

# A rapid phospholipase D assay using zirconium precipitation of anionic substrate phospholipids: application to N-acylethanolamine formation in vitro

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**Abstract** Activation of phospholipase D (PLD) is involved in a number of signal transduction pathways in eukaryotic cells. The most common method for determination of PLD activity in vitro involves incubation with a radiolabeled substrate and lipid extraction followed by thin-layer chromatography in order to separate and quantify substrate and product(s). A more rapid assay can be used when utilizing phosphatidylcholine as a substrate because one of the products, choline, is water soluble and therefore easily separated from the substrate. However, this separation principle is not applicable in evaluating N-acylphosphatidylethanolamine (NAPE)-hydrolyzing PLD activity, which produces two lipophilic products, N-acylethanolamine (NAE) and phosphatidic acid. Therefore, we developed a rapid assay for the routine detection of NAPE-hydrolyzing PLD activity. This assay is based on precipitation of radiolabeled substrate (NAPE) in the presence of ZrOCl<sub>2</sub>, followed by quantification of radiolabeled NAE released into a methanolic supernatant. The precipitation involves a chemical reaction of the zirconyl cation with the phosphate anion. Conditions were optimized for the complete precipitation of NAPE, whereas N-acylsphosphatidylethanolamine and glycerophospho(N-acyl)ethanolamine were precipitated at least 95%. Furthermore, this precipitation method can be extended to assays of other anionic phospholipid-hydrolyzing PLD activities by selecting an optimal pH of the precipitation solution. For example, 98–99% precipitation of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine was achieved. Consequently, this new assay allows for a convenient examination of PLD activities toward a variety of phospholipid substrates, and in particular allows for the analysis of NAE formation from NAPE in vitro, a feature that will facilitate a more complete biochemical characterization of this anandamide-generating enzyme.—Petersen, G., K. D. Chapman, and H. S. Hansen. A rapid phospholipase D assay using zirconium precipitation of anionic substrate phospholipids: application to N-acylethanolamine formation in vitro. *J. Lipid Res.* 2000. 41: 1532–1538.

**Supplementary key words** N-acylphosphatidylethanolamine • N-acylethanolamine • anandamide • N-acylsphosphatidylethanolamine • glycerophospho(N-acyl)ethanolamine • phosphatidylethanolamine • phosphatidylglycerol • phosphatidylserine • zirconyl chloride • zirconium oxide chloride

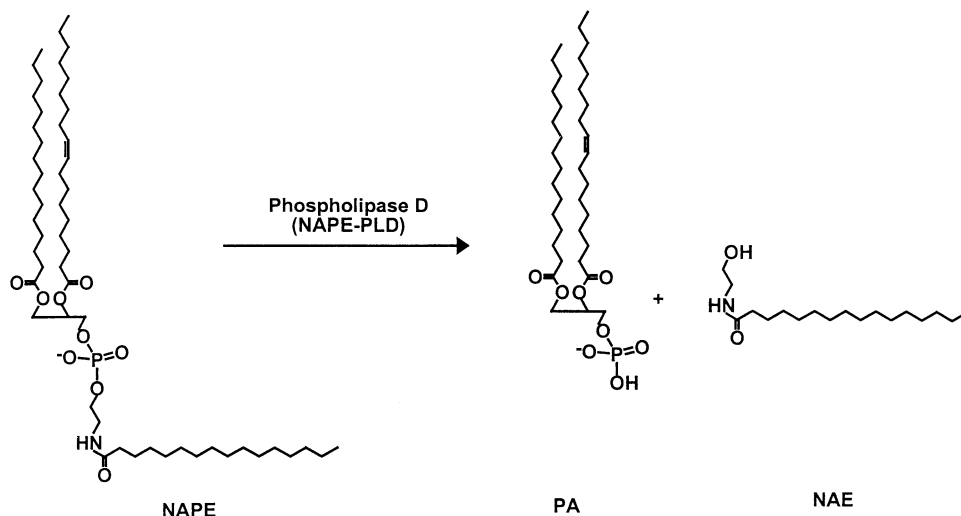
Phospholipase D (PLD) activity has been detected in bacterial (1), plant (2, 3), yeast (4), and animal cells (5–11). Different PLDs can catalyze the hydrolysis of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (lysoPC), sphingomyelin, phosphatidylglycerol (PG), or N-acylsphosphatidylethanolamine (NAPE; Fig. 1), generating phosphatidic acid (PA) and a free head group of the phospholipid substrate. Mammalian PLD1 (5) and PLD2 (6), which are both PC specific, have been cloned. A PC-specific oleate-stimulated PLD has been partially characterized (7) and one group has suggested that it is identical to PLD2 (8). In addition, a PI-specific PLD (9), a mitochondrial PLD hydrolyzing PE (10), and an NAPE-hydrolyzing PLD (NAPE-PLD) (11) have been identified in various mammalian tissues. These PLD enzymes may fulfill several different roles in cells, for example, transmembrane signaling, vesicle trafficking, and modulation of cytoskeletal dynamics (12).

