N-acylphosphatidylethanolamine-hydrolysing phospholipase D lacks the ability to transphosphatidylate

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Abstract The *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) generates *N*-acylethanolamines, including *N*-arachidonoyl-ethanolamine (anandamide), that may be neuroprotective and analgesic. The properties of NAPE-PLD from rat heart and brain microsomes are investigated and compared to those of other PLDs. NAPE-PLD is inhibited by the fatty acid aminohydrolase inhibitor MAFP in high concentrations ($\geq$100 μM) while PMSF in high concentrations (10 mM) tends to stabilise NAPE-PLD activity. Oleate inhibits NAPE-PLD but the enzyme is not affected by PIP_2, α-synuclein or mastoparan. Furthermore, it is for the first time reported that NAPE-PLD is not capable of catalysing a transphosphatidylation reaction like most other known PLDs.

Key words: Phospholipase D; *N*-acylphosphatidylethanolamine; *N*-acylethanolamine; Anandamide; Transphosphatidylation

1. Introduction

Phospholipase D (PLD) enzymes are widely distributed in mammalian cells, where some of them are regulated by a variety of hormones, growth factors, and other extracellular signals [1]. The phosphatidylcholine-specific PLDs have been widely investigated. Both the human PLD1 [2] and PLD2 [3] have recently been cloned. The major substrate phosphatidylcholine (PC) is hydrolysed by these PLDs as well as by an oleate-dependent PLD [4] to phosphatidic acid (PA) and choline. These PLDs are identified and quantified in cells and tissues by their ability to catalyse a transphosphatidylation reaction in the presence of a primary alcohol, producing phosphatidylalcohol instead of phosphatidic acid [5–7].

N-acylphosphatidylethanolamine (NAPE) is hydrolysed by a PLD subtype (NAPE-PLD) to PA and *N*-acylethanolamine (NAE) [8,9]. NAPE is only present in very small amounts in mammalian tissue. A significant increase in the formation of NAPE and NAE, including *N*-arachidonoyl-ethanolamine (anandamide), is seen when neurones are exposed to a neurotoxic environment [10–12]. It has been suggested that NAPE and NAE may serve a cytoprotective role as a response to the high intracellular calcium concentrations that are seen in injured cells [13].

Anandamide has been proposed to have a physiological role as an endogenous ligand for the cannabinoid receptor CB_1 [14,15]. It has recently been shown that cannabinoid receptor agonists can protect neurones against excessive NMDA receptor stimulation via presynaptic CB_1 receptor-mediated inhibition of glutamate release [16]. *N*-palmitoyl-ethanolamine (PEA) can also be formed by NAPE-PLD-catalysed hydrolysis and may act on CB_2-like receptors [17,18].

NAPE-PLD may play an important role for the formation of anandamide, PEA as well as other NAEs, e.g. in terms of protecting neurones against excitotoxicity and peripheral in the formation of local analgesics [18]. Mammalian NAPE-PLD has previously been preliminary characterised [19]. However, it has not been known whether this NAPE-PLD can transphosphatidylate or whether it resembles the other known mammalian PLDs in terms of inhibitors and activators.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine (56 mCi/mmol) and [1-^{14}C]palmitic acid (55 mCi/mmol) were from Amersham Pharmacia Biotech (Amersham, UK). Phosphatidylethanol was from Avanti Polar Lipids (Alabaster, AL, USA). Methyl arachidonyl fluorophosphonate (MAFP) and phoshatidylbutanol were from Biomol (Plymouth Meeting, MA, USA). Synthetic mastoparan from *Vespula lewisii* and PLD from *Streptomyces* species (*St.* sp.) were from Sigma Chemical Co. (St. Louis, MO, USA). Karina Petersen, Lundbeck A/S (Valby, Denmark) kindly provided purified recombinant human α-synuclein.

2.2. Synthesis of *N*-acylphosphatidylethanolamines

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl) ethanolamine and 1,2-dilauroyl-*sn*-glycero-3-phospho- (*N*-[1′-^{14}C]-palmitoyl)ethanolamine were prepared essentially as described by Schmid et al. [19] with a specific activity of 5000 dpm/nmol. 1-Palmitoyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)ethanolamine was synthesised according to Shangguan et al. [20] with a specific activity of 500 dpm/nmol. The products were purified by thin-layer chromatography (TLC) and developed in chloroform: methanol:ammonium hydroxide (80:20:2 v/v/v).

2.3. Subcellular fractionation

Hearts and brains from 13–17 weeks old Sprague-Dawley rats were homogenised with an UltraTurrax in 10 v/w 25 mM Tris-HCl buffer, 0.25 M sucrose, 2 mM EDTA, pH 7.3. Microsomal fractions were prepared from the 1000×*g* supernatant by centrifugation at 109 000×*g*. The resultant pellet (microsomes) was resuspended in 60 mM bis-Tris propane, 2 mM 1,4-dithiothreitol, pH 8.0 at a con-

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Abbreviations: DAG, diacylglycerol; DMSO, dimethylsulfoxide; FAAH, fatty acid aminohydrolase; MAFP, methyl arachidonyl fluorophosphonate; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D; NMDA, *N*-methyl-D-aspartate; PA, phosphatidic acid; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, *N*-palmitoyl-ethanolamine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; PMSF, phenylmethylsulfonylfluoride; PS, phosphatidylserine; *St.* sp., *Streptomyces* species; TLC, thin-layer chromatography

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centration of 3–19 mg of protein/ml. Protein was determined by the method of Bradford [21].

2.4. Assay of PLD activity

The substrate (NAPE) was dried under a stream of nitrogen, resuspended in 60 mM bis-Tris propane, 2 mM 1,4-dithiothreitol, pH 8.0 and sonicated for 10 min. 200 μg of protein from rat heart or rat brain microsomes was added to a final volume of 200 μl. Incubations were carried out at 37°C. When indicated the incubation mixture also consisted of Triton X-100, phenylmethylsulfonylfluoride (PMSF) and dimethylsulfoxide (DMSO). The reactions were terminated by adding 1.5 ml of ice-cold chloroform:methanol (2:1 v/v) and the lipids were extracted. Separation of the lipids was carried out by TLC, developing the plates in chloroform:methanol:ammonium hydroxide (80:20:2 v/v/v). After development, the reaction products were located and quantified by a PhosphoImager scanner (STORM, Molecular Dynamics, Sunnyvale, CA, USA).

3. Results and discussion

3.1. Time-dependent NAE formation

The amount of NAE (PEA) formed under the standard assay conditions including 0.6 mM Triton X-100, 10 mM PMSF and 5 μl DMSO was linear for at least 8 h of incubation at 37°C. PEA was formed at a rate of 700 pmol per h per mg protein using rat heart microsomes while incubations with rat brain microsomes resulted in the lower rate of 520 pmol PEA per h per mg protein (data not shown).

