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COMMENTARY

Formation of *N*-Acyl-phosphatidylethanolamines and *N*-Acylethanolamines

PROPOSED ROLE IN NEUROTOXICITY

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ABSTRACT. The formation of *N*-acyl-phosphatidylethanolamine (NAPE) and *N*-acylethanolamine (NAE), including anandamide, in mammals in relation to neurotoxicity is discussed. Data on the characterization of the NAPE-forming *N*-acyltransferase, the NAPE-hydrolyzing phospholipase D, and the NAE-hydrolyzing amidase are reviewed. We suggest that NAPE and NAE, including anandamide, are formed in neurons in response to the high intracellular calcium concentrations that occur in injured neurons, e.g. due to glutamate excitotoxicity. NAPE may have functions of its own besides being a precursor for NAE. The formation of both of these lipids may serve as a cytoprotective response, whether mediated by physical interactions with membranes or enzymes, or mediated by activation of cannabinoid receptors. This suggestion implies that NAPE and NAE may have pathophysiological roles in the brain. Whether these lipids also have physiological roles is uncertain. BIOCHEM PHARMACOL **55**;6:719–725, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *N*-acyl-phosphatidylethanolamine; *N*-acylethanolamine; anandamide; neurotoxicity; cannabinoid; 2-arachidonoylglycerol; brain ischemia; glutamate; neuroprotection

In the 1970s, it was discovered that the opioid receptor bound endogenous ligands having the same pain-relieving effect as morphine, i.e. the endorphins. There has also been a search for receptors for other bioactive plant-derived compounds and their related endogenous ligands. In 1988, Howlett and coworkers [1] discovered that the brain contains a receptor for the active principle of cannabis, Δ^9-tetrahydrocannabinol. This was then followed by the cloning of the brain-type cannabinoid receptor (CB1†) and the peripheral-type cannabinoid receptor (CB2) as described in recent reviews [2–6]. The CB1 receptor and the splice variant CB1a [7] are localized mainly in the brain, whereas the CB2 receptor is localized mainly in spleen and hemopoietic cells [2–4]. In 1992, *N*-arachidonoylethanolamine was isolated from bovine brain as an endogenous ligand for the CB1 receptor, and the compound was given the name anandamide [8]. Other polyunsaturated NAEs have also been shown to function as ligands for the CB1 receptor. Recently, another compound, 2-AG, was suggested as an endogenous ligand for the CB1 receptor as well as for the CB2 receptor [9–12]. The formation of 2-AG has been described in the brain as well as peripherally [9, 10, 12–16], and it is found in human plasma [17].

The CB1 receptor has high affinity for NAEs containing C20-polyunsaturated fatty acids [18, 19]. K_i values for anandamide have been reported to be around 100 nM [see, for example, Refs. 8, 18, and 19], depending on the experimental conditions. Two studies have compared the affinity of anandamide with that of 2-AG and found K_i values of 252 vs 472 nM [9] and 89 vs 2400 nM [10]. The CB2 receptor has K_i values for anandamide and 2-AG of 581 and 1400 nM, respectively [9]. The interesting report that *N*-palmitoylethanolamine shows higher affinity for the CB2 receptor than does anandamide [20] needs to be confirmed in more elaborate studies. *N*-Palmitoylethanolamine shows low affinity for the CB1 receptor [10, 21].

The present commentary will be restricted to a discussion of the synthesis of NAE and its precursor molecule, NAPE, in the brain and the possible biological function of these lipids in relation to neurotoxicity. On the basis of present knowledge, we suggest that NAPE and NAE, including anandamide, are formed in response to the high intracellular calcium concentrations that are seen in injured cells. This occurs in certain mammalian tissues, especially neurons. The formation of NAPE and NAE may serve as a cytoprotective response. NAPE accumulates in much higher amounts than NAE, and NAPE *per se* may have important cytoprotective functions besides being a precursor for NAE.

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† *Abbreviations:* 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; NAE, *N*-acylethanolamine; NAPE, *N*-acyl-phosphatidylethanolamine; NMDA, *N*-methyl-D-aspartate; PtdCho, phosphatidylcholine; and PtdEtn, phosphatidylethanolamine.

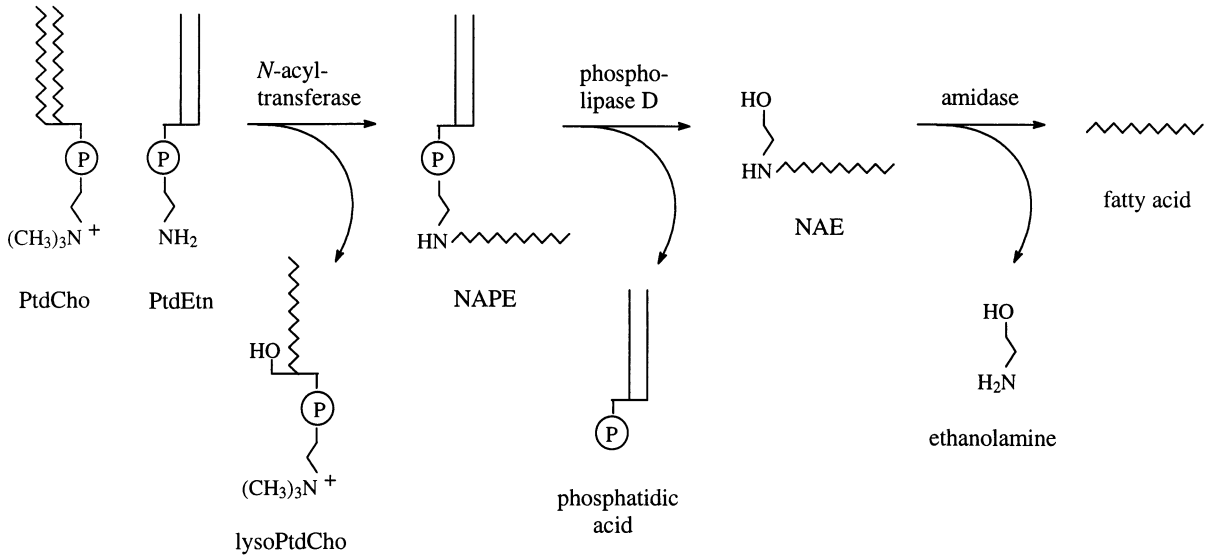

FIG. 1. Formation of *N*-acyl-phosphatidylethanolamine (NAPE) from phosphatidylethanolamine (PtdEtn) with phosphatidylcholine (PtdCho) as acyl donor, and the following enzyme-catalyzed hydrolysis of NAPE and *N*-acylethanolamine (NAE) by phospholipase D and amidase, respectively.

FORMATION AND METABOLISM OF NAPE AND NAE

It is now generally accepted that NAEs, including anandamide, are formed from the unusual precursor phospholipid NAPE by cleavage of the phosphodiester bond catalyzed by an NAPE-specific phospholipase D [5, 22–26] (Fig. 1).

Direct formation of NAE via condensation of a fatty acid with ethanolamine (i.e. the reversed amidase reaction) seems not to be of physiological relevance [5, 22–26]. By the reactions shown in Fig. 1, several different molecular species of NAPEs and NAEs are formed, and anandamide accounts for only $< 15\%$ of the NAEs formed in tissues post mortem [27, 28], in tissue homogenates [22, 23], and in cell cultures [25, 29–33]. In one case, Bisogno *et al.* [30] reported without further comments that in antigen-stimulated RBL-2H3 cells anandamide accounts for 37.5% of the NAEs formed.

NAPE Formation

For several decades NAPE has been known to occur in plant tissues, and its formation has been suggested to be a cytoprotective response to different stress stimuli [see, for example, Ref. 34]. Since 1973 [35], NAPE has been reported to occur in mammalian tissues, but as Schmid *et al.* [27] concluded in a very comprehensive review in 1990: "*N*-acylethanolamine phospholipids and NAEs are not formed in measurable amounts in normal mammalian cells and tissues, but they can accumulate under conditions of degenerative change involving disintegration and/or digestion of membranes." A cytoprotective function of these lipids has been suggested, especially in ischemic heart tissue [27]. The *N*-acyltransferase activity was first described in dog heart preparations [27, 36]. The enzyme uses diacyl-PtdEtn, plasmalogen, and lyso-PtdEtn as acceptor substrates, and the *sn*-1 acyl group of PtdEtn, PtdCho, cardiolipin, and to a lesser extent lyso-PtdCho as donor substrates [27]. The enzyme does not seem to discriminate much between different fatty acids, i.e. any fatty acids in the *sn*-1 position will be a donor substrate [23]. Sugiura *et al.* [23] and Di Marzo *et al.* [37] confirmed that arachidonoyl in the *sn*-1 position of PtdCho also is a donor substrate. Curiously, PtdEtn and lyso-PtdCho with arachidonoyl in the *sn*-1 positions were not found to be donor substrates [23]. The reaction has an alkaline pH optimum, is sensitive to inhibition by thiol-reactive agents, and is highly activated by millimolar concentrations of Ca^{2+} or Mn^{2+} [27]. Half-maximal stimulation was obtained between 0.2 and 0.5 mM Ca^{2+} [38, 39]. Protein kinase C does not seem to be involved in the regulation of NAPE formation [24, 33], but cyclic AMP may play a role. In one study [24], increased intracellular cyclic AMP was found to enhance NAPE formation, whereas in another study [33] dibutyryl-cyclic AMP was without effect. The biosynthetic activity in dog brain was associated with membrane fractions, primarily microsomes, synaptosomes, and mitochondria, but not with myelin [39]. Cadas *et al.* [32] have reported that approximately 50% of the NAPE in cultured neurons was localized on the outer surface of the cells, since this percentage of cellular NAPE was accessible for hydrolysis by exogenous *Streptomyces chromofuscus* phospholipase D. However, this conclusion is only valid if all the cells in the culture were intact. The *N*-acyltransferase activity has been studied especially in dog heart and brain, but it is also found in a number of other tissues and cells [22, 24, 27, 29, 36, 39]. A thorough study of the tissue distribution of the enzyme has not been reported. Interestingly, rat heart appears to contain no or very low *N*-acyltransferase activity [26]. The *N*-acyltransferase has not been purified.

NAE Formation

NAPE can be hydrolyzed by an NAPE-specific phospholipase D, thereby generating NAE and phosphatidic acid. It is not known whether this phospholipase D in the presence of ethanol can catalyze the formation of phosphatidylethanol instead of phosphatidic acid, i.e. a transphosphatidylation reaction. All other known mammalian phospholipase D enzymes [40–43] catalyze this type of reaction, and the transphosphatidylation reaction is generally used to identify activation of phospholipase D in cell cultures. Phosphatidic acid may be a second messenger, as it has been proposed to regulate the function of several different proteins within the cell [44]. The NAPE-hydrolyzing phospholipase D has been found in a number of different tissues and cells [27]. In the rat, the specific activity of the enzyme in the tissues is of the following order: heart > brain > testis > kidney > spleen > lung > liver [45]. NAPE-hydrolyzing phospholipase D is specific for NAPE and lyso-NAPE, has an alkaline pH optimum, is inhibited by thiol-reactive agents, and is localized to a microsomal fraction and, to a lesser extent, to a mitochondrial fraction [45, 46]. The enzyme is inhibited by 5 mM Zn^{2+} [45]. Two different K_m (*N*-palmitoyl-PtdEtn) values have been reported: 150 μM [46] and 21 μM [45]. However, these two values cannot be compared directly because they have been obtained under different conditions of incubation. Furthermore, kinetic constants for phospholipases and other lipid-metabolizing enzymes may be described better in mol% using "surface dilution kinetics" [47]. The NAPE-hydrolyzing phospholipase D does not seem to discriminate between different *N*-acyl groups [22, 23], i.e. the species of NAEs formed seem to be equivalent to the *N*-acyl composition of NAPE. If there is no selectivity in the diesterase reaction catalyzed by the NAPE-hydrolyzing phospholipase D, then any anandamide formation is dependent on the formation of the unusual *sn*-1 arachidonoyl-phospholipid species that can serve as acyl donors in the *N*-acyltransferase reaction. Such species can be found in very small amounts in tissues, and they are especially formed when cells are supplied with high amounts of arachidonic acid [48–50]. Arachidonic acid belongs to the essential fatty acids of the n-6 family [51], but the n-3 fatty acid docosahexaenoic acid can also be incorporated into NAPE and NAE from rat brain [23]. Interestingly, high amounts of dipolyene-PtdEtn species, including didocosahexaenoyl-PtdEtn, are found in the retina [52]. However, it is not known whether NAPE or NAE can be formed in the retina.

The NAPE-hydrolyzing phospholipase D is not influenced markedly by calcium ions or EGTA [22, 23, 27, 37, 45, 46]. Bisogno *et al.* [30] have reported data suggesting that the NAPE-hydrolyzing phospholipase D in mouse J774 macrophages was stimulated by the calcium influx mediated by a calcium ionophore, and Cadas *et al.* seem to believe that as well, as judged by their illustration in Ref. 24. However, in our experiments with mouse cortical neurons, we have always observed that a large increase in NAPE formation was accompanied by a lesser increase in NAE formation. This suggests that an increase of intracellular [Ca^{2+}] stimulated the *N*-acyltransferase and that whenever the amount of NAPE increased, it was slowly hydrolyzed by a constitutively active NAPE-hydrolyzing phospholipase D [31, 33]. The lesser increase in NAE formation was not due to rapid NAE degradation, since the presence of bovine serum albumin in the cell culture medium ensured a long half-life (hours) of anandamide [31]. We have not found agents that selectively stimulated the NAPE-hydrolyzing phospholipase D [31, 33]. The NAPE-hydrolyzing phospholipase D has not been purified.

NAE Degradation

Earlier literature on enzymatic hydrolysis of NAE has been reviewed [5, 26, 27]. Here we will concentrate on the recent studies characterizing the enzymes involved. Schmid *et al.* [53] have partly characterized an amidase from rat liver. The enzymatic activity was found in both the mitochondrial fraction and the microsomes, it was inhibited by thiol-reactive agents and oleate, it had no requirement for divalent cations, it showed increased activity between pH 7.5 and 10.5, and it hydrolyzed *N*-oleoylethanolamine and saturated NAEs. No polyunsaturated NAEs were tested for hydrolysis in that study [53]. In 1993, Deutsch and Chin [54] reported that anandamide also was a substrate for an amidase found in several rat tissues and in N18TG2 and C6 cells. The enzyme was inhibited by phenylmethylsulfonyl fluoride [54], a well known protease inhibitor. A number of papers describe partial purification and/or characterization of an amidase activity from different tissues and cells [30, 55–59]. The general picture is that many tissues contain an amidase that degrades NAEs and fatty acid amides. It should be noted that the amidase activity is extremely low in mammalian heart [27]. The preferred substrate seems to be long-chain polyunsaturated NAEs and short-chain saturated NAEs, but generally all NAEs are substrates [30, 53, 55, 57–59]. The likely cause for the differences in the rate of hydrolysis is the physical state of the different substrates in the incubation medium, i.e. solubility and micellar formation [53]. K_m values for anandamide have been reported to be in the range of 3.4 to 67 μM for amidases from different tissues [23, 30, 56–58, 60], and it is difficult to decide whether the wide range of K_m values is caused by different assay conditions or different enzyme properties. Some data seem to indicate the existence of amidases that hydrolyze anandamide far better than *N*-palmitoylamide [30, 55, 58], but this needs to be confirmed. The sleep-inducing compound oleamide [61] is also hydrolyzed by the amidase [58, 59] with a K_m value of 9 μM [58]. Recently, amidases from rat, mouse, and human liver have been cloned and expressed in Cos-7 cells [59, 62]. All three enzymes consist of 579 amino acids, mouse and rat amidases share 91% amino acid identity, and the human amidase shares 82 and 84% identity with the two former amidases, respectively. These are integral membrane proteins with

one transmembrane region, and the calculated molecular masses range between 63.0 and 63.4 kDa [62]. Northern blot analysis with the human amidase cDNA probe and mRNA from various human tissues identified a single major mRNA transcript that was most abundant in pancreas, brain, kidney, and skeletal muscle, with lesser amounts in liver and placenta [62]. Mouse amidase mRNA was most abundant in liver and brain, with lesser amounts in spleen, lung, kidney, and testes [59]. A number of different synthetic inhibitors of the amidase, some of which also inhibit phospholipase A_2 enzymes, have been described [63–67].

PROPOSED BIOLOGICAL FUNCTION OF NAPE AND NAE IN NEUROTOXICITY

Neither NAPE nor NAE seems to occur in normal tissues, but they accumulate in some tissues during cell injury as well as post mortem [27, 28]. Anandamide, being one of the NAE species, also accumulates post mortem in animal and human tissues [28, 68, 69]. This accumulation raises the question as to whether anandamide is present at all in fresh tissue, since some anandamide is expected to accumulate during the process of tissue sampling [28, 68, 69]. Kempe *et al.* [68] suggested that the precursor for anandamide, *N*-arachidonoyl-PtdEtn, accounted for less than 1 in 10^6 phospholipid molecules in fresh rat brain. This seems to be the same situation as when researchers in the 1970s estimated prostaglandin levels in tissues. Prostaglandins also accumulate during tissue sampling [70, 71], and they are usually not found in tissues unless their synthesis has been initiated by a stimulus. Most stimuli are pathological stimuli, like those occurring during inflammation or platelet aggregation, but prostaglandins can also be formed in physiological processes like labor [71, 72].

Compounds that increase intracellular [Ca^{2+}] are likely to induce the synthesis of NAPE and NAE [24, 29, 31]; perhaps rather high or prolonged increases in intracellular [Ca^{2+}] are needed. We have found that the neurotransmitter glutamate will induce a steadily increasing formation of NAPE and NAE in mouse cortical neurons in culture via activation of the ionotrophic NMDA receptor [33] that is a glutamate receptor subtype. In our primary cell culture, glutamate also exerts a cytotoxic effect, which is mediated via an increase in the intracellular [Ca^{2+}] [73]. Killing the cortical neurons with the mitochondrial respiratory chain inhibitor sodium azide was an even better stimulus for NAPE/NAE formation than was glutamate [33]. This is in keeping with increased membrane leakage and Ca^{2+} influx in energy-deprived cells. Such formation of NAPE and NAE may be a biological response to cellular injury [27]. It has been demonstrated that NAPE stabilizes phospholipid membranes [74, 75] and *N*-oleoylethanolamine protects against increased Ca^{2+} permeability of isolated damaged mitochondria from rat heart [76]. Mitochondrial dysfunction has been suggested to play an important role in initiating glutamate-induced neurotoxicity [77, 78]. Furthermore, *N*-palmitoylethanolamine has been reported recently to protect cerebellar granule neurons against excitotoxic death in a narrow time window following glutamate exposure [79]. The saturated NAEs have anti-inflammatory properties [5, 27, 80], and *N*-palmitoylethanolamine inhibits serotonin release from mast cells, probably via interaction with the CB2 receptor [20]. The formation of saturated NAEs by injured neurons may thereby attenuate an inflammatory response mediated by brain mast cells [81]. Furthermore, formation of NAE by injured neurons may induce apoptosis in adjacent neurons, thereby reducing the spread of a local neuronal injury that otherwise could induce necrosis in the surrounding neurons. This is suggested from a study in which *N*-oleoylethanolamine potentiated staurosporine-induced apoptosis in embryonic chick neuronal cultures, probably via an increase in the intracellular level of ceramide [82]. *N*-Oleoylethanolamine is an inhibitor of ceramidase [27, 83], and ceramide can induce apoptosis [84]. Exogenous short-chain ceramide can also be neuroprotective in hippocampal neurons via a mechanism involving *de novo* protein synthesis [85]. Anandamide, via interaction with cannabinoid receptors, can inhibit synaptic release of glutamate by a mechanism that is mediated by an inhibitory G-protein [86].

All of these effects of NAPE and different molecular species of NAE tend to reduce the impact of a local neuronal injury within the brain, whether mediated by physical interactions with membranes or by activation of cannabinoid receptors. Since glutamate excitotoxicity may be a possible pathogenic mechanism in chronic neurodegenerative diseases like Parkinson's disease, Huntington's disease, and Alzheimer's disease [87], it raises the possibility that formation of NAPE and NAE is increased in these diseases.

A number of papers have reported biological effects of a selective CB1 receptor antagonist (SR 141716A), thereby suggesting the existence of a possible endogenous cannabinoid tone in a putative anandamidergic system [88–91]. An alternative explanation for these biological effects of SR 141716A may be that this compound can function like an inverse agonist, i.e. by inhibiting the signalling of the receptor *per se* in the absence of an endogenous ligand [92]. Whether 2-AG [9–12] is a candidate for being a mediator in such an endogenous system must await further research.

CONCLUSION

The scenario described above implies that NAPE and NAE, including anandamide, may have pathophysiological roles in the brain (and in other tissues like the heart [27]). Whether these lipids, including anandamide, also have physiological roles is uncertain.

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Note added in proof:

Very recent evidence indicates that anandamide may have a physiological function in the mouse uterus, being involved in regulating embryo implantation [93]. Schmid *et al*. [93] suggest that uterine anandamide may be formed, not by the reactions shown in Fig. 1, but instead by a special anandamide synthase catalyzing the direct condensation of arachidonic acid and ethanolamine. The uterine anandamide synthase has been shown to be active in the presence of phenylmethylsulfonyl fluoride as opposed to the amidase [94], suggesting that formation and catabolism of anandamide in the uterus are catalyzed by two different enzymes.

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