NAPE-PLD plays an important role in the formation of various species of N-acylethanolamine (NAE), which are potent lipid mediators, for example, N-arachidonylethanolamine (anandamide), involved in regulating a variety of cellular processes (13–15). In addition, NAE accumulation is associated with cellular injury. It has been suggested that NAE generation may serve a cytoprotective role (16, 17).

Mammalian NAPE-PLD has been characterized in rat heart and rat brain microsomes (11, 18). The enzyme has

Abbreviations: BTP, bis-Tris propane; C:M, chloroform–methanol; DTT, dithiothreitol; LSC, liquid scintillation counting; NAE, N-acylethanolamine; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, NAPE-hydrolyzing PLD; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLD, phospholipase D; PS, phosphatidylserine; TLC, thin-layer chromatography.

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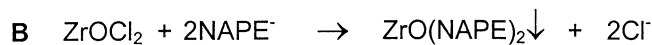
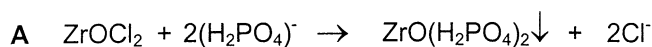


**Fig. 1.** Formation of *N*-acylethanolamine (NAE) and phosphatidic acid (PA) from *N*-acylphosphatidylethanolamine (NAPE) catalyzed by the enzyme *N*-acylhydrolyzing phospholipase D (NAPE-PLD).

not been purified or cloned. The simple PC-specific PLD assay using measurements of radiolabeled release of choline into the aqueous supernatant (19, 20) cannot be transferred to the NAPE-PLD assay because of the lipophilic head group product, NAE, of this reaction. The purpose of the current study was to develop a simple and rapid PLD assay that can be used for detection of NAPE-PLD activity during purification and biochemical characterization of the enzyme. The principle of the described NAPE-PLD assay is based on a chemical reaction of the zirconyl ( $ZrO^{2+}$ ) ion with the phosphate group of NAPE (**Fig. 2**).

Tetravalent zirconium forms insoluble salts with phosphates even in strongly acidic solutions (21). Addition of the inorganic precipitating agent  $(NH_4)_2HPO_4$  to a solution of zirconium results in precipitated  $ZrO(H_2PO_4)_2$  (22), like in Fig. 2A. Oxy acids of sulfur and phosphorus react with zirconium salts in aqueous solution, and are bonded to the zirconium atom through oxygen. Both water-soluble and water-insoluble organic compounds of this kind are known (23). Organic acids as a class are more selective for zirconium precipitation than organic bases. In most cases, however, a precipitate of indefinite composition separates out (22). The reaction scheme in Fig. 2B is therefore considered a simplified model of the actual reaction complex.

The new PLD assay developed here was compared with conventional measurements of PLD-mediated NAPE hydrolysis in various mammalian microsomal membrane preparations and was found to be reliable, sensitive, and



**Fig. 2.** Tetravalent zirconium forms insoluble salts with phosphates. Precipitation of phosphate (A) and NAPE (B) by zirconyl chloride.

more rapid. This new assay could be adapted to study the hydrolysis of other anionic phospholipids and so provide a novel means to determine class and molecular species preferences for PLD enzymes *in vitro*.

## MATERIALS AND METHODS

### Materials

[1- $^{14}C$ ]Palmitic acid (55 mCi/mmol) and 1,2-di[1'- $^{14}C$ ]decanoyl-*sn*-glycero-3-phosphocholine (67 mCi/mmol) were from Amersham Pharmacia Biotech (Amersham, UK). 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, palmitic acid, bis-Tris propane (BTP), phospholipase  $A_2$  ( $PLA_2$ ) from bovine pancreas, PLD from *Streptomyces* species, and zirconyl chloride were from Sigma (St. Louis, MO). Zirconium oxide chloride (zirconyl chloride) was also purchased from Riedel-de Haën (Seelze, Germany). Silica gel 60 thin-layer chromatography (TLC) plates were from either Merck (Darmstadt, Germany) or Fisher Scientific (Fairlawn, NJ). Three-milliliter  $C_{18}$  end-capped SPE columns (200 mg sorbent mass) were from International Sorbent Technology (Mid Glamorgan, UK).

