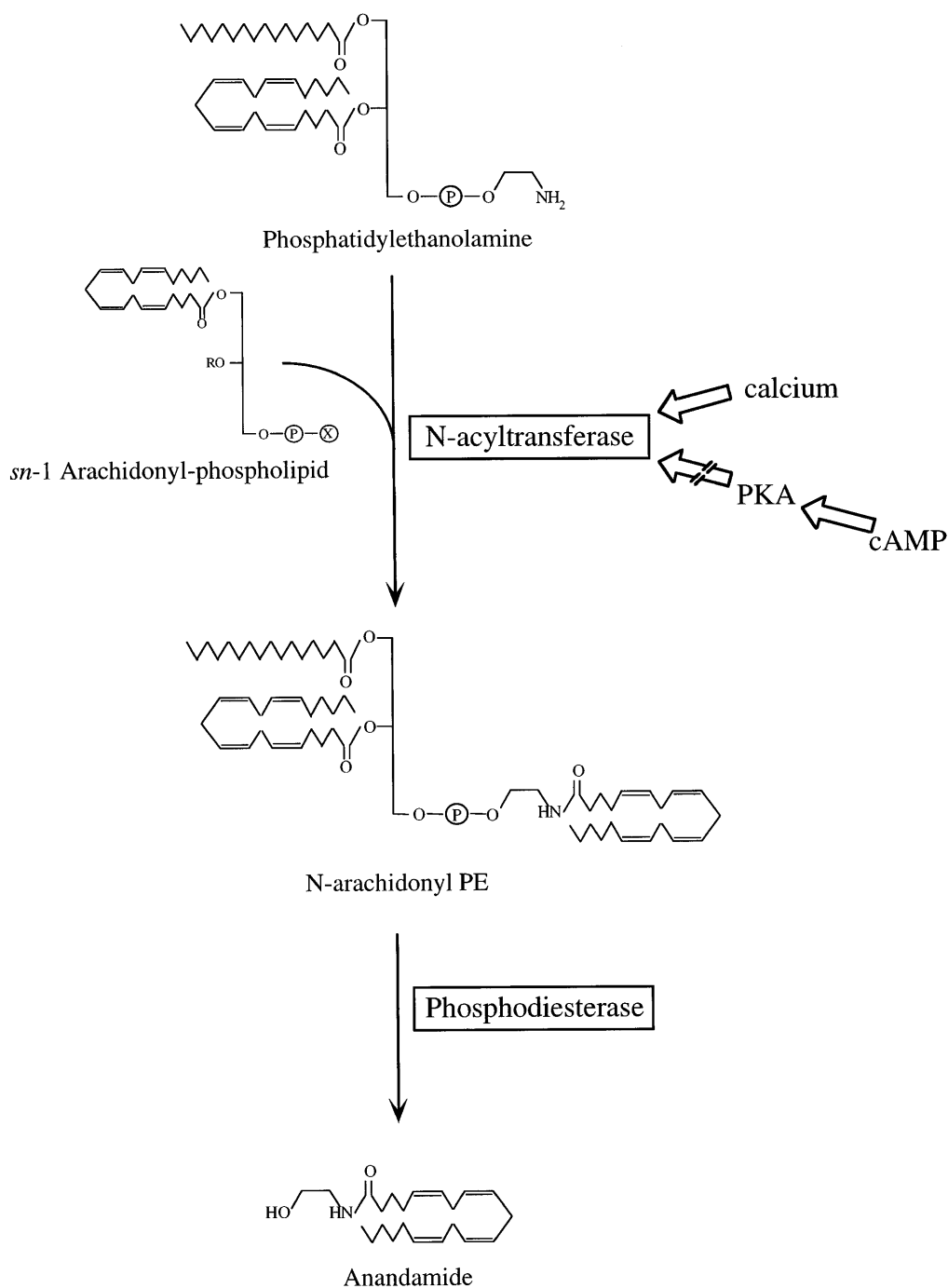
FIG. 2. This hypothetical model illustrates the two main enzymatic reactions implicated in anandamide formation: (1) anandamide may be generated by the hydrolysis of *N*-arachidonyl phosphatidylethanolamine (PE), catalyzed by a D-type phosphodiesterase activity (PLD); (2) the resynthesis of *N*-arachidonyl PE depleted in anandamide formation may be mediated by *N*-acyltransferase (NAT) activity; this enzyme detaches arachidonate from the *sn*-1 position of a phospholipid such as phosphatidylcholine (PC), and transfers it to the primary amino group of PE. Stimuli that raise intracellular Ca^{2+} ($[Ca^{2+}]_i$) stimulate simultaneously PLD and NAT activities, causing the release of anandamide and the resynthesis of its precursor. In addition to Ca^{2+}, NAT activity may also be regulated by receptors (R) that are linked to the stimulation of adenylyl cyclase and cAMP-dependent protein kinase A (PKA).

damide formation proceeds from the cleavage of the phospholipid precursor, *N*-arachidonyl phosphatidylethanolamine (PE), catalyzed by a phosphodiesterase activity such as phospholipase D. The precursor consumed in this reaction may be rapidly resynthesized by a second enzyme activity, *N*-acyltransferase, which cleaves arachidonate from the *sn*-1 glycerol ester position of phospholipids and transfers it to the primary amino group of PE.[1] Under physiological conditions, formation of anandamide and resynthesis of its precursor may be initiated at the same time, when neurons are depolarized and intracellular Ca^{2+} levels are elevated (20–25).

The anandamide precursor, *N*-arachidonyl PE, belongs to a family of N-acylated PEs, the ethanolamine moiety of which is linked to different saturated or unsaturated fatty acids. Like *N*-arachidonyl PE, other *N*-acyl PEs are also synthesized by *N*-acyltransferase and, when cleaved by phosphodiesterase, give rise to acylethanolamides. In addition to their lack of activity on CB1 receptors (4,26), we know very little about the pharmacological effects of these acylethanolamides and even less about their possible biological functions (see for review 27, 28). One notable exception may be palmitylethanolamide, which is produced by cleavage of *N*-palmityl PE. This compound exerts significant analgesic and anti-inflammatory effects *in vivo* (29,30) which have been attributed to its ability to interact with a CB2-like receptor sensitive to the compound SR144528 (31). The molecular identity of this putative receptor subtype is unknown, although it is likely to be distinct from the cloned CB2 receptor (3) for which palmitylethanolamide shows little or no binding affinity (32) (but see 33, for contrasting results). The fact that both *N*-acyl PEs and acylethanolamides are also present in plants, where their synthesis is regulated by extracellular stimuli (34,35), suggests that this signaling mechanism has been established early in the evolution of multicellular organisms.

2-Arachidonylglycerol

Textbook lipid biochemistry predicts two most plausible pathways of 2-AG biosynthesis, which are depicted in Fig. 3. Phospholipase C (PLC) hydrolysis of membrane phospholipids produces 1,2-diacylglycerol, which is converted to 2-AG by diacylglycerol lipase

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[1]Although arachidonate is generally stored in the *sn*-2 position of phospholipids, a small pool in which this fatty acid is esterified to the *sn*-1 position has been recognized(23,24,78,79).

activity. Alternatively, phospholipase A_1 (PLA_1) generates a lysophospholipid, which is hydrolyzed to 2-AG by lyso-PLC activity (36–41). In addition to these phospholipase-mediated pathways, 2-AG accumulation may result from the breakdown of triacylglycerols, catalyzed by neutral lipase activity (42), or from the dephosphorylation of lysophosphatidic acid (LPA). The fact that 2-AG formation in cortical neurons in culture is prevented by various PLC and diacylglycerol lipase inhibitors suggests a predominant involvement of the PLC pathway (43). However, the phospholipid substrate and PLC isoform implicated in this reaction remain to be discovered. Also, it cannot be excluded that multiple enzyme pathways may participate in generating 2-AG, an event that is not uncommon in lipid metabolism (see 44, for example).

Regardless of its precise mechanism, 2-AG biosynthesis appears to be triggered by rises in intracellular Ca^{2+} elicited during neuronal activity. This was shown in hippocampal slices by applying electrical stimulations to the Schaffer collaterals, a glutamatergic fiber tract in the Ammon's horn region that projects from CA3 to CA1 neurons. High-frequency stimulation of these fibers produced a fourfold increase in 2-AG accumulation compared to controls, which was prevented by the Na^+ channel blocker tetrodotoxin or by removing Ca^{2+} from the superfusing solution. Noteworthy, the local concentrations reached by 2-AG after stimulation were calculated to be approximately 1–2 μM (43). Since 2-AG binds to CB1 receptors with a K_d of 0.7–2 μM (7,6,43), the levels of 2-AG found after stimulation are expected to cause a substantial activation of the dense CB1 receptor population expressed in hippocampus (45,46).

RELEASE UPON DEMAND

An essential feature of these models is that both anandamide and 2-AG may be produced and released upon demand, through a mechanism that may not require vesicle neurosecretion. In the case of anandamide, this hypothesis is supported by a variety of experimental evidence. First, the concentration of anandamide in neurons is exceedingly low under basal conditions (5–10 pmol/g) (22,24,47,48), about 100 to 10,000 times lower than those of amino acid and amine neurotransmitters which are stored in synaptic vesicles (49). Second, stimulus-dependent release of anandamide from neurons is associated with anandamide formation and with *de novo N*-arachidonyl PE biosyn-
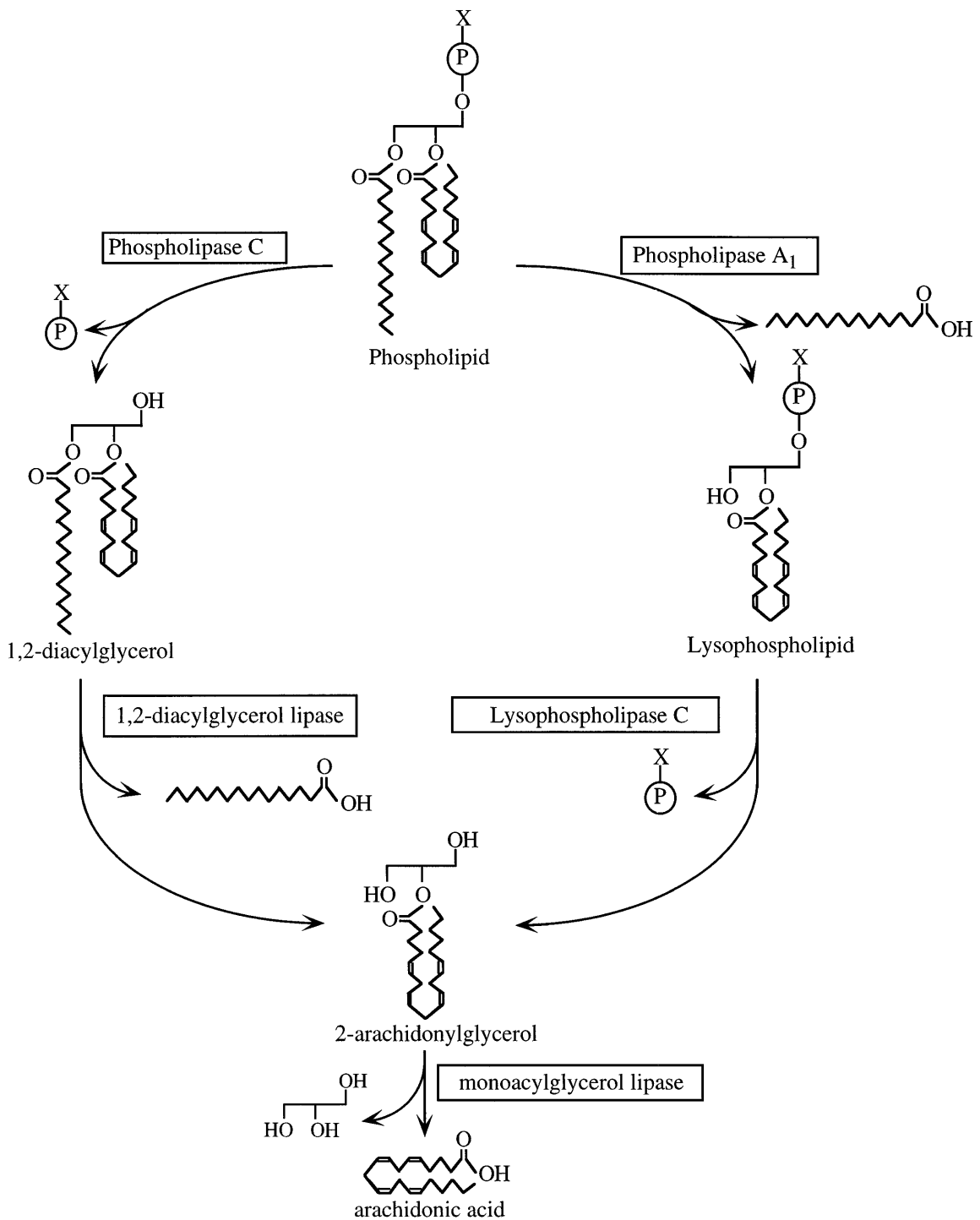
FIG. 3. Two main pathways of 2-AG formation in neurons may be predicted on the basis of our current understanding of phospholipid metabolism. Phospholipid hydrolysis by phospholipase C activity may produce 1,2-diacylglycerol which, in turn, may be cleaved by 1,2-diacylglycerol lipase to yield 2-AG. Alternatively, phospholipase A_1 activity may give rise to a lysophospholipid, which may be converted to 2-AG by lysophospholipase C. In addition to these pathways, 2-AG may be produced by triacylglycerol hydrolysis, catalyzed by neutral lipase activity (modified from Ref. 10).

thesis (20,21,24). Third, the release of anandamide may be dissociated experimentally from that of classical neurotransmitters; for example, although striatal neurons in culture rapidly take up radioactively labeled anandamide, they do not release it in a Ca^{2+}-dependent manner, as it can be readily demonstrated with labeled amino acids or biogenic amines (S. Schinelli and D. Piomelli, unpublished observations). A parsimonious interpretation of these findings is that anandamide may be produced when need arises and immediately dispatched outside cells, without an intermediate step of vesicle storage.

MECHANISM OF ANANDAMIDE RELEASE

How is newly formed anandamide released, and how does it reach its cellular targets? Water-soluble neurotransmitters that are released by secretion can diffuse unhindered through the aqueous compartment of the synaptic cleft to their postsynaptic receptors. But this is probably not the case with anandamide, the hydrophobic nature of which may favor its association with lipid membranes and introduce considerable constraints to its extracellular movements. Nevertheless, we know that anandamide does exit neurons because it can be found in incubation media of brain slices or perfusates of brain microdialysis experiments (50). We also know that certain cells, such as striatal astrocytes, respond to anandamide but do not have the enzymatic machinery to produce it (20,21,51). This implies that anandamide may travel from one cell to another, overcoming its tendency to partition in the lipid bilayer. We do not know yet how this may occur, but enough clues are available to offer the working hypothesis illustrated in Fig. 4.

Evidence suggests that about 40% of cellular *N*-acyl PEs are found on the neuronal plasmalemma (52). This indicates that anandamide may be produced within the cell membrane and may be able to move into the extracellular space immediately after cleavage of *N*-arachidonyl PE has taken place. As with other lipid compounds, the actual release step may be mediated either by membrane transporters (such as P-glycoproteins) or by lipid-binding proteins (such as lipocalins). The latter may also facilitate the movement of anandamide in the aqueous environment surrounding cells and help it attain its cellular targets.

NEUROTRANSMITTER OR LOCAL MODULATOR?

A close link between activity-dependent formation and extracellular release is a feature that distinguishes anandamide from classical neurotransmitters and underscores certain functional properties that may be characteristic of this endogenous cannabinoid. From a kinetic standpoint, anandamide release is unlikely to be as rapid and discrete as that of established neurotransmitters, implying that anandamide may act as a slow messenger molecule. Moreover, since anandamide release does not appear to be mediated by vesicle secretion, it is unlikely to be exclusively localized to synaptic nerve endings. As a result, anandamide may serve both synaptic and extrasynaptic signaling functions, a possibility that finds support in the presence of CB1 cannabinoid receptors in neuronal cell bodies and dendrites (46).

Slow and diffuse local effects are not unique to anandamide. To a certain extent they are also seen with neuroactive peptides, but they are especially characteristic of the growing family of phospholipid-derived bioactive mediators. It is perhaps not surprising that compounds like the eicosanoids and LPA would share with anandamide a number of functional properties such as release upon demand and spatially circumscribed actions. More intriguing is the fact that LPA and CB1 receptors exhibit a significant degree of sequence homology (53), which suggests the existence of a connection between these two apparently unrelated messenger systems. The functional significance of this link, if any, remains at present unknown.

The dissimilarities with classical neurotransmitters on the one hand, and the analogies with lipid mediators on the other, emphasize the idea that anandamide may act primarily as a local modulatory substance. In agreement with this, as we have seen, may be the time course of anandamide release and the extrasynaptic range of its biological effects. But also the possibility that many cell types that express cannabinoid receptors may also be capable to synthesize anandamide (20), which is consistent with a localized feedback (i.e., autacoid) action of this lipid messenger. A critical test of this hypothesis is still lacking, however. This will have to come from the anatomical localization of the enzymes involved in anandamide formation, as well as from detailed studies on the kinetics and distribution of anandamide release and inactivation *in vivo.*
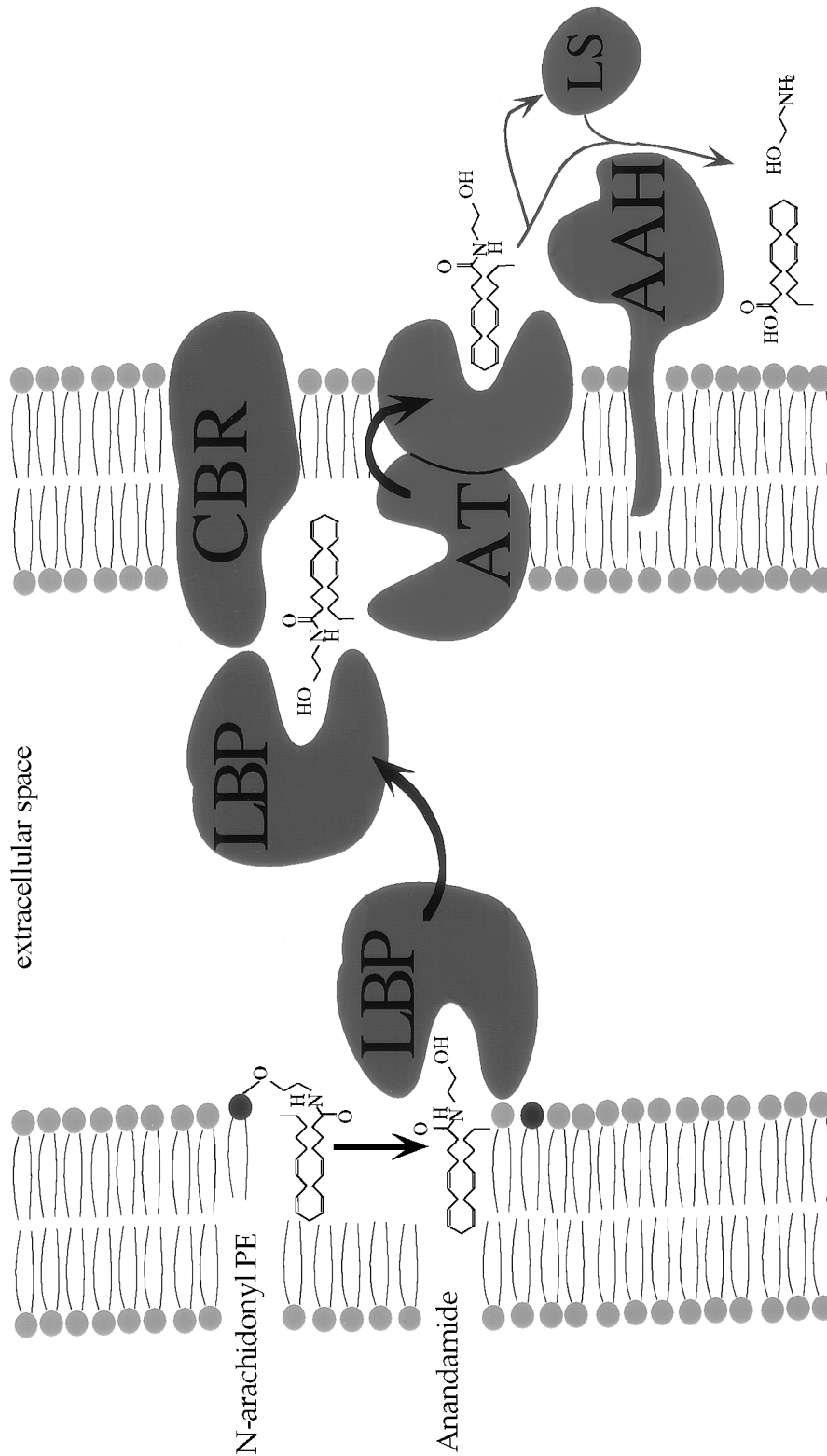
FIG. 4. A plausible model for the extracellular release and inactivation of anandamide. Evidence indicates that a large fraction of neuronal N-acyl PEs may be present in the plasmalemma (52), where the N-acyl group may be embedded in the lipid bilayer (77). Although the membrane leaflet localization of N-arachidonyl PE is not known, for clarity we depicted it in the outer leaflet. Newly formed anandamide may be extruded from cells by active transport (not shown) and/or association with a chaperone protein (lipid-binding protein, LBP). The binding with LPB may help anandamide reach cannabinoid receptors (CBR) located on neighboring cells or, not shown, on the same neurons by which anandamide is produced. Anandamide may be removed from its sites of action by high-affinity carrier-mediated transport (anandamide transporter, AT). Uptake may be followed by sequestration into as yet unidentified lipid stores (LS) and by intracellular hydrolysis catalyzed by membrane-bound anandamide amidohydrolase (AAH). The arachidonic acid produced by the AAH reaction may be rapidly reincorporated into phospholipid and is unlikely to undergo further metabolism (55).

ANANDAMIDE INACTIVATION

The inactivation of anandamide, with its two subsequent steps of high-affinity transmembrane transport and intracellular degradation, generally resembles that of amino acid and amine neurotransmitters (Fig. 4). A closer examination reveals nevertheless mechanistic differences that may be the reflection of subtle but important divergences in biological functions.

High-Affinity Transport

Neurons and astrocytes in primary culture avidly internalize exogenous anandamide through a process that meets all key criteria of a carrier-mediated transport. These criteria have been defined in detail by experiments with other membrane transporters (54), and include time and temperature dependence as well as high substrate affinity and selectivity (55,56).

Strong support to the existence of an anandamide transporter has also come from the development of a compound, *N*-(4-hydroxyphenyl)-arachidonylethanolamide (AM404), which blocks anandamide transport competitively (55). Using this inhibitor, it has been possible to demonstrate that high-affinity transport participates in the inactivation of exogenously administered anandamide both *in vitro* and *in vivo* (55,57). It is unclear whether AM404 also blocks the biological disposition of endogenously released anandamide; if this were the case, AM404 may be helpful to understand the physiological functions of anandamide, and may serve as a scaffold to develop therapeutic agents acting as indirect agonists at central and peripheral cannabinoid receptors.

A mechanistic feature of anandamide transport is its lack of Na^+ dependence, which suggests that anandamide is accumulated in cells via a carrier-mediated diffusion process driven by the concentration gradient of anandamide across the lipid bilayer. This feature differentiates anandamide from all known neurotransmitters but associates it with prostaglandin E_2, the membrane transporter of which is also Na^+ independent (58,59). Thus, even from the standpoint of its inactivation route, anandamide appears to behave more as a local mediator than as a bona fide neurotransmitter. In addition, the fact that anandamide may be transported by facilitated diffusion suggests that the kinetics of its biodisposition may be slower than that of amino acid or amine transmitters. To be able to address this question, however, we must await the cloning and functional expression of the anandamide transporter protein.

Hydrolysis

If cellular energy does not propel the transfer of anandamide across cell membranes, what does? Intracellular degradation may be one possibility. Indeed, a membrane-bound enzyme that catalyzes the breakdown of anandamide to arachidonic acid and ethanolamine has been identified (13,15,60,61) and cloned (62). The intracellular localization of this enzyme, termed anandamide amidohydrolase or fatty acid amide hydrolase, is supported by studies with subcellular membrane fractions (61), by its deduced amino acid sequence (62), and by the fact that anandamide hydrolysis takes place after this lipid has been accumulated in cells (20) (M. Beltramo and D. Piomelli, unpublished observations). Nevertheless, pharmacological inhibition of anandamide amidohydrolase activity which can be achieved by using a number of covalent inhibitors with varying degrees of selectivity (63–65) does not appear to affect the uptake of radioactive anandamide, at least when this is measured over brief time intervals (55). These findings indicate that, in the short-term, anandamide hydrolysis does not provide the driving force for anandamide transport. Importantly, however, they do not rule out an involvement of anandamide amidohydrolase in the long-term biodisposition of anandamide: it is conceivable, in fact, that anandamide transport into cells may be transiently driven by passive sequestration in lipid storage sites, as it may happen with free fatty acids (66), followed by a step of enzymatic hydrolysis. If this hypothesis is correct, prolonged inhibition of amidohydrolase activity would result in the intracellular accumulation of unmetabolized anandamide, and may eventually compromise its transmembrane transport and biodisposition. Thus, drugs that block anandamide amidohydrolase activity may cause long-term modifications of anandamide homeostasis, which might in turn be exploited therapeutically in an approach analogous to that used with monoamino oxidase inhibitors (for review, see 67).

To summarize, the biological actions of anandamide may be terminated by two temporally distinct, but functionally linked mechanisms. A high-affinity transporter protein may combine with extracellular anandamide and remove it from its sites of action by facilitated diffusion. Inside cells, anandamide may be

temporarily collected in lipid stores to be then disposed of by enzymatic hydrolysis (Fig. 4).

2-AG: ENDOGENOUS CANNABINOID OR METABOLIC INTERMEDIATE?

Pharmacological experiments with cortical neurons in culture indicate that 2-AG formation is mediated by the PLC/DAG lipase pathway. According to this hypothesis, illustrated in Fig. 3, PLC activity may catalyze the cleavage of a membrane phospholipid producing 1,2-diacylglycerol, which may be converted to 2-AG by DAG lipase activity. Thus 2-AG may be an intermediate component of a well known and ubiquitous enzymatic pathway that generates sequentially three distinct signaling molecules: 1,2-diacylglycerol, 2-AG, and arachidonic acid (by monoacylglycerol lipase-mediated cleavage of 2-AG, Fig. 3). Two of these molecules, 1,2-diacylglycerol and arachidonic acid, serve a host of regulatory functions, most of which have no apparent relationship with cannabinoid signaling. The question arises, therefore, of what biochemical checkpoints may be in place to control the traffic of these widely different messenger molecules. For several reasons, the consecutive reactions catalyzed by DAG lipase and monoacylglycerol lipase are likely to be essential.

It is generally thought that the second messenger effects of 1,2-diacylglycerol, which include protein kinase C activation, are terminated by its conversion to phosphatidic acid, catalyzed by DAG kinase (68). This suggests that the presence of an active DAG lipase may be a critical factor in determining whether 2-AG will be formed under given circumstances, i.e., that a high DAG lipase/DAG kinase ratio may be characteristic of cells that have the need to produce substantial amounts of 2-AG from 1,2-diacylglycerol.

Yet, the ability to generate 2-AG is not sufficient to conclude that a cell may use it as a signaling molecule. In platelets, for example, 2-AG is produced in large quantities but it is also immediately converted to arachidonic acid by monoacylglycerol lipase activity (36–39). This very short life span suggests that 2-AG may serve primarily as an intermediate in arachidonate release (for an alternative view, see 69). In neurons, by contrast, 2-AG appears to escape immediate metabolism and to accumulate in response to Ca^{2+}-mobilizing stimuli (43) (N. Stella and D. Piomelli, unpublished observations). Is such accumulation the consequence of a specific regulation of DAG lipase, DAG kinase, and monoacylglycerol lipase activities? The molecular characterization of neuronal DAG lipase and monoacylglycerol lipase should be instrumental to answer this question.

TONIC RELEASE OF ENDOGENOUS CANNABINOIDS

The dependence of anandamide and 2-AG formation on neural activity suggests that endogenous cannabinoids may be released from neurons *in vivo* and may contribute to the control of behavior. This possibility has not been confirmed directly yet, but finds support in pharmacological experiments with the CB1 antagonist SR141716A. The administration of SR141716A to naïve animals causes a series of effects that may be accounted for by the reversal of an endogenous cannabinoid tone. For example, SR141716A produces hyperalgesia (30,70,71), arousal (72), memory improvement (73), anxiety-like responses (74), and increased acetylcholine release (75,76). Unfortunately, the inverse agonist properties of SR141716A (8) and the current lack of biochemical information on the modalities of endogenous cannabinoid release *in vivo* make these pharmacological data difficult to interpret. One notable exception may be the pronociceptive effect of SR141716A administration in skin. The high levels of anandamide found in this tissue suggest that local CB1 receptors may be activated by the native ligand at least under certain conditions (30). An alternative experimental approach may be provided by the anandamide uptake inhibitor AM404. If this drug acts effectively *in vivo*, as initial studies appear to suggest, it may cause the accumulation of endogenous anandamide at its sites of action, and produce a selective "hypercannabinoid state" that should be reversed by CB1 receptor antagonists.

CONCLUSIONS AND PERSPECTIVES

Many aspects of the natural history of anandamide and 2-AG are still hypothetical. While we have a general idea of how these compounds may be produced and inactivated, we still know very little of the molecular mechanisms involved in these processes. If we want to place the endocannabinoid system in its anatomical context and in its relationship with other neural signaling molecules, these gaps need to be filled. Meanwhile, as new analytical tools become

available, we may also begin to tackle experimental questions that have remained intractable thus far, particularly those concerned with the possible roles of intrinsic cannabinoids in regulating behavioral responses under normal and pathological conditions.

ACKNOWLEDGMENTS

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