Chapter 13

Assay of NAPE-PLD Activity

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Abstract

N-acyl-phosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) is a prominent enzyme involved in the biosynthesis of fatty acid amides (FAAs), a family of bioactive lipids including anandamide (AEA) as the prototypical member. Here, we describe a NAPE-PLD assay based on radioactive substrates and product separation by thin-layer chromatography (TLC).

Key words *N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, Fatty acid amides, Anandamide, *N*-palmitoylethanolamine, *N*-acyl-phosphatidylethanolamines, Thin-layer chromatography, Radioactive assay

1 Introduction

N-acyl-phosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD; EC 3.1.4.4) belongs to the zinc metallo-β-lactamase protein fold family, has no homology with the classical PLDs, and is highly conserved from rodents (396 amino acids) to human (393 amino acids) [1]. It is responsible for the release of fatty acid amides (FAAs, also called *N*-acylethanolamines) from membrane phospholipids, and therefore its activity is very important in determining the in vivo concentrations of these important bioactive lipids. *N*-acyl-phosphatidylethanolamines (NAPE) represent the natural substrates of NAPE-PLD, so that the C20:4-NAPE (called NArPE) is the precursor of *N*-arachidonoylethanolamine (anandamide, AEA).

Changes in FAA levels have been documented under different physiological and pathological conditions [2]; thus it appears of paramount importance to better understand the contribution of NAPE-PLD activity to the regulation of the endogenous tone of these compounds (in particular of AEA).

The activity of NAPE-PLD is not so easy to measure, because it requires a radiochromatographic approach based on reversed-phase high-performance liquid chromatography (HPLC) coupled to online scintillation counting [3].

Mauro Maccarrone (ed.), *Endocannabinoid Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1412, DOI 10.1007/978-1-4939-3539-0_13, © Springer Science+Business Media New York 2016

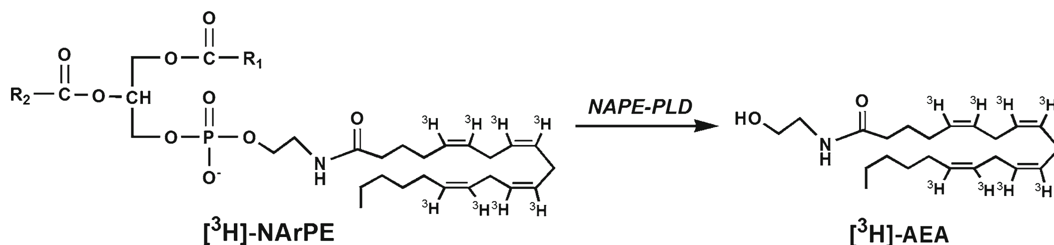

Fig. 1 NAPE-PLD activity using [^{3}H]-NArPE as a substrate

Here, we report a simple method that works well with both tissues and cell lines, and that is based on a radiolabeled NAPE. The latter leads to the release of radiolabeled FAA upon incubation with a biological extract that contains NAPE-PLD (Fig. 1). To separate the reaction products, we use a simple TLC that allows separation and quantitative determination of many samples at once.

2 Materials

Prepare all buffers and solutions by using ultrapure water and analytical grade reagents. Prepare and store all reagents at −20 °C (unless indicated otherwise).

2.1 Radiolabeled Components

1. Phosphatidylethanolamine, *N*-arachidonyl [arachidonyl-5,6,8,9,11,12,14,15-^{3}H(*N*)] ([^{3}H]-NArPE) (ARC, American Radiolabeled Chemicals, Inc, Saint Louis, MO, USA).
2. *N*-palmitoyl [1-^{14}C] phosphatidyl ethanolamine ([^{14}C]-NPPE) (ARC, American Radiolabeled Chemicals, Inc, Saint Louis, MO, USA).

2.2 Unlabeled Components

1. Buffer 1: Phosphate-buffered saline (PBS), 0.32 M sucrose. Make it fresh as required.
2. Buffer 2: 50 mM Tris–HCl, 0.1 % Triton, pH 7.4.
3. Chloroform/methanol (2:1, vol/vol).
4. Mobile phase: Methanol/chloroform/NH_4OH (85:15:1, vol/vol/vol).
5. Bromothymol blue solution (BBS): 0.1 % Bromothymol blue in 10 % aqueous ethanol, made just alkaline with NH_4OH (*see* **Note 1**).
6. *N*-arachidonoyl-phosphatidylethanolamine (NArPE) (*see* **Note 2**).
7. *N*-palmitoyl-phosphatidylethanolamine (NPPE) (*see* **Note 2**).
8. Anandamide (AEA) (*see* **Note 2**).

9. *N*-palmitoylethanolamine (PEA) (*see* **Note 2**).
10. URB597 (inhibitor of AEA-hydrolyzing enzyme fatty acid amide hydrolase).
11. Iodine (*see* **Note 3**).
12. Liquid scintillation cocktail (Ultima Gold™ XR).

2.3 Equipment

1. Aluminum TLC plates (20 × 20).
2. 2 mL Eppendorf microtubes.
3. TLC developing chamber.
4. Iodine vapor chamber.
5. Teflon-glass homogenizer.
6. Vortex mixer.
7. UltraTurrax T25.
8. Vacuum concentrator.
9. Centrifuge.
10. Thermoblock or water bath (37 °C).
11. Scintillation vials (20 mL).
12. Liquid scintillation β-counter (LCS).

3 Methods

All procedures need to be performed on ice, and all solutions should be prepared fresh just before processing biological samples.

3.1 Substrate Preparation

To prepare the substrate solution, it is necessary to add unlabeled (cold) NArPE or NPPE (*see* **Note 4**) to [^{3}H]-NArPE or [^{14}C]-NPPE, respectively, in order to bring the molar concentration up to the needed value (*see* **Note 5**). The final concentration is 100 μM/0.045 μCi (or 100,000 dpm, disintegrations per min; 1 μCi = 2.22×10^{6} dpm) for each experimental point (*see* **Note 5**).

3.2 Preparation of Tissue and Cell Samples

1. Homogenize tissue (fresh weight/volume ratio = 1/10, g/mL) (*see* **Note 6**) or cell samples (30×10^{6} cells/mL) with a glass/Teflon Potter homogenizer in buffer 1, precooled at 4 °C (*see* **Note 7**).
2. Centrifuge the homogenate at 1000 × *g* and 4 °C for 5 min.
3. Collect supernatant and determine protein concentration with any commercially available assay (i.e., Bradford colorimetric assay).

3.3 Enzyme Assay

NAPE-PLD assay can be performed by using [^{3}H]-NArPE or [^{14}C]-NPPE as substrate, and by separating the corresponding

products (AEA or PEA, respectively) under the same experimental conditions.

1. In 2 mL microtubes (*see* **Note 8**), preincubate for 10 min tissue or cell homogenates (*see* **Note 9**) with 0.1 nM URB597 (*see* **Note 10**), in a final volume of 200 μL of pre-warmed buffer 2.
2. Add [^{3}H]-NArPE or [^{14}C]-NPPE (each at the final concentration of 100 μM), to start the reaction.
3. Incubate for 30 min at 37 °C.
4. Stop the reaction by adding 600 μL of ice-cold chloroform/methanol, and shake.
5. Centrifuge the mixture at 3000 × *g* for 5 min (*see* **Note 11**).
6. Remove the upper aqueous layer by suction, and dry the lower organic phase (*see* **Note 12**).

3.4 TLC

1. Put 100 mL of mobile phase in TLC developing chamber (*see* **Note 13**).
2. Draw the TLC (*see* **Note 14** and Fig. 2a).
3. Load standards (AEA and NArPE, or PEA and NPPE), at the origin of the TLC (*see* **Note 15** and Fig. 2a).
4. Dissolve the dried lipids into 30 μL of chloroform/methanol, shake, centrifuge, and load on the TLC to the corresponding line (Fig. 2a).
5. Repeat **step 4** with additional 25 μL of chloroform/methanol.
6. Wait until the solvent is dried out, and put the TLC in the developing chamber.
7. Remove the TLC from the chamber when the solvent reaches approximately 1 cm below the top (Fig. 2b), and immediately draw with a pencil a line across the solvent front.
8. Once dried out, develop the TLC with BBS (*see* **Note 16**), and immediately circle the standards with a pencil (Fig. 2b).
9. Draw and cut out the TLC (*see* **Note 17** and Fig. 2c).
10. Place the clippings in the corresponding scintillation vials, and add 3 mL of methanol and 15 mL of scintillation cocktail (*see* **Note 18**).
11. Measure radioactivity in a liquid scintillation counter (*see* **Note 19**).

4 Notes

1. BBS is a stain used for the detection of lipids and phospholipids. When sprayed on the dried plate, (phospho)lipids give blue-green colors.
2. To make stock solutions (10 mM AEA, 2 mM NArPE, and 5 mM NPPE), aliquot them (10–50 μL) and store at −20 °C.