### Synthesis of *N*-acylphospholipids

1,2-Dilauroyl-*sn*-glycero-3-phospho(*N*-[1'- $^{14}C$ ]palmitoyl)ethanolamine (NAPE) was prepared essentially as described by Schmid et al. (11) with a specific activity of 5,000 dpm/nmol. The product was purified by TLC ( $R_f = 0.31$ ), developed in chloroform-methanol-ammonium hydroxide 80:20:2 (v/v/v).

1-Lauroyl-*sn*-glycero-3-phospho(*N*-[1'- $^{14}C$ ]palmitoyl)ethanolamine (lysoNAPE) was prepared by adding 60 mM BTP, 2 mM 1,4-dithiothreitol (DTT), Triton X-100 (0.4 mg/ml), pH 8.0, to 2 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1'- $^{14}C$ ]palmitoyl)ethanolamine and sonicating the mixture for 10 min before incubation with 2 units of phospholipase  $A_2$  for 24 h in a total volume of 200  $\mu$ l. The reaction was terminated by adding 1.5 ml of ice-cold chloroform-methanol 2:1 (v/v) or 1 ml of ice-cold methanol. LysoNAPE was detected by TLC ( $R_f = 0.14$ ) after development in chloroform-methanol-ammonium hydroxide 80:20:2 (v/v/v) or by liquid scintillation counting (LSC) after zirconium precipitation.

*sn*-Glycero-3-phospho(*N*-[1-<sup>14</sup>C]palmitoyl)ethanolamine (GP-NAE) was prepared by alkaline hydrolysis of 400 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1-<sup>14</sup>C]palmitoyl) ethanolamine in 1 ml of chloroform–methanol 1:1 (v/v) and 200 μl of 0.35 M methanolic NaOH for 4 h. Addition of 80 μl of 10% (v/v) formic acid in methanol terminated the incubation. GP-NAE was purified by the use of a C<sub>18</sub> end-capped SPE column (200 mg sorbent mass) mounted on a vacuum system. The column was activated with 10 ml of methanol and equilibrated with 10 ml of methanol–water 1:9 (v/v) before application of the sample in 2 ml of methanol–water 1:9 (v/v). GP-NAE was eluted with 10 ml of methanol after the column had been washed with 10 ml of water. The product was purified by TLC (*R*<sub>f</sub> = 0.01), developed in chloroform–methanol–ammonium hydroxide 80:20:2 (v/v/v).

### Synthesis of phospholipids by transphosphatidylolation

The transesterification capacity of PLD from *Streptomyces* species (24, 25) was utilized to obtain radiolabeled PE, PG, and PS from radiolabeled PC. To 190 μl of 0.1 M sodium acetate buffer, pH 5.6, containing 0.1 M CaCl<sub>2</sub> and a 1 M concentration of the appropriate alcohol (ethanolamine, glycerol, or L-serine) was added 2 units of PLD from *Streptomyces* species in 10 and 100 μl of diethyl ether containing 2 μmol of 1,2-di[1-<sup>14</sup>C]decanoyl-*sn*-glycero-3-phosphocholine (5,000 dpm/nmol). The incubation was carried out for 60 min at 30°C and was terminated by addition of 1.5 ml of ice-cold chloroform–methanol 2:1 (v/v). The resulting 1,2-di[1-<sup>14</sup>C]decanoyl-*sn*-glycero-3-phosphoethanolamine (*R*<sub>f</sub> = 0.61), 1,2-di[1-<sup>14</sup>C]decanoyl-*sn*-glycero-3-phosphoglycerol (*R*<sub>f</sub> = 0.63), and 1,2-di[1-<sup>14</sup>C]decanoyl-*sn*-glycero-3-phosphoserine (*R*<sub>f</sub> = 0.34) were purified by TLC, developed in chloroform–methanol–acetic acid–water 50:30:8:4 (v/v/v/v).

### Subcellular fractionation

Hearts from 13- to 14-week-old Sprague-Dawley rats and bovine organs obtained from a local slaughterhouse (in Texas) were homogenized with an UltraTurrax<sup>®</sup> or a Polytron<sup>®</sup> in 10 volumes of 25 mM Tris-HCl buffer, 0.25 M sucrose, 2 mM EDTA, pH 7.3. Microsomal fractions were prepared from the 1,000 *g* supernatant by centrifugation at 109,000 *g*. The resultant pellet (microsomes) was resuspended in 60 mM BTP, 2 mM DTT, pH 7.0 or 8.0 at a concentration of 10–20 mg of protein/ml. Protein was determined by the method of Bradford (26).

### Assay of PLD activity

The substrate, NAPE, was dried under a stream of N<sub>2</sub> and resuspended in 60 mM BTP, 2 mM DTT, Triton X-100 (0.4 mg/ml) pH 8.0. A final concentration of 10 mM PMSF was added in 5 μl of DMSO after 2–10 min of sonication. Microsomal protein (200 μg) was added to a final volume of 200 μl. Incubations were carried out at 37°C. The reactions were terminated by adding 1.5 ml of ice-cold chloroform–methanol 2:1 (v/v) or 1.0 ml of ice-cold methanol.

### Precipitation of phospholipids

After termination of the PLD assay by the addition of 1.0 ml of ice-cold methanol, 100 μl of 0.2 M ZrOCl<sub>2</sub>–0.1 M NaOH and 150 μl of 0.1 M NaH<sub>2</sub>PO<sub>4</sub> were added to the mixture, unless otherwise stated; the mixture was then vortexed thoroughly (5 sec) and precipitated for 15 min, followed by a 15-min centrifugation at 1,000 *g*. Supernatant (700 μl) was counted by LSC or evaporated to dryness under a stream of N<sub>2</sub> for TLC detection.

### TLC detection of the NAPE-PLD-catalyzed reaction

The lipids were extracted from the lower phase of samples terminated with chloroform–methanol and reextracted with 1 ml

of chloroform. From samples precipitated with zirconium, 700 μl of the supernatant was evaporated to dryness and redissolved in 50 μl of chloroform–methanol 19:1 (v/v). Separation of the lipids was carried out using TLC, developed 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) or by a radiometric scanner (System 200 Imaging Scanner; Bioscan, Washington, D.C.).

## RESULTS AND DISCUSSION

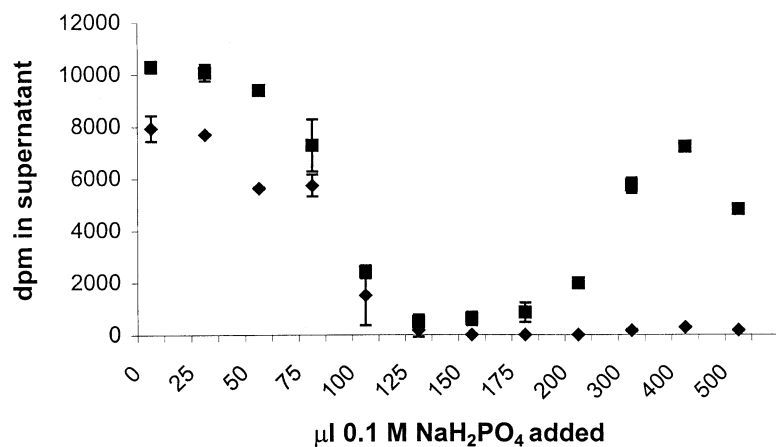
### Preliminary observations

It was important to control the pH of the precipitation mixture both because NAPE at low pH (<1.5) is hydrolyzed and because NAPE must be negatively charged in order to precipitate with zirconium. A solution of zirconyl chloride has a pH about equal to that of HCl of the same molarity (27). Addition of 5–20 μmol of zirconyl chloride to 2 nmol of NAPE in 200 μl of assay buffer and 1.0 ml of methanol resulted in a pH from 1.9 to 2.4. Application of 700 μl of the supernatant on a TLC plate resulted in detection of NAPE. The reason for the lack of precipitation of NAPE is most likely that the p*K*<sub>a</sub> of this phospholipid is above pH 2.4. NAPE molecules are therefore neutral at this acidic pH and cannot be precipitated by zirconium.

Various amounts of NaOH were added to the precipitation mixture either directly or in solution along with zirconyl chloride in order to obtain a pH above the p*K*<sub>a</sub> of NAPE. Addition of 100 μl of 0.2 M ZrOCl<sub>2</sub>–0.1 M NaOH was found to be the optimal combination. This solution takes 4 days to become clear. Addition of more concentrated NaOH to 0.2 M ZrOCl<sub>2</sub> resulted in solutions that stayed milky white. To test the system, rat heart microsomes were incubated for 1 h with 2 nmol of NAPE in 200 μl of assay buffer before adding 1.0 ml of methanol and 100 μl of 0.2 M ZrOCl<sub>2</sub>–0.1 M NaOH. TLC fractionation of 700 μl of the resultant supernatant revealed no NAPE whereas NAE was readily detectable. The pH of the final mixture was approximately 5 on addition of 100 μl of 0.2 M ZrOCl<sub>2</sub>–0.1 M NaOH. Therefore, NAPE presumably has a p*K*<sub>a</sub> above 2.4 and below 5. In comparison, phosphatidylethanol was reported to have a p*K*<sub>a</sub> of 1.43 whereas other phosphodiester have p*K*<sub>a</sub> values in the range of 1–2 (28).

### Addition of a carrier

A phosphate carrier was added to assays to promote a complete and reproducible precipitation of NAPE in the presence of ZrOCl<sub>2</sub>. This was noted to improve the yield of zirconium phosphate precipitation (29). NaH<sub>2</sub>PO<sub>4</sub> was added in various amounts (Fig. 3) and did not influence the pH of the final solution of the mixture. ZrOCl<sub>2</sub> (20 μmol) was added in these experiments. Addition of 12.5–15 μmol of NaH<sub>2</sub>PO<sub>4</sub> resulted in at least 94% precipitation. A possible explanation for the increasing level of radioactivity in the supernatant when adding more than 15 μmol of NaH<sub>2</sub>PO<sub>4</sub> (Fig. 3) is that the phosphate displaces binding of the radiolabeled NAPE to zirconium. Complete



**Fig. 3.** Carrier phosphate and protein improve zirconium precipitation of NAPE. To a solution consisting of 2 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1'-<sup>14</sup>C]palmitoyl)ethanolamine (NAPE; 5,000 dpm/nmol) in 200  $\mu$ l of 60 mM BTP, 2 mM DTT, Triton X-100 (0.4 mg/ml) pH 8.0, 10 mM PMSF, and 5  $\mu$ l of DMSO, with 200  $\mu$ g of microsomal protein (solid diamonds) or without (solid squares), were added (without incubation) 1.0 ml of methanol and 100  $\mu$ l of 0.2 M  $ZrOCl_2 \cdot 0.1$  M NaOH. To the mixture was then added increasing amounts of 0.1 M  $NaH_2PO_4$ . Supernatant (700  $\mu$ l) was counted by LSC after 15 min of centrifugation at 1,000 *g*. The results are depicted as disintegrations per minute in the total volume of the mixtures. Data represent means  $\pm$  SEM of one experiment performed in duplicate. The results shown are representative of three independent experiments.

precipitation was reproducibly obtained by addition of 200  $\mu$ g of microsomal protein and a minimum of 15–20  $\mu$ mol of  $NaH_2PO_4$ . Only 10% of NAPE was precipitated under the same conditions without addition of zirconium chloride.

#### Correlation between TLC and LSC

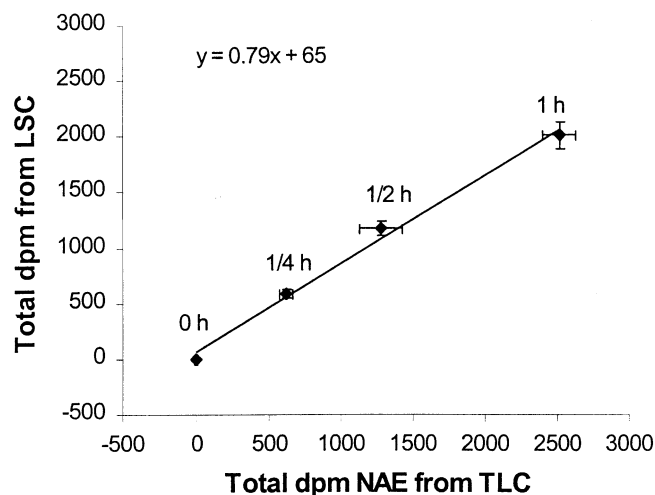
To replace TLC detection of NAPE-PLD activity with detection by LSC, comparative experiments were carried out using both methods (Fig. 4; Table 1). A time course of measurements of NAPE-PLD-catalyzed formation of NAE from NAPE was carried out (Fig. 4). There was an excel-

lent correlation between assays to quantify NAPE hydrolysis by TLC or by LSