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The stimulatory effect of phosphatidylethanolamine on *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD)

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Abstract

N-Acylphosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) is a membrane-bound enzyme which releases the endocannabinoid anandamide and other bioactive *N*-acylethanolamines from their corresponding NAPEs in animal tissues. Our previous studies showed that NAPE-PLD solubilized from the membrane is remarkably stimulated by millimolar concentrations of Ca^{2+} while the membrane-bound form is much less sensitive to Ca^{2+}. This finding suggested that certain membrane constituents diminished the stimulatory effect of Ca^{2+}. In the present studies, we examined the effects of membrane fractions from COS-7 cells and brain tissue on the purified recombinant rat NAPE-PLD, and found that heat-stable membrane component(s) dose-dependently activated NAPE-PLD up to 4.8–5.0 fold. In the presence of the membrane fractions, however, the stimulatory effect of Ca^{2+} on the purified NAPE-PLD was considerably reduced. When it was examined if the membrane fractions can be replaced with various pure phospholipids, phosphatidylethanolamine activated NAPE-PLD up to 3.3 fold, which was followed by decrease in the stimulatory effects of Ca^{2+} and several other divalent cations. These results suggest that membrane components including phosphatidylethanolamine keep the membrane-associated form of NAPE-PLD constitutively active.

Keywords: *N*-Acylethanolamine; Anandamide; Endocannabinoid; Phosphatidylethanolamine; Phospholipase D; Phospholipid

1. Introduction

N-Acylethanolamines (NAEs) are ethanolamides of long-chain fatty acids and exist in various organisms including animals and plants (Schmid et al., 1990; Hansen et al., 2000). NAEs are currently considered to be a class of endogenous lipid mediators. Anandamide (*N*-arachidonoylethanolamine) is the most extensively characterized NAE, which functions as an endogenous ligand of cannabinoid receptors and transient receptor potential vanilloid 1 (TRPV1) channels (Devane et al., 1992; van der Stelt and Di Marzo, 2004), and actually exerts a variety of central and peripheral activities through these receptors (Pacher et al., 2006). On the other hand, saturated and monounsaturated NAEs are inactive with cannabinoid receptors, but show various biological activities. For example, *N*-palmitoylethanolamine exhibits anti-inflammatory, neuroprotective, and analgesic effects (Skaper et al., 1996; Calignano et al., 1998; Lambert et al., 2002), *N*-oleoylethanolamine shows anorexic action (Fu et al., 2003), and *N*-stearoylethanolamine is known as a pro-apoptotic (Maccarrone et al., 2002) and anorexic mediator (Terrazzino et al., 2004). The anti-inflammatory effect of *N*-palmitoylethanolamine and the anorexic action of *N*-oleoylethanolamine were suggested to be mediated through peroxisome proliferator-activating receptor α (Lo Verme et al., 2005; Fu et al., 2003). *N*-Oleoylethanolamine was also reported to be a ligand of the G protein-coupled receptor GPR119 (Overton et al.,

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2006). In addition, *N*-palmitoylethanolamine as well as anandamide was shown to act as an agonist of GPR55, another G protein-coupled receptor (Baker et al., 2006).

It is generally accepted that in animal tissues NAEs are principally formed from membrane phospholipids by two steps of enzyme reactions (Schmid et al., 1990; Di Marzo et al., 1994; Hansen et al., 2000; Schmid, 2000). In the first reaction, *N*-acylphosphatidylethanolamine (NAPE) is produced from phosphatidylethanolamine (PE) by calcium-dependent *N*-acyltransferase, and in the second reaction, NAE is released from the resultant NAPE by NAPE-hydrolyzing phospholipase D (NAPE-PLD). We cloned cDNA of NAPE-PLD from mouse, rat, and human, and found that the enzyme belongs to the metallo-β-lactamase family (Okamoto et al., 2004). Analysis on the highly purified recombinant NAPE-PLD from *Escherichia coli* revealed its high substrate specificity for NAPEs among various glycerophospholipids (Wang et al., 2006). Moreover, metal analysis suggested the existence of catalytically important zinc in NAPE-PLD (Wang et al., 2006).

Previously, we found that the partially purified NAPE-PLD from rat heart is potently activated by millimolar concentrations of Ca^{2+} and several other divalent cations (Ueda et al., 2001; Liu et al., 2002). Recently, we confirmed this finding with recombinant NAPE-PLD highly purified from *E. coli* (Wang et al., 2006). However, NAPE-PLD is a membrane-associated protein, and the membrane-bound form from dog brain, rat brain, and rat testis was inhibited or only slightly activated by Ca^{2+} (Natarajan et al., 1984; Sugiura et al., 1996a,b). The potent stimulation by Ca^{2+} was observed only after the enzyme was solubilized from the membrane using detergent (Liu et al., 2002). These results suggested that certain membrane components including phospholipids stimulate NAPE-PLD instead of Ca^{2+} and/or reduce the stimulatory effect of Ca^{2+}. However, further investigation has not been performed. Thus, in the present study, we examined stimulatory or inhibitory effects of crude membrane fractions and various pure phospholipids on the highly purified recombinant NAPE-PLD from *E. coli*.

2. Methods

2.1. Materials

[1-^{14}C]Palmitic acid (2.06 GBq/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA); arachidonic acid was from Nu-Chek-Prep (Elysian, MN); bovine serum albumin (BSA), oleic, stearic, and palmitic acids, sphingomyelin (SM) from bovine brain, 1,2-dioleoyl-PE, 1,2-dipalmitoyl-phosphatidylcholine (PC), 1,2-dioleoyl-PC, 1,2-dioleoyl-phosphatidylserine (PS), and 1,2-dioleoyl-phosphatidic acid (PA) were from Sigma (St. Louis, MO); phosphatidylinositol (PI) from bovine liver and cardiolipin (CL) from heart were from Avanti Polar Lipids (Alabaster, AL); Dulbecco's modified Eagle's medium, LipofectAMINE, fetal calf serum, pcDNA3.1(+), and *E. coli* BL21 cells were from Invitrogen (Carlsbad, CA); [1-^{14}C]arachidonic acid (2.07 GBq/mmol), [1-^{14}C]oleic acid (2.22 GBq/mmol), [1-^{14}C]stearic acid (2.15 GBq/mmol), glutathione-Sepharose 4B, PreScission protease, and pGEX6P-1 were from Amersham Biosciences (Piscataway, NJ); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and *n*-octyl-β-D-glucoside were from Dojindo (Kumamoto, Japan); EDTA and 3(2)-*t*-butyl-4-hydroxyanisole (BHA) were from Wako Pure Chemical (Osaka, Japan); protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA); and precoated Silica Gel 60 F254 aluminum sheets for thin-layer chromatography (TLC) (20 × 20 cm, 0.2 mm thickness) were from Merck (Darmstadt, Germany). *N*-[^{14}C]Acyl-PEs were prepared from their corresponding 1-^{14}C-labeled fatty acids (arachidonic, oleic, stearic and palmitic acids) and 1,2-dioleoyl-PE according to the method of Schmid et al. (1983), and was purified by TLC with a mixture of chloroform/methanol/28% ammonium hydroxide (40:10:1, v/v).

2.2. Expression and purification of recombinant NAPE-PLD from E. coli

The expression and purification of recombinant rat NAPE-PLD in *E. coli* were performed as we described before (Wang et al., 2006). Briefly, a full-length rat NAPE-PLD cDNA was inserted into a pGEX6P-1 prokaryotic expression vector to generate a fusion protein of NAPE-PLD and glutathione *S*-transferase (GST). This fusion protein was expressed in *E. coli* BL21 cells together with the chaperone proteins GroEL and GroES. The expressed fusion protein was solubilized with 1% CHAPS, and was purified by glutathione-Sepharose 4B chromatography. The GST tag was cleaved by PreScission protease, followed by its removal using the second cycle of glutathione chromatography. The GST-free NAPE-PLD was further purified by hydroxyapatite chromatography. The purified enzyme was stored in the presence of 1% octyl glucoside at −80 °C until use. Protein concentration was determined by the method of Bradford (1976) with BSA as standard.

2.3. Expression of NAPE-PLD in COS-7 cells

COS-7 cells were grown at 37 °C to 70% confluency in a 100-mm dish containing Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum in a humidified 5% CO_2, 95% air incubator. The cells were then treated with 8 μg of the pcDNA3.1(+) plasmid harboring mouse NAPE-PLD cDNA (Okamoto et al., 2004) and LipofectAMINE, and cultured at 37 °C for 48 h, with one change of medium at 12 h. The harvested cells were sonicated 3 times each for 3 s in 20 mM Tris–HCl (pH 7.4) containing 5 mM EDTA (buffer A). The resultant homogenates were centrifuged at 105,000 × *g* at 4 °C for 15 min, and the obtained pellet was suspended in buffer A and centrifuged again at 105,000 × *g* at 4 °C for 15 min. Thus prepared pellet (membrane fraction) was suspended in 20 mM Tris–HCl (pH 7.4) and was subjected to enzyme assay. To examine the effect of membrane components on NAPE-PLD, membrane fractions were similarly prepared from control COS-7 cells. Membrane fractions were also prepared from the brain of C57BL/6 mice as described previously (Okamoto et al., 2004).

2.4. Enzyme assay

The activity of NAPE-PLD was assayed as previously reported (Wang et al., 2006). The enzyme was incubated with 25 μM *N*-[^{14}C]acyl-PEs (2500 cpm/2.5 nmol) in 100 μl of 50 mM Tris–HCl (pH 7.4) at 37 °C for 10 min. EDTA, $CaCl_2$, BSA, octyl glucoside, membrane fractions from COS-7 cells or mouse brain, and phospholipid were added in the reaction mix as indicated. The concentration of octyl glucoside derived from the stock solution of the purified NAPE-PLD did not exceed 0.01%. The enzyme reaction was terminated by the addition of 0.3 ml of a mixture of chloroform/methanol (2:1, v/v) containing 5 mM BHA. Thereafter, 100 μl of the organic phase was spotted on a silica gel thin-layer plate (10 cm height) and developed in chloroform/methanol/28% ammonium hydroxide (40:10:1, v/v) at 4 °C for 25 min. Distribution of radioactivity on the plate was quantified by a BAS1500 bioimaging analyzer (Fujix, Tokyo, Japan). Since the highly purified recombinant NAPE-PLD formed NAE as a sole product, the specific enzyme activity was calculated on the basis of the radioactivity of the produced [^{14}C]NAE and the remaining [^{14}C]NAPE. All the enzyme assays were performed in triplicate. K_m and V_{max} were determined according to the method of Lineweaver and Burk (1934). Lipids were extracted from the membrane fractions of control COS-7 cells and mouse brain by the method of Bligh and Dyer (1959). Phospholipid concentrations were then determined by the method of Rouser et al. (1966).