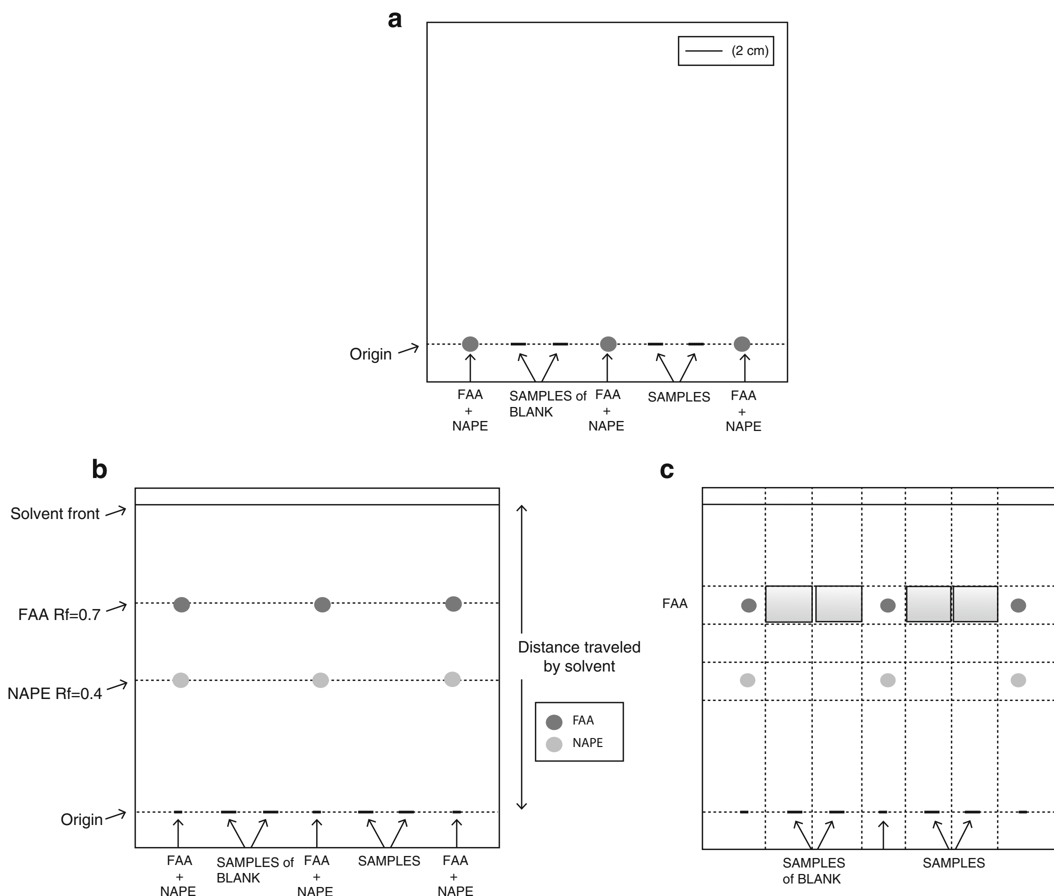

Fig. 2 Scheme of TLC preparation (**a**), standards and samples loading at baseline (**b**), TLC after the run and Rf corresponding to NAPE and FAA standards (**c**)

3. Iodine vapor is a universal and (relatively) unspecific reagent for many organic compounds. Put some crystals of iodine into the chamber, and then place the developed, dried chromatogram in iodine vapor. Spots will turn tan-brown in color.
4. To evaluate NAPE-PLD activity, it is possible to use either NArPE or NPPE as substrates, but in tissues or cell lines that express low amounts of enzyme it is better to use the latter compound. Indeed, NAPE-PLD prefers *N*-palmitoyl over *N*-arachidonoyl moiety as a substrate [5].
5. The "appropriate" concentration should be obtained from Michaelis–Menten kinetics. In our procedure, 100 μM NArPE (or NAPPE) works well [1, 5]. It should be stressed that radioactive compounds usually have high specific activity, and yet they are at very low concentration. Since, for enzymatic assay, higher substrate concentrations are required, it is necessary to add "cold" compound to prepare an appropriate substrate solution. Therefore, in order to obtain 100 μM NArPE (or NPPE)

in 0.2 mL, 0.02 μmol NArPE (or NPPE) is needed for each experimental point. Of course, addition of cold compound will change the specific activity; therefore, the specific activity of substrate solution has to be recalculated. To estimate the corrected specific activity, you have to make a ratio between the total amount of radioactive compound and the total amount of cold compound added in solution. An example of the calculation of [^{3}H]-NArPE molar concentration follows.

Radioactive concentration (r.c.): 1 Ci/L (indicated by the supplier).

Specific activity (s.a.): 200 Ci/mmol (indicated by the supplier).

[3H-NArPE] = r.c./s.a. = 1 Ci/L/200 Ci/mmol = 0.005 mM.

An example of substrate solution preparation for ten experimental points follows.

Total volume of substrate solution: 2.5 μL × 10 = 25 μL – volume of cold and radiolabeled substrate.

Total amount of NArPE moles: 0.02 × 10 = 0.2 μmol.

Total amount of radiolabeled [^{3}H]-NArPE: 0.045 × 10 = 0.45 μCi. Calculation of specific activity of the substrate solution: 0.45 μCi/0.2 μmol = 2.25 μCi/μmol.

6. With tissues and other fibrous materials, it is more appropriate to use UltraTurrax T 25 to facilitate homogenization before using the glass/Teflon Potter homogenizer. An alternative procedure to prepare cell homogenates is via a Vibracell sonifier, which provides three cycles of sonication for 10 s each, interspersed by 10s breaks.
7. The best results are obtained with fresh cells and tissues.
8. Prepare also 2 mL microtubes with the same buffer used for the samples, but without proteins (blank). Prepare a suitable number of blanks, based on the number of TLC that you have to load.
9. For unknown biological samples it is appropriate to conduct a dose-response curve. The effect of protein content on NAPE-PLD assay in mouse brain is shown in Fig. 3.
10. URB597 is an inhibitor of fatty acid amide hydrolase (FAAH), a major FAAs-hydrolyzing enzyme [4]. Addition of this compound is important to prevent hydrolysis of FAAs generated by NAPE-PLD.
11. The centrifuge must be programmed in order to exclude the brake.
12. Use a vacuum concentrator for 30 min at 37 °C, or a nitrogen flow.
13. The chamber should contain enough solvent to simply cover the bottom.

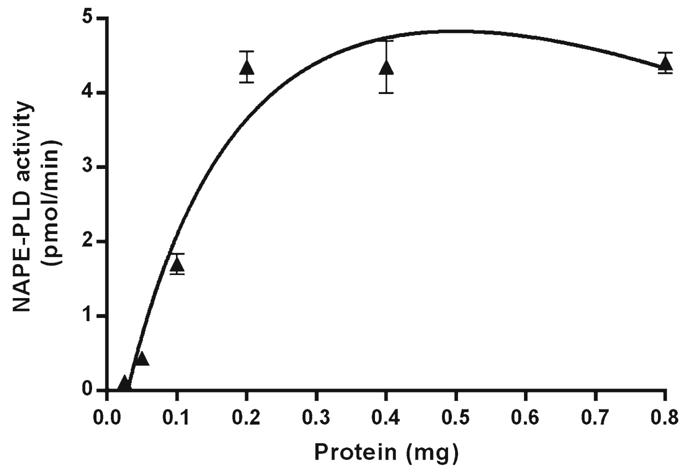

Fig. 3 Dependence of NAPE-PLD activity on mouse brain protein concentration

14. Use a pencil (never use a pen!), and leave at least 2 cm from the bottom and side edges.
15. Make sure that a sufficient amount of standard is spotted on the plate (20–40 nmol for each compound).
16. For NArPE and AEA, but not for NPPE and PEA, an alternative way for visualization can be the use of iodine vapor.
17. Count only the boxes that correspond to FAA spots (squares in gray in Fig. 2c).
18. The optimal methanol/scintillation cocktail ratio depends on the characteristics of the scintillation liquid (i.e., ULTIMA Gold XR is a liquid scintillation cocktail with very high sample load capacity and an extended range of sample holding capacity, up to 50 %).
19. To convert cpm (counts per min) into dpm, you need to know the efficiency of your β-counter for the given radioisotope ($[^3H]$ or $[^{14}C]$), subtract the average of the blanks from each sample and transform dpm to moles based on the substrate-specific activity. To calculate NAPE-PLD-specific activity, divide the moles of product by the reaction time (min) and the protein content of the reaction mixture (mg of protein), as moles/(min × mg of protein).

    Example:

    dpm obtained – dpm average blanks:
    $3000 \text{ dpm} = 9.09 \times 10^{-4}\ \mu\text{Ci}\ (1\ \mu\text{Ci} = 2.22 \times 10^{6} \text{ dpm})$.
    μmol formed: $9.09 \times 10^{-4}\ \mu\text{Ci}/2.25\ \mu\text{Ci}/\mu\text{mol} = 4.04 \times 10^{-4}\ \mu\text{mol}$.
    NAPE-PLD-specific activity $= 4.04 \times 10^{-4}\ \mu\text{mol}/30 \text{ min} \times 0.1 \text{ mg} = 1.34 \times 10^{-4}\ \mu\text{mol}/(\text{min} \times \text{mg of protein})$.

Acknowledgements

This study was supported by the Italian Ministry of Education, University and Research (Ministero dell'Istruzione, dell'Università e della Ricerca, grant PRIN 2010–2011 to M.M., and grant PRIN 2012 to F.F.).

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