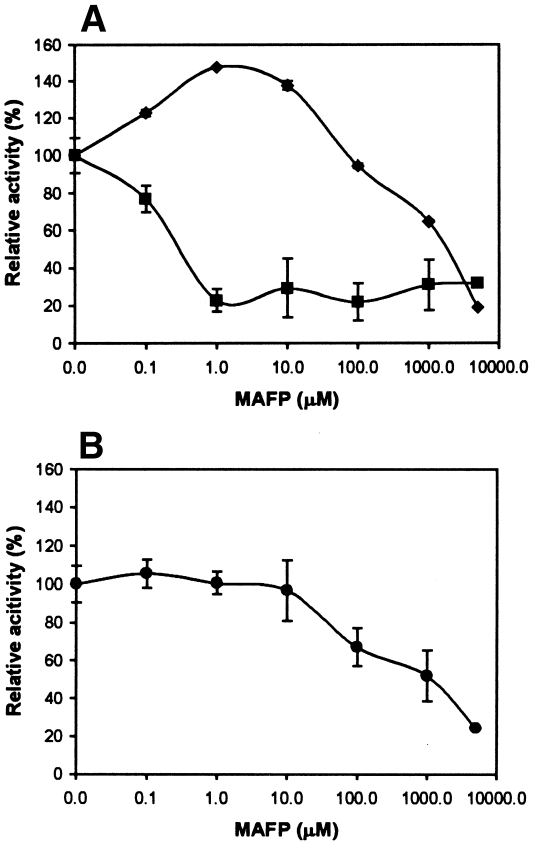

Fig. 1. Effect of increasing concentrations of MAFP on NAPE-PLD activity and FAAH activity. 10 μM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine was incubated for 3 h under the described conditions, various concentrations of MAFP added in 2 μl of DMSO and protein from rat brain microsomes. A: The relative activity of NAPE-PLD-catalysed PEA (♦) formation and FAAH-catalysed palmitic acid (■) formation. B: NAPE-PLD activity calculated as the sum of PEA and palmitic acid formation. 100% activity in A equals 127 pmol PEA/h/mg protein and 77 pmol palmitic acid/h/mg protein, respectively. Data represent means ± S.E.M. of one experiment performed in duplicate. Another experiment with concentrations of MAFP up to 100 μM showed the same inhibition as the above data.

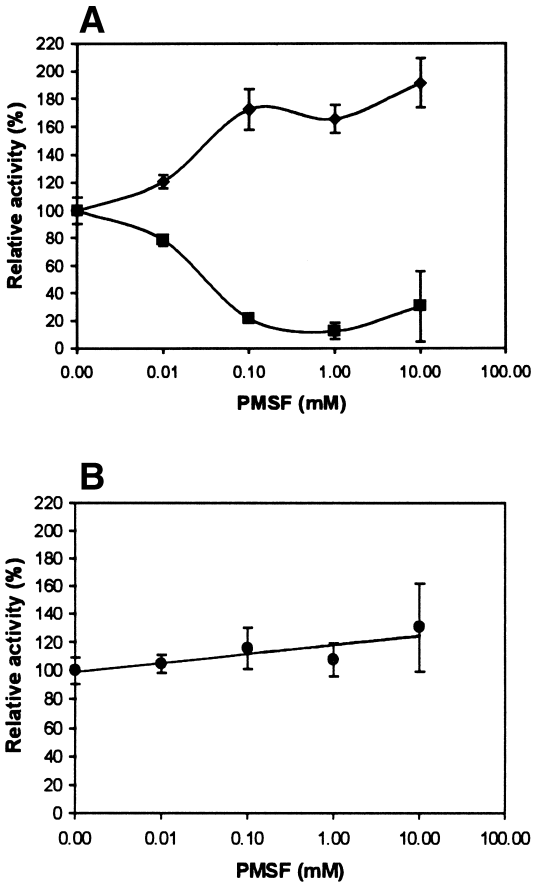

Fig. 2. Effect of increasing concentrations of PMSF on NAPE-PLD activity and FAAH activity. 10 μM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine was incubated for 3 h under the described conditions, various concentrations of PMSF added in 2 μl of DMSO and protein from rat brain microsomes. A: The relative activity of NAPE-PLD-catalysed PEA (♦) formation and FAAH-catalysed palmitic acid (■) formation. B: NAPE-PLD activity calculated as the sum of PEA and palmitic acid formation. 100% activity in A equals 127 pmol PEA/h/mg protein and 77 pmol palmitic acid/h/mg protein, respectively. Data represent means ± S.E.M. of one experiment performed in duplicate. Another experiment was carried out with PMSF added in 10 μl of DMSO with the same results.

3.2. Effects of FAAH inhibitors on NAPE-PLD activity

In order to avoid hydrolysis of NAE during the NAPE-PLD assay two different fatty acid aminohydrolase (FAAH) inhibitors were investigated. MAFP has recently been reported to be a very potent irreversible FAAH inhibitor [22,23]. Fig. 1A shows that 1 μM MAFP increases PEA formation due to inhibition of FAAH activity. Complete inhibition of FAAH has earlier been reported by Schmid et al. [24] to occur at similar concentrations, i.e. 0.1–1 μM MAFP dependent on the dilution of the membrane preparation. NAPE-PLD activity is significantly inhibited adding 100 times higher concentration of MAFP (Fig. 1B). It is therefore concluded that it is safe to use 1 μM MAFP in the NAPE-PLD assay.

PMSF, which has been known to inhibit serine proteases and some thiol proteases, was in 1993 also reported to abolish FAAH activity [25]. Fig. 2A shows that 0.1 mM PMSF effectively inhibits FAAH activity and thereby increases PEA formation. These results imply that MAFP is 100 times more potent than PMSF in inhibiting FAAH. This is in good agreement with results obtained by Deutsch et al. [22]. Higher concentrations of PMSF tend to increase NAPE-PLD activity (Fig. 2B).

The potentiating effect of PMSF on NAPE-PLD activity was studied in more detail. An experiment was carried out pre-incubating the microsomes 1–8 h at 37°C with PMSF and without substrate. The results showed a significant decrease of 40% in NAPE-PLD activity in rat brain microsomes after 1 h of pre-incubation while only 13% decrease in NAPE-PLD activity was seen in rat heart microsomes after 8 h of pre-incubation (data not shown). These results suggest that the potentiating effect of PMSF is conducted via prevention of NAPE-PLD degradation due to inhibition of proteases by PMSF.

3.3. Detergent effects of Triton X-100

Triton X-100 has earlier been reported to be the only detergent stimulating NAPE-PLD activity in in vitro assays. The optimum NAPE-PLD activity was found to be 2.0 mg/ml Triton X-100 in rat brain microsomes [19] and 4 mM Triton X-100 in dog brain microsomes [26]. 0.6 mM Triton X-100 was here found to be the optimal concentration of detergent while a 10-fold increase in concentration of the detergent resulted in 69% inhibition of NAPE-PLD activity (data not shown).

3.4. NAPE-PLD exposed to known activators/inhibitors of PLD activity

It has been suggested that PLD1 and PLD2 act through similar catalytic mechanisms since both enzymes have an absolute requirement for phosphatidylinositol 4,5-bisphosphate (PIP_2) for activation [3]. Experiments performed using rat heart microsomes showed that mammalian NAPE-PLD activity is not stimulated by PIP_2, neither when PIP_2 is included in lipid vesicles in 0.1, 1 or 10 mol% with PC (Fig. 3) or PE (data not shown). The decrease in NAPE-PLD activity seen when 9-fold phospholipid was added can most likely be ascribed to a non-specific surface dilution of the substrate [27] since the same decrease was seen whether PC or PE was added. Addition of PA or PS seems to inhibit NAPE-PLD activity while PI stimulates the enzyme.

α-Synuclein and β-synuclein proteins have been reported to selectively inhibit mammalian PLD2 [28]. It was suggested that PLD2 might be involved in synaptic vesicle cycling and that the synucleins are important regulatory components of this process. Jenco et al. [28] showed that 100 nM of α-synuclein inhibited PLD2 completely. No inhibitory effect was seen when 0.1–400 nM human recombinant α-synuclein was tested on NAPE-PLD activity using rat brain microsomes (data not shown).

Mammalian tissues also contain PLD activities that can be stimulated by unsaturated fatty acids such as oleate, linoleate, linoleneate and arachidonate (oleate-dependent PLD) [4,29,30]. The most effective in vitro activator concentrations were found to be around 4 mM [31]. In contrast, rat heart NAPE-PLD activity was inhibited by oleate (Fig. 3), as are PLD1 and PLD2 enzymes [2,32].

Mastoparans exhibit a wide variety of biological effects, including affecting the activity of PLA_2 and PLC, see [33]. Stimulation of PLD activity has been reported in carnation petals [34] as well as in L1210 cells [35]. Fensome et al. [36] have on the contrary observed that GTPγS-stimulated PLC-β and PLD activity were inhibited by mastoparan. All these mechanisms have been related to the activation of G-proteins [37,38] or induction of membrane perturbation [33]. 25 μM mastoparan has been reported to stimulate NAPE-PLD activ-

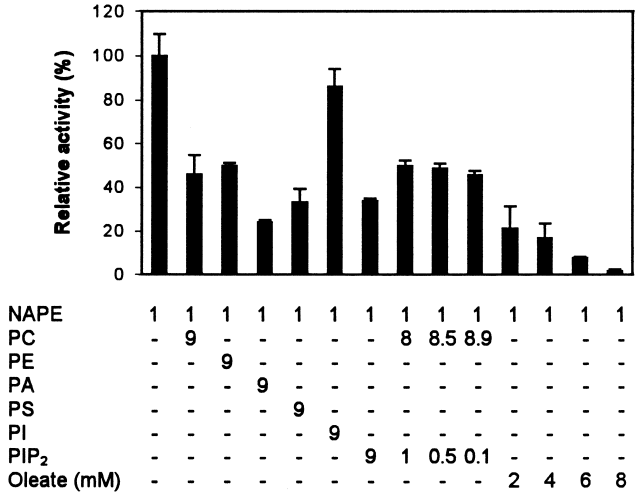

Fig. 3. Effect of phospholipids and oleate on NAPE-PLD activity. 10 μM 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)-ethanolamine was incubated for 30 min under the described conditions, including 0.6 mM Triton X-100, 10 mM PMSF, 5 μl DMSO and protein from rat heart microsomes. Data represent means ± S.E.M. of one experiment performed in duplicate. A second experiment was carried out with 1 h of incubation with similar results. 100% activity equals 3148 pmol PEA/h/mg protein. Numbers indicate the molar ratio except for oleate.

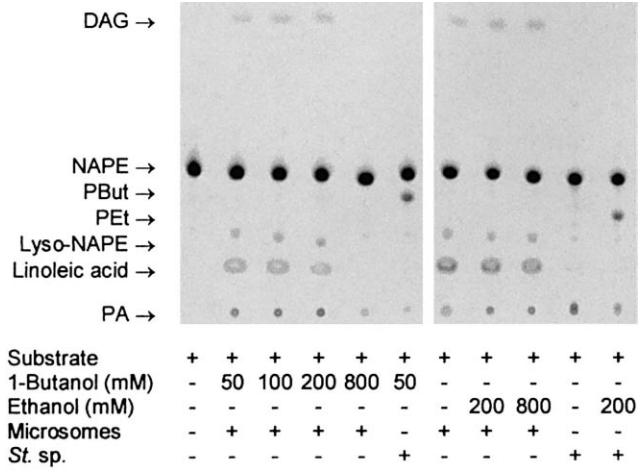

Fig. 4. Effect of different concentrations of alcohols on NAPE-PLD activity. 100 μM of the substrate 1-palmitoyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phospho(*N*-oleoyl)ethanolamine was incubated for 3 h (microsomes) or 1 h (PLD from *St.* sp.) under the described conditions, various concentrations of alcohols added before sonication and protein from rat brain microsomes or 2 units of PLD from *St.* sp. PhosphoImaging of the TLC plates identified the reaction products. The same lack of transphosphatidylation was seen in five other experiments.

ity from microsomes isolated from elicitor-treated tobacco (*Nicotiana tabacum* L.) cells [39]. In similar experiments using rat brain microsomes and 0.5–100 μM mastoparan from *Vespula* no stimulation was seen (data not shown).

3.5. Transphosphatidylation activity of NAPE-PLD

PLD from higher plants was first shown to catalyse a phosphatidyl transfer reaction in which a primary alcohol acts as nucleophilic acceptor in place of water [40,41]. Later this transphosphatidylation reaction was also recognised in rat brain synaptosomes, catalysed by an oleate-dependent PLD [31]. Mammalian PLD1 and PLD2 are also capable of catalysing this reaction [2,3]. Today the transphosphatidylation reaction is generally used to identify activation of PLD in cell cultures and tissues.

The ability of alcohols to serve as substrates for an NAPE-PLD-catalysed transphosphatidylation reaction is shown in Fig. 4. NAPE-PLD from rat brain and rat heart microsomes is not capable of catalysing a transphosphatidylation reaction using either 1-butanol, ethanol, 1-propanol or 2-propanol as substrates (Fig. 4 and data not shown). A positive control of the reaction is shown using PLD from *Streptomyces* species.

Since most classes of PLDs are able to catalyse a transphosphatidylation reaction the lack of this ability is seen as a distinct feature of mammalian NAPE-PLD. So far only one PLD activity, in yeast, has been described not to be capable of catalysing a transphosphatidylation reaction. Therefore this Ca^{2+}-dependent PLD with preference for hydrolysing PE and PS over PC was suggested to be part of a new PLD gene family [42,43]. The findings reported here open the possibility that mammalian NAPE-PLD may be part of this new gene family.

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