ELSEVIER

Chemistry and Physics of Lipids
108 (2000) 71–87

CPL
CHEMISTRY AND PHYSICS OF LIPIDS

www.elsevier.com/locate/chemphyslip

Review

Pathways and mechanisms of *N*-acylethanolamine biosynthesis: can anandamide be generated selectively?

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Received 21 April 2000; received in revised form 19 June 2000; accepted 19 June 2000

Abstract

Long-chain *N*-acylethanolamines (NAEs) and their precursors, *N*-acylethanolamine phospholipids, are ubiquitous trace constituents of animal and human cells, tissues and body fluids. Their cellular levels appear to be tightly regulated and they accumulate as the result of injury. Saturated and monounsaturated congeners which represent the vast majority of cellular NAEs can have cytoprotective effects while polyunsaturated NAEs, especially 20:4n-6 NAE (anandamide), elicit physiological effects by binding to and activating cannabinoid receptors. It is the purpose of this article to review published data on the pathways and mechanisms of NAE biosynthesis in mammals and to evaluate this information for its physiological significance. The generation and turnover of NAE via *N*-acyl PE through the transacylation-phosphodiesterase pathway may represent a novel cannabinoid receptor-independent signalling system, analogous to and possibly related to ceramide-mediated cell signalling.

Keywords: *N*-acyl PE; *N*-acylethanolamine; Anandamide; Mammals

1. Introduction

It is now commonly accepted that *N*-acylethanolamines (NAEs) and their precursors, *N*-acylphosphatidylethanolamines (*N*-acyl PEs), are ubiquitous constituents of mammalian cells. They have also been identified in fish (Natarajan et al., 1985), invertebrates such as sea urchins and mollusks (Bisogno et al., 1997b; Sepe et al., 1998), higher plants (Bomstein, 1965; Dawson et al., 1969), and in certain microorganisms such as *Butyrivibrio sp*. (Clarke et al., 1976) and slime molds (Ellingson, 1980).

Under normal conditions, the levels of NAE and *N*-acyl PE in mammalian cells are quite low

Abbreviations: NAE, *N*-acylethanolamine; *N*-acyl PE, *N*-acylphosphatidylethanolamine (natural PE and *N*-acyl PE may consist of diacyl as well as alkylacyl and alk-1-enylacyl subclasses); PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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0009-3084/00/$ - see front matter
PII: S0009-3084(00)00188-2

and appear to be tightly regulated. However, relatively high levels of both lipids were detected in undifferentiated neuroblastoma cells; they were found to decrease significantly upon cell differentiation (Gulaya et al., 1989). They can also accumulate under physiological conditions in cells undergoing degeneration and phospholipid degradation, such as the granular cells of the epidermis (Gray, 1976), or as the result of injury (reviewed in Schmid et al., 1990). Under conditions of necrosis, such as myocardial infarction, where extensive membrane degradation occurs, this accumulation can be massive (Epps et al., 1979, 1980). Pronounced increases in these lipids were also observed in degenerating BHK cells (Somerharju and Renkonen, 1979), post-decapitative cerebral ischemia (Natarajan et al., 1986), glutamate-induced neuronal cytotoxicity (Hansen et al., 1995), and cadmium-induced testicular inflammation (Kondo et al., 1998) (see also the article by Hansen et al. in this issue). Since both *N*-acyl PE (Newman et al., 1986; Domingo et al., 1993; Mercadal et al., 1995) and NAE (Ambrosini et al., 1993) exhibit membrane stabilizing effects in vitro and because NAE was shown to inhibit the injury-induced mitochondrial permeability transition (Epps et al., 1982), their production was proposed to represent a defense mechanism aimed at minimizing injury and promoting survival (Schmid et al., 1990). This hypothesis was also in agreement with the reported anti-inflammatory effects of *N*-palmitoylethanolamine (Kuehl et al., 1957; Berdyshev et al., 1997, 1998) and its anti-oxidant effect in biological membranes (Parinandi and Schmid, 1987). However, the mechanism by which NAE inhibited the mitochondrial permeability transition was never identified and, although it was also shown that 16:0 NAE can protect cerebellar granule neurons against glutamate-induced excitotoxic death (Skaper et al., 1996), the low levels of *N*-acyl PE and NAE in intact cells and tissues argued against an interpretation of their functions in terms of their physical properties.

The discovery that *N*-arachidonoylethanolamine (20:4n-6 NAE, anandamide) is present in the pig brain and binds to the central (CB1) cannabinoid receptor (Devane et al., 1992; Felder et al., 1993) has fundamentally changed our perception of the potential role of NAE in mammalian cells and has had a profound effect on NAE research.

In recent years it has been shown that anandamide and, very likely, other polyunsaturated NAEs, bind to and activate not only 'central' (CB1) but also 'peripheral' (CB2) cannabinoid receptors and that it elicits virtually all of the in vitro and in vivo effects of Δ^9-tetrahydrocannabinol (Δ^9 THC). It has therefore received widespread attention as an endogenous cannabinoid receptor agonist and the regulation of its biosynthesis and degradation has been the subject of intensive investigation (reviewed in Schmid et al., 1996; Hillard and Campbell, 1997; Di Marzo, 1998). Since anandamide is usually accompanied by much larger amounts of cannabinoid receptor-inactive saturated and monounsaturated NAEs, it is also of interest to determine to what extent it is selectively synthesized, transported and degraded. Finally, it is very likely that different NAEs, including anandamide, can mediate biological processes through targets other than cannabinoid receptors (see also the articles by Howlett and Mukhopadhyay and by Berdyshev in this issue).

It is the purpose of the present review to summarize all presently available information on the biosynthesis and turnover of anandamide and other NAEs in mammalian systems and to interpret it in terms of its biological significance.

2. The transacylation-phosphodiesterase pathway

In the late 1970s, while investigating the effects of ischemic injury on the membrane lipids of mammalian heart, we observed not only the expected accumulation of unesterified fatty acids in the developing infarction but also relatively large amounts of a novel phospholipid and neutral lipid (Epps et al., 1979, 1980). The myocardial infarct had been induced in dogs in vivo by coronary artery ligation and, after 24 h, the amounts of the novel lipids in the infarcted portion of the myocardium were sufficient for isolation and chemical structure analysis. The phospholipid was identified as a mixture of *N*-acylated

ethanolamine phospholipids consisting of diacyl, alk-1-enylacyl and alkylacyl subclasses in approximately the same proportions as the ethanolamine phospholipids of normal myocardium. The appearance of *N*-acyl PE in the infarcted areas of the myocardium was accompanied by a reduction in PE, suggesting a precursor-product relationship. However, while the alk-1-enyl and 2-*O*-acyl compositions of the alk-1-enylacyl ethanolamine phospholipids were the same as those of their *N*-acylated analogs, the *O*-acyl composition of 1,2-diacyl PE was quite different from the corresponding *N*-acyl PE isolated from the same infarct tissue. At first, this structural difference between postulated precursor and product was difficult to rationalize (Epps et al., 1980) but was explained later when the mechanism of *N*-acylation was established (see below).

2.1. The transacylase reaction

Initial attempts to accomplish PE *N*-acylation with preparations of normal or infarcted canine myocardium failed when labeled fatty acids or acyl CoAs were used under a variety of conditions. However, when we decided to incorporate labeled ethanolamine into the phospholipids of minced tissue by base-exchange, we obtained both labeled PE and *N*-acyl PE (Natarajan et al., 1981). Since phospholipid base-exchange reactions require Ca^{2+} but no energy, as had been demonstrated with dog heart membranes (Filler and Weinhold, 1980), it was logical to propose that a Ca^{2+}-dependent, energy-independent transacylation was involved in PE *N*-acylation. We also observed that preparations of normal myocardium catalyzed *N*-acylation of PE more effectively than those of infarct tissue and that both normal and infarct tissue catalyzed the hydrolysis of *N*-acyl PE to NAE (Natarajan et al., 1981). The addition of millimolar concentrations of Ca^{2+} to homogenates or subcellular preparations of dog heart muscle resulted in the efficient generation of *N*-acyl PE, *N*-acyl lyso PE and NAE from endogenous lipids. Using this approach, as well as specifically radiolabeled lipid precursors, we characterized the *N*-acyltransferase of dog heart (Natarajan et al., 1982; Reddy et al., 1983a,b, 1984; Schmid and Schmid, 1987) and other mammalian tissues and identified the following properties.

The enzyme is tightly membrane bound and present in both sarcoplasmic reticulum (microsomes) and mitochondria. These organelles were isolated, purified and characterized by established procedures, and cross-contamination was estimated as less than 10% (Natarajan et al., 1982). Pulse experiments with [1,2-^{14}C]ethanolamine-labeled minced tissue clearly established PE as the precursor of *N*-acyl PE, *N*-acyl lyso PE and NAE. While neither palmitic acid in the presence or absence of acyl CoA-generating systems, nor palmitoyl CoA, could serve as acyl donor, both PE and PC as well as their lysolipids were effective *N*-acyl donors with dog heart homogenates (Natarajan et al., 1982).

As previously observed with the in vivo infarct, *N*-acyl PE produced from endogenous substrates in dog heart homogenate exhibited a significantly different *O*-acyl composition than its precursor, PE. Positional analysis revealed that this difference was due to changes of the fatty acids present at the *sn*-1 position. While the 1-*O*-acyl groups of dog heart PE were 16:0, 18:0, 18:1, 18:2n-6, 20:3n-6 and 20:4n-6 in percentages of 4.2, 83.4, 8.2, 1.8, 1.3 and 0.3, the percentages of the same 1-*O*-acyl groups in *N*-acyl PE were 23.5, 52.6, 7.5, 11.3, 0.0 and 1.2, respectively (Natarajan et al., 1982).

The reason for this alteration in the 1-*O*-acyl composition due to PE *N*-acylation became clear when we could show that only 1-*O*-acyl groups could serve as acyl donors and that a substantial portion of the acyl transfer was intramolecular, resulting in the production of a hypothetical intermediate, 2-*O*-acyl-*sn*-glycero-3-phospho(*N*-acyl)-ethanolamine (or *N*-acyl lyso PE) whose free *sn*-1 hydroxy group would be subject to reacylation, most likely by the same transacylase system (Reddy et al., 1983a,b) as summarized in Fig. 1.

This assumption is consistent with the observation that the major *N*-acyl groups in both *N*-acyl PE and NAE generated by dog heart preparations in vitro, as well as in vivo, were 16:0, 18:0, 18:1 and 18:2n-6. In dog heart 16:0 and 18:0 are the principal 1-*O*-acyl groups of PC and PE, respectively, while 18:1 is commonly present at both the

sn-1 and *sn*-2 position of glycerolipids. In contrast, 18:2n-6 is primarily present in the *sn*-2 position, except in cardiolipin (diphosphatidylglycerol) which contains four fatty acids and is known to be rich in linoleate (18:2n-6). Linoleate amounts to over 90% of the constituent fatty acids of dog heart cardiolipin, one of the major mitochondrial phospholipids. It was therefore logical to assume that the 18:2n-6 used for *N*-acylation came from the *sn*-1 position of cardiolipin and that mitochondria participated in the transacylation reaction both in vivo and in vitro. Indeed, incubation of isolated mitochondria with Ca^{2+} resulted in the generation of *N*-acyl PE whose *N*-acyl groups contained about 14% 18:2n-6, whereas the *N*-acyl PE generated by microsomes contained less than 4%. In these preparations, cardiolipin amounted to 18.5% of total phospholipids in mitochondria but only to 3.3% in microsomes (Reddy et al., 1983a,b). The assumption that cardiolipin can serve as acyl donor in the *N*-acylation of PE was confirmed with radiolabeled substrates which allowed the firm conclusion that only 1-*O*-acyl groups of glycerophospholipids or lysophospholipids can serve

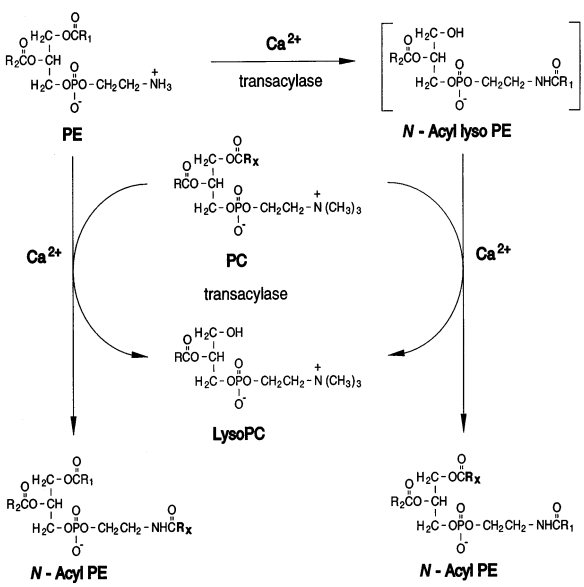


Fig. 1. Intra- and intermolecular transacylation to PE from PC. Other intermolecular acyl donors can be lysoPC, PE and cardiolipin.

as the source of the amide-linked fatty acids of dog heart *N*-acyl PE. When dog heart mitochondria were incubated with Ca^{2+} in media containing 40% $H_2^{18}O$, the resulting *N*-acyl PE did not contain ^{18}O in either its *N*-acyl or 1-*O*-acyl groups (Schmid and Schmid, 1987). This indicated that acyl transfer had occurred without hydrolysis, most likely through an acyl-enzyme complex which may be covalently linked. Analogous transacylation reactions were also established in dog brain (Natarajan et al., 1983), rat brain (Natarajan et al., 1986), bovine heart (unpublished) and other mammalian systems (see below).

Although our early attempts to solubilize the *N*-acyltransferase and to reconstitute the transacylation system failed, we were able to partially characterize the enzyme. Using crude membrane preparations as the source, we found the enzyme to be heat sensitive, inhibited by sulfhydryl reagents and sensitive to pretreatment with trypsin. Interestingly, Sr^{2+} and Mn^{2+} could fully substitute for Ca^{2+} in bringing about PE *N*-acylation, at the same optimum ion concentration (5 mM) and pH optimum (9.0), and yielding exactly the same molecular species of *N*-acyl PE from endogenous substrates (Reddy et al., 1984). While Ba^{2+} was equally effective, other divalent cations were less effective or ineffective. We proposed that the loss of Ca^{2+} homeostasis in the developing infarct was responsible for the progressive *N*-acylation of PE and the accumulation of both *N*-acyl PE and NAE (Epps et al., 1979, 1980), but it is still unclear how PE *N*-acylation may be regulated under physiological conditions. When we first observed the effects of divalent cations other than Ca^{2+}, we postulated that they may be required in order to induce phase transitions from a bilayer to a hexagonal (H_{II}) phase in mixtures of acidic phospholipids and phosphatidylethanolamine (Cullis et al., 1983). An understanding of the role of Ca^{2+} in PE *N*-acylation is further complicated by the observation that different systems have different optimal ion concentrations. For example, preparations of young rat brain require only about one-tenth the Ca^{2+} concentration (0.5 mM) than those of dog heart for optimal activity and the apparent enzyme activity decreases with

age (Natarajan et al., 1986). Moreover, even though current technology has allowed the detection of both *N*-acyl PE and NAE in virtually all mammalian cells and tissues, many do not respond to Ca^{2+} treatment with measurable PE *N*-acylation (unpublished observations).

The discovery of anandamide (*N*-arachidonoylethanolamine) led to intensive investigations of the transacylation-phosphodiesterase pathway as the most likely route of its biosynthesis. Initially, treatment of cultured, [^{3}H]-ethanolamine-labeled, rat brain neurons with high [K^+], the glutamate receptor agonist kainate, or the K^+ channel blocker 4-aminopyridine in the presence of Ca^{2+} resulted in the release and selective re-uptake of labeled anandamide as well as the release of other NAEs (Di Marzo et al., 1994). These authors also provided evidence for the presence of *N*-arachidonoyl PE among neuronal *N*-acyl PE and for the enzymatic conversion of *N*-acyl PE to NAE by neuronal homogenates. Although individual NAEs were separated by HPLC and characterized by mass fragmentography, quantitative data about their relative abundance were not reported. Subsequently, evidence was presented for the generation of anandamide and other NAEs via *N*-acyl PE in mouse J774 macrophages and N_{18} neuroblastoma cells (Di Marzo et al., 1996), and leukocytes (Bisogno et al., 1997a) as well as in rat cortical neurons (Cadas et al., 1996a). In these studies, Ca^{2+} was found to be required for *N*-acylation to occur and treatment with the Ca^{2+} ionophore was among the conditions that resulted in enhanced *N*-acyl PE synthesis (see below). Cadas et al. (1996a) also determined that neural homogenates catalyze PE *N*-acylation from various [^{14}C]labeled glycerophospholipids and that neither fatty acids nor acyl CoAs could serve as acyl donors. Although these data suggested that anandamide can be produced by the transacylation-phosphodiesterase pathway, definitive proof for the relevance of this reaction sequence for anandamide synthesis was provided by Sugiura et al. (1996a,b) using preparations of both rat testis and brain. These investigators reported not only the actual levels and *N*-acyl compositions of NAE and *N*-acyl PE, including the percentages of *N*-arachidonoyl moieties, in these tissues, but they offered convincing evidence of the biosynthetic mechanism as a Ca^{2+}-dependent acyl transfer from the *sn*-1 position of PC to the amino group of PE. They also analyzed the composition of the 1-*O*-acyl groups of brain and testis phospholipids. In brain, arachidonate amounted to 0.3% of the *sn*-1 fatty acids of PC, 0.4% of the *N*-acyl groups of *N*-acyl PE and 0.7% of NAE. In testis, the 1-*O*-acyl groups of PC and PE contained 3.4 and 6.8%, respectively, of arachidonate and *N*-arachidonoyl groups were 10.9 and 6.0% of *N*-acyl PE and NAE, respectively. Cadas et al. (1997) also identified *N*-arachidonoyl PE in rat brain, at a level of 0.6% of total *N*-acyl PE, and characterized the mechanism of PE *N*-acylation. They determined that arachidonate amounts to 0.5% of fatty acids at the *sn*-1 position of brain glycerophospholipids and that *N*-acylation of PE occurs by transfer of 1-*O*-acyl groups including arachidonate. The reaction required Ca^{2+} but Ba^{2+} and Sr^{2+} were also effective in bringing about transacylation. Interestingly, Be^{2+}, Cd^{2+}, Zn^{2+} and Ag^{2+} were strongly inhibitory when added together with Ca^{2+}. Also, agents that alkylate serine, cysteine and histidine were found to be inhibitory, as was (E)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP), an inhibitor of Ca^{2+}-independent phospholipase A_2.

In summary, all evidence regarding the mechanism of PE *N*-acylation in mammalian cells supports the concept of a Ca^{2+}-dependent, energy-independent transacylation of fatty acids from the *sn*-1 position of PC and other glycerophospholipids to the amino group of PE (or lyso PE). So far, there exists no evidence for any acyl selectivity of this reaction. Arachidonate and other polyunsaturated fatty acids appear to be used only to the extent that they are esterified at the *sn*-1 position of a donor phospholipid.

2.2. The phosphodiesterase reaction

In our early work, we demonstrated not only the accumulation of both *N*-acyl PE and NAE in canine myocardial infarct but also the enzymatic hydrolysis of *N*-acyl PE to NAE. This enzyme activity was widespread in mammalian tissues and

we characterized it in preparations of rat heart (Schmid et al., 1983). Since 2-*O*-acyl-labeled *N*-acyl PE yielded both radioactive phosphatidic acid and diacylglycerol (DAG), it was important to determine whether both phospholipase D and phospholipase C activities are involved in the phosphodiesterase hydrolysis of *N*-acyl PE. Using synthetic, doubly labeled substrates, as well as NaF as an inhibitor of phosphatidic acid phosphatase, we established that the phosphodiesterase is a phospholipase D type enzyme. We also determined that NAE phosphate was not produced from *N*-acyl PE, thereby ruling out phospholipase C type activity. The enzyme was present in both microsomes and mitochondria of rat heart and was active over a wide range of pH with the activity at lower pH enhanced after repeated freezing and thawing. The enzyme hydrolyzed *N*-acyl PE and its plasmalogen (alk-1-enylacyl) analog as well as the corresponding lysophospholipids but showed little activity towards PC and PE. Enzyme activity did not require Ca^{2+} or other divalent cations and Zn^{2+} was inhibitory. Among detergents, Triton X-100 enhanced enzyme activity, whereas sodium dodecyl sulfate and alkyltrimethylammonium bromide were strongly inhibitory (Schmid et al., 1983). Among rat tissues, heart muscle exhibited the highest specific activity, followed, depending on incubation conditions (pH, detergent), by brain, testis, kidney, spleen, liver and lung. Additional work with dog brain preparations confirmed many characteristics observed with the rat heart phosphodiesterase, including the wide pH range and stimulatory effect of Triton X-100. With dog brain microsomes, Ca^{2+} was somewhat stimulatory at low concentrations but inhibitory above 1 mM. In the absence of detergents, *N*-acyl lyso PE was a better substrate than *N*-acyl PE but by far the best substrate was the deacylated analog, glycerophospho(*N*-acyl)ethanolamine. Hence, the phosphodiesterase is both a phospholipase D and lysophospholipase D, as well as an enzyme hydrolyzing a lipid that has no *O*-acyl groups. In contrast to dog heart, dog brain microsomes also hydrolyzed PE and PC at rates comparable to *N*-acyl PE (Natarajan et al., 1984). As observed with rat heart, dog brain did not contain any phosphodiesterase of the phospholipase C type acting upon *N*-acyl PE. In studies on *N*-acyl PE generation and turnover in young rat brain, we observed that *N*-acyl PE phosphodiesterase activity was about twice as high in microsomes from 30-day-old brain than from 10-day-old brain (Natarajan et al., 1986).

When the generation of anandamide from *N*-arachidonoyl PE became of interest, it was assumed that the relevant enzyme is a phosphodiesterase of the phospholipase D type present in neuronal cells (Di Marzo et al., 1994, 1996; Bisogno et al., 1997a,b; Cadas et al., 1997), macrophages (Di Marzo et al., 1996) and microsomal preparations of rat testis (Sugiura et al., 1996a) and brain (Sugiura et al., 1996b). The brain enzyme was tested against *N*-acyl PE having different *N*-acyl groups and found to be equally effective against the major *N*-acyl groups (16:0, 18:0, 18:1 and 18:2) but somewhat less active against *N*-arachidonate (Sugiura et al., 1996b). Interestingly, the rat brain or rat heart enzyme was not capable of catalyzing the transphosphatidylation of phosphatidic acid to short-chain primary alcohols, a reaction observed with all other mammalian phospholipase D enzymes (Petersen and Hansen, 1999). Bovine retina, a tissue rich in docosahexaenoic acid (22:6n-3), was found to contain 22:6n-3 *N*-acyl groups in both *N*-acyl PE and NAE, and to contain the relevant phosphodiesterase and amidase activities (Bisogno et al., 1999). Finally, the presence of *N*-acyl PE and its hydrolysis to NAE were also established in the ovaries of sea urchins (Bisogno et al., 1997a,b) and in bivalve mollusks (Sepe et al., 1998). Although the mechanism of *N*-acyl PE synthesis in these organisms remains to be established, the phosphodiesterase activity was clearly shown to produce anandamide from *N*-arachidonoyl PE in vitro.

It is important to consider that so far no fatty acid selectivity for either the biosynthesis or hydrolysis of *N*-acyl PE could be demonstrated. Any possible selectivity in anandamide generation must therefore rely on other mechanisms. However, the movement of anandamide across cellular membranes appears to exhibit selectivity for anandamide and other unsaturated NAEs over their

saturated congeners (Di Marzo et al., 1994; Hillard et al., 1997) (see also the article by Hillard and Jarrahian in this issue).

2.3. The amidohydrolase reaction

In our earlier studies we observed the hydrolysis of NAE to fatty acid and ethanolamine with preparations of virtually all mammalian tissues. Since we found the highest amidase activity in rat liver (Schmid et al., 1983), we investigated the rat liver enzyme in some detail and determined its location, properties and substrate specificity. As observed for the transacylase and phosphodiesterase (see above), the enzyme was present in both microsomes and mitochondria. The NAE amidohydrolase was active over a wide range of pH with an alkaline pH optimum and did not require divalent cations. It was inhibited by sulfhydryl-reactive agents and several detergents. However, sodium taurodeoxycholate had little effect on enzyme activity and was therefore used to solubilize it. The solubilized enzyme exhibited high substrate specificity for long-chain amides of ethanolamine. Amides of hydroxypropanolamine, propanolamine or its higher homologs were hydrolyzed at drastically slower rates and the same substrate selectivity was observed when the enzyme was acting in reverse, catalyzing amide synthesis. Neither ceramide nor *N*,*O*-diacylethanolamine were substrates or inhibitors of the enzyme (Schmid et al., 1980). The amidase present in dog brain microsomes exhibited a pH optimum of 10.0 and hydrolyzed 16:0 NAE with an apparent $V_{max} = 667$ nmol/h per mg protein and $K_m = 53$ μM (Natarajan et al., 1984).

After the discovery of anandamide (Devane et al., 1992), the amidase was the first, and so far the only, relevant enzyme to be studied in detail (Deutsch and Chin, 1993; Koutek et al., 1994; Desarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Maurelli et al., 1995; Katayama et al., 1997; Omeir et al., 1999), purified and cloned (Cravatt et al., 1995; Goparaju et al., 1999). It has been named fatty acid amide hydrolase (FAAH) and is discussed by Ueda et al. in this issue.

2.4. Agonist-induced generation of N-acyl PE and NAE

While the injury-induced accumulation of *N*-acyl PE and NAE, including the effects of glutamate on cultured neuronal cells (Hansen et al., 1995, 1997), as well as the post-mortem generation of these lipids (Schmid et al., 1995a,b; Kempe et al., 1996; Felder et al., 1996) could be attributed to a loss of Ca^{2+} homeostasis, it is less clear how their levels are regulated in intact cells. In this context, it is not only important to consider the regulation of the transacylation-phosphodiesterase pathway as such but how this sequence of reactions could permit the selective synthesis of the cannabinoid agonist, anandamide. Furthermore, if anandamide is the messenger, what exactly is the physiological trigger to elicit the message?

From the beginning (Di Marzo et al., 1994), work with neuronal cells has suggested that a combination of Ca^{2+} (treatment with the Ca^{2+} ionophore ionomycin) and membrane depolarizing agents results in enhanced anandamide synthesis via *N*-arachidonoyl PE. Similar effects of ionomycin treatment were also observed with mouse J774 macrophages, N_{18} neuroblastoma cells (Di Marzo et al., 1996) and RBL-2H3 basophils (Bisogno et al., 1997a,b) but selectivity for anandamide was not evident in these experiments. Interestingly, the Ca^{2+}-dependent *N*-acyl PE synthesis in cortical neurons was potentiated by agents that increase cAMP levels, including forskolin and vasoactive intestinal peptide (Cadas et al., 1997) but, again, no selectivity for anandamide was observed. Also, many experiments on stimulated *N*-acyl PE/NAE generation were carried out with prelabeled cells, the measured levels of radioactivity in the lipids of interest were usually low and in some cases amidase inhibitors such as arachidonyltrifluoromethyl ketone had to be included in incubations of both stimulated and control cells in order to see an effect (Di Marzo et al., 1996). It had previously been shown that treatment of intact neuroblastoma cells with this inhibitor results in a 12-fold increase of cellular anandamide levels (Koutek et al., 1994). Also, treatment with ionomycin did not enhance *N*-acyl

PE and NAE generation in human breast cancer EFM-19 cells (Bisogno et al., 1998). On the other hand, recent studies with [^{3}H]arachidonic acid-labeled neuroblastoma (SK-N-SH) cells showed not only enhanced generation of anandamide and its precursor, *N*-arachidonoyl PE, by treatment with veratridine, 4-aminopyridine and the ionophore A32187 but further enhancement by ethanol exposure. Interestingly, the ethanol-enhanced synthesis of both anandamide and its precursor phospholipid was inhibited by exogenous anandamide, whereas the CB1 receptor antagonist SR141716A and pertussis toxin inhibited only anandamide synthesis. The authors suggested that the CB1 receptor and $G_{i/o}$ protein mediated the regulation of anandamide levels in these cells (Basavarajappa and Hungund, 1999). Due to the [^{3}H]arachidonate label used in these experiments, potential effects on NAEs other than anandamide (and their precursor) could not be determined.

At this time it must, therefore, be concluded that the lack of specificity exhibited for the enzymes of the transacylation-phosphodiesterase pathway is reflected in both constitutive and agonist-induced NAE biosynthesis. However, there are also reports regarding the induction of anandamide synthesis in cells of the immune system by arachidonate mobilizing agents (Pestonjamasp and Burstein, 1998), in the blood during hemorrhagic shock (Wagner et al., 1997) and in the brain as the result of electrical stimulation or formalin treatment (Walker et al., 1999). However, neither the mechanism of anandamide generation nor the degree of its selectivity is apparent from these studies.

If the transacylation-phosphodiesterase pathway should be the primary and perhaps the only mechanism of NAE synthesis in mammalian cells, changes in the fatty acid composition of the acyl donor phospholipid could be an important determinant of which NAEs are produced. For example, exogenous addition or stimulated liberation of endogenous arachidonate can result in its reuptake through de novo phospholipid synthesis resulting in the generation of 1,2-diarachidonoylglycerol molecular species (Schmid et al., 1997c; Kuwae et al., 1997). Hence, a temporary increase in arachidonic acid at the *sn*-1 position of PE or PC could lead to a proportional increase in anandamide among the NAEs generated via *N*-acyl PE. However, treatment of mouse peritoneal macrophages with the Ca^{2+} ionophore A23187, which had previously been shown to result in the generation of 1,2-diarachidonoyl PC (Kuwae et al., 1997), had no effect on the levels or compositions of NAE (Kuwae et al., 1999).

Interestingly, when these cells were incubated in media containing $H_2^{18}O$ the amide bonds of both *N*-acyl PE and NAE became labeled very rapidly, suggesting a very rapid constitutive turnover through the transacylation-phosphodiesterase pathway (Kuwae et al., 1999). The incorporation of ^{18}O from the media into the amide-linked fatty acids of *N*-acyl PE also appeared to be in conflict with previous data obtained with cell-free preparations of dog heart and rat testis which showed that the *N*-acyltransferase reaction does not involve hydrolysis, i.e. the uptake of ^{18}O from $H_2^{18}O$-containing media (Schmid and Schmid, 1987; Schmid et al., 1998). Although it is possible that mouse macrophages generate *N*-acyl PE by a different mechanism, we proposed that the observed rapid *N*-acylation of PE is accompanied by an equally rapid generation of a small precursor pool of one or more acyl donor phospholipids (Fig. 2). Continuous resynthesis of these lipids, most likely through deacylation-reacylation cycles, would thus provide ^{18}O-containing 1-*O*-acyl groups for PE *N*-acylation when intact cells are incubated in $H_2^{18}O$-containing media.

The concept that the turnover of PE → *N*-acyl PE → NAE → fatty acid + ethanolamine is extremely rapid, by far exceeding the average phospholipid acyl turnover in these cells (Kuwae et al., 1990) needs to be confirmed in other cells and needs to be quantified by determining the time-dependence of ^{18}O-incorporation. If proven to be a common phenomenon, it appears possible that the entire transacylation-phosphodiesterase system could be easily up- or downregulated by a variety of agonists or environmental conditions (see below). While this concept is logical, it would be in conflict with previous proposals that *N*-acyl PE represents a relatively stable membrane component from which anandamide and/or other

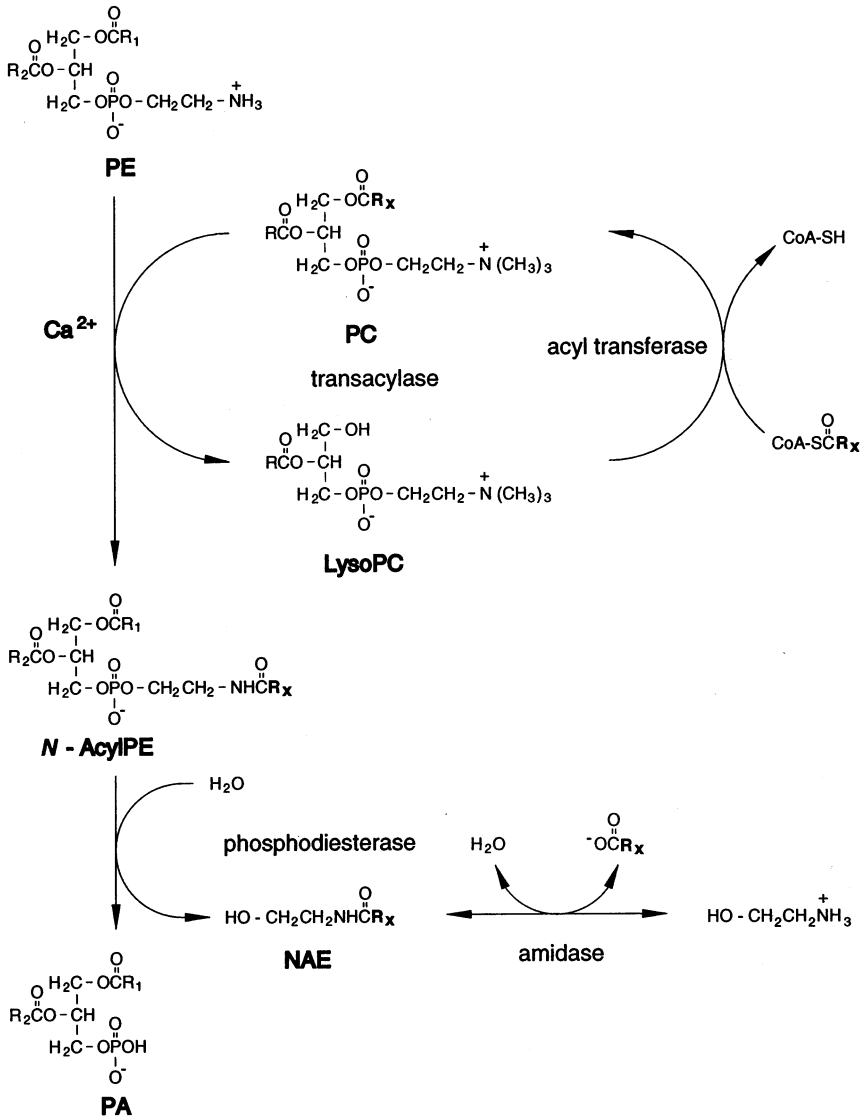


Fig. 2. Proposed acyl turnover through the transacylation-phosphodiesterase pathway.

NAE can be liberated by agonist-(or Ca^{2+})-activated phospholipase D activity (Di Marzo et al., 1996; Cadas et al., 1996b). However, it would offer an opportunity for arachidonate, selectively liberated by phospholipase A_2 activity, to be rapidly and selectively converted to anandamide by the transacylation-phosphodiesterase pathway. However, such a reaction sequence has yet to be demonstrated in intact cells.

3. Direct *N*-acylation of ethanolamine

The energy-independent enzyme-catalyzed condensation of fatty acid and ethanolamine was first reported by Udenfriend and coworkers (Colodzin et al., 1963; Bachur and Udenfriend, 1966) who argued that the biosynthetic activity was different from NAE hydrolysis. However, our initial observation regarding the reversibility of the amidohy-

drolase was confirmed in considerable detail after the discovery of anandamide (see above and the article by Ueda et al. in this issue). Hence, the 'anandamide synthase' activities reported for mammalian brain which required relatively high K_m values for both arachidonic acid and ethanolamine, as well as an alkaline pH optimum (Kruszka and Gross, 1994; Devane and Axelrod, 1994) may actually have represented reverse amidase activity (Ueda et al., 1995). Interestingly, anandamide synthesis by an enzyme that was not inhibited by an amidase inhibitor was observed in mouse uterus (Paria et al., 1996), an organ which not only contains very high levels (up to 1.3 nmol/μmol P) but also very high percentages (up to 95%) of anandamide while *N*-arachidonate is only a minor component of *N*-acyl PE (Schmid et al., 1997b) (see also the article by S.K. Dey in this issue).

Since both ethanolamine and arachidonic acid could be generated by phospholipase D and phospholipase A_2 activity, respectively, and since their direct condensation could represent an effective and potentially highly selective biosynthesis of anandamide, we carried out a series of studies in order to determine the likelihood of such a pathway.

We incubated various preparations of rat testes membranes with either 5 mM Ca^{2+} or with 5 mM EGTA plus 10 mM ethanolamine. As illustrated for membranes of the 7000 × *g* pellet (Table 1), the Ca^{2+} activated transacylation-phosphodiesterase pathway strongly favored the production of saturated and monounsaturated NAE, reducing the relative amount of anandamide from the original 8% to less than 3% of NAE, and *N*-arachidonoyl PE from 11 to 1%. In contrast, the presence of ethanolamine (in the absence of Ca^{2+}) resulted in a relative increase of anandamide from 8% to over 32% while having little effect on the level or composition of *N*-acyl PE (Schmid et al., 1998).

Incubations in media containing both [1,1,2,2-$^2H_4$]ethanolamine and 40% $H_2^{18}O$ showed that the Ca^{2+}-independent NAE synthesis occurred by direct condensation of the exogenous ethanolamine with endogenous fatty acids. This biosynthetic activity occurred at ethanolamine concentrations as low as 50 μM and selectivity for arachidonate increased with increasing ethanolamine concentrations. Results of inhibitor experiments suggested that this mechanism of NAE synthesis represented the reverse activity of NAE amidohydrolase (Schmid et al., 1998).

By using deuterated ethanolamine and incubations of mouse peritoneal macrophages in the presence or absence of Ca^{2+} plus A23187, we were able to determine that direct *N*-acylation of ethanolamine can also occur in intact cells. However, no selectivity for arachidonate was observed in this system and the physiological relevance of this pathway of anandamide synthesis remains questionable (Kuwae et al., 1999).

4. The emerging relevance of cannabinoid receptor-independent NAE signalling

As of now, all available evidence points to the fact that the production of NAE through *N*-acyl PE by the transacylation-phosphodiesterase pathway is a widespread, perhaps ubiquitous, aspect

Table 1
N-acyl PE and NAE, pmol/μmol P (%), in rat testes membranes before (A) and after incubation with Ca^{2+} (B) or EGTA plus ethanolamine (C)[a,b]

N-acyl	A	B	C
	N-acyl PE		
16:0	155.47 (60.9)	3713.28 (85.0)	173.17 (63.5)
18:0	46.70 (18.2)	300.26 (6.9)	44.75 (16.5)
18:1n-9	11.06 (4.3)	167.50 (3.8)	11.53 (4.2)
18:1n-7	4.26 (1.7)	64.63 (1.5)	4.11 (1.5)
18:2n-6	9.94 (3.9)	80.96 (1.9)	10.02 (3.7)
20:4n-6	27.92 (11.0)	41.70 (1.0)	28.60 (10.6)
	NAE		
16:0	13.28 (47.6)	783.43 (82.0)	388.86 (30.2)
18:0	5.88 (21.1)	58.10 (6.1)	42.45 (3.3)
18:1n-9	3.76 (13.5)	57.80 (6.1)	197.53 (15.4)
18:1n-7	1.98 (7.1)	19.76 (2.1)	32.43 (2.5)
18:2n-6	0.76 (2.7)	29.36 (3.1)	210.94 (16.4)
20:4n-6	2.25 (8.0)	7.09 (0.7)	414.56 (32.2)

[a] Membrane preparations (7000 × *g* pellet) were extracted and analyzed (A) or incubated in Hepes buffer, pH 7.4 at 37°C for 30 min with either 5 mM Ca^{2+} (B) or 5 mM EGTA plus 10 mM ethanolamine (C). Lipids were extracted, fractionated, derivatized and assayed by GC-MS.

[b] Data from Schmid et al., 1998.

of mammalian lipid metabolism. This pathway, now also referred to as the Schmid pathway (e.g. Farooqui et al., 2000), appears to be designed to produce primarily saturated and monounsaturated NAEs which do not bind to and activate cannabinoid receptors. It is reasonable to assume that these NAEs serve other functions, possibly in intracellular signalling.

In this context, it is also important to consider that the anti-inflammatory effects of 16:0 NAE regarding the down regulation of mast cell activity are not due to their interaction with CB2 receptors (Showalter et al., 1996; Ross et al., 1997) as originally reported (Facci et al., 1995) and that even anandamide itself can elicit biological effects that are not mediated by cannabinoid receptors (Derocq et al., 1998; Hampson et al., 1998). Additional complexity regarding the endocannabinoid signalling system arises from the increasing awareness that both CB1 and CB2 cannabinoid receptors may be designed to respond to 2-arachidonoylglycerol (2-AG) rather than anandamide (Sugiura et al., 1999, 2000). It also appears that 2-AG can be generated selectively and is often present in cells at levels far exceeding those of anandamide (see the article by Sugiura and Waku in this issue).

In order to distinguish among the biologic properties of different NAEs, it is important to use precise and sensitive analytical techniques for the assay of both *N*-acyl PE and NAE. During our original studies on the lipids of myocardial infarct (Epps et al., 1979, 1980), we used a variety of chemical and enzymatic degradations for the identification of *N*-acyl PE, and gas chromatography for the analysis of both ester and amide linked fatty acids. We later published a simplified procedure for the unequivocal identification of *N*-acyl PE and *N*-acylphosphatidylserine (*N*-acyl PS) through sequential chemical and enzymatic degradation (Schmid et al., 1986). However, the methods used at the time were not sensitive enough to detect and quantify *N*-acyl PE and NAE in normal cells and tissues. After the discovery of anandamide, we developed a sensitive technique based on stable isotope dilution and gas chromatography/mass spectrometry (GC-MS) in selected ion monitoring mode. Internal standards were synthesized with [1,1,2,2-$^2H_4$]ethanolamine and the fatty acids of interest (16:0, 18:0, 18:1n-9, 18:2n-6 and 20:4n-6). Although other polyunsaturated NAEs have been identified in biological materials (Sugiura et al., 1996a; Kondo et al., 1998; Bisogno et al., 1999), their levels in most cells are so low that we have not chosen to include their corresponding internal standards in our assay mixture. With our method we can measure all the major NAEs and anandamide as well, at a level of less than 0.2 ng in a lipid extract (Schmid et al., 1995a,b, 1997a). Very recently, we have synthesized sn-2 monoacylglycerols from [1,1,2,3,3-$^2H_5$]glycerol and 18:1, 18:2 and 20:4 fatty acids. Monoacylglycerols are isolated and derivatized to *t*-butyldimethylsilyl derivatives together with NAEs and can be analyzed in the same lipid extract (Schmid et al., 2000). We thus have the methodology to assay simultaneously for anandamide and 2-AG, as well as their major cannabinoid receptor-inactive congeners.

Using this approach, we have undertaken a systematic study of the dynamics of *N*-acyl PE and NAE generation in intact cells. We have found that certain stress conditions such as UVB irradiation and serum deprivation result in significantly increased levels of both *N*-acyl PE and NAE in mouse epidermal JB6 P^+ cells (Berdyshev et al., 2000a). Although experimentally relevant levels of *N*-acyl PE and NAE were present in serum-containing incubation media, as expected from previously published serum analyses (Giuffrida and Piomelli, 1998), we found that their levels in the media remained unchanged during incubation of cells and they did not appear to have any effects on cellular *N*-acyl PE and NAE levels and compositions. Perhaps most importantly, the increases in cellular *N*-acyl PE and NAE levels induced by serum deprivation were fully reversible when serum was added to the incubation media after 36 h of starvation (Fig. 3). This represents the first documented evidence that the elevation of cellular *N*-acyl PE and NAE levels can be reversible and suggests that they are tightly regulated under physiological conditions. The JB6 P^+ cells used in these experiments are sensitive to tumor promoters and can therefore serve as models for the study of neoplastic trans-

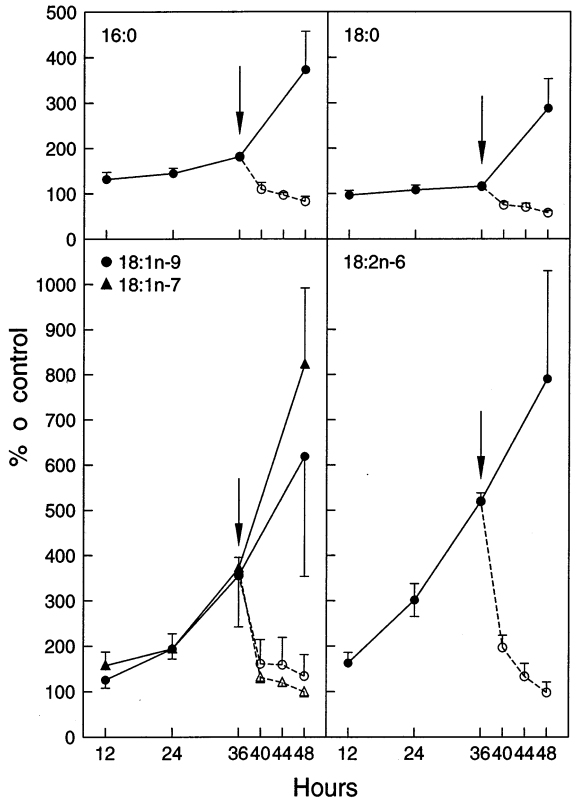
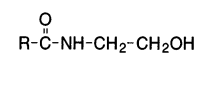


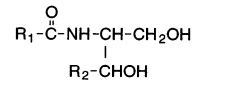
Fig. 3. Effect of serum deprivation on the levels of certain NAEs in JB6 P^+ cells. Cells were grown in EMEM containing 5% (v/v) FBS. After achieving 95% cell confluence, the medium was poured off and replaced by a medium without serum; cells were starved for up to 48 h (●, ▲) Some cells were returned to medium containing 5% (v/v) FBS (○, △) after 36 h of starvation (indicated by arrow). Lipids were extracted from cells only, fractionated and analyzed by GC-MS. Reprinted from Berdyshev et al. (2000a) Berdyshev et al. (2000b), by permission.

formation induced by UVB irradiation or treatment with other agonists (Huang et al., 1997a; Dong et al., 1997). Although it is not clear at this time whether or not NAE generation is related to the induction of apoptosis, it is important to consider that UVB irradiation induces apoptosis through ceramide-mediated signalling (Verheij et al., 1996) and that its role as a tumor initiator and promoter may also be ceramide-mediated (Huang et al., 1997b).

The functional group of NAE exhibits a certain structural similarity to the polar group of the ceramide molecule:



NAE (*N*-acylethanolamine)



Ceramide (*N*-acylsphingosine)

We have therefore proposed that the saturated and monounsaturated NAEs could have signalling functions analogous to ceramide by interacting either directly or indirectly with the molecular targets of ceramide signalling pathways (Hannun, 1996). It has also been known for a long time that 18:1n-9 NAE can act as an inhibitor of ceramidase (Sugita et al., 1975) and very recent results show that it can inhibit ceramide glucosylation as well (Spinedi et al., 1999). Interestingly, in our experiments, UVB irradiation had the greatest effect on the levels of 18:1n-9 NAE (Berdyshev et al., 2000a).

Our most recent results suggest that ceramide treatment of JB6 P^+ cells stimulates cellular NAE synthesis. When cells were grown to 90% confluence and then maintained for up to 8 h in medium containing low (0.1%) serum, in the presence or absence of 20 μM C_2-ceramide, cellular levels of all saturated and monounsaturated NAEs were increased. The largest relative increase due to ceramide (400% of untreated control after 8 h) occurred in *N*-vaccenoyl-ethanolamine (18:1n-7 NAE), while the increase in *N*-oleoylethanolamine (18:1n-9 NAE) was only 150% of control (Berdyshev et al., 2000b). When 18:1n-9 NAE or 18:1n-7 NAE (10 μM) were added at the beginning to cells incubated in low serum media for up to 48 h, the stress-induced ceramide accumulation in these cells was completely prevented (Berdyshev et al., manuscript in preparation).

Work on the identification of potential targets of NAE mediated cell signalling and on possible relationships between NAE generation and ceramide signalling is currently underway in our laboratory.

Acknowledgements

Over the years, work in the author's laboratory was supported in part by United States Public Health Service grants HL08214, HL24312,

NS14304 and GM45741 and The Hormel Foundation. Major contributions to the work summarized here were made by a number of postdoctoral associates, primarily V. Natarajan, now a professor at Johns Hopkins University, and also by D.E. Epps, P.V. Reddy, J.L. Newman, N.L. Parinandi, T. Kuwae and my most recent colleague, E.V. Berdyshev. Special thanks are due to P.C. Schmid who provided not only special expertise but also the necessary continuity for the entire 20 years of this project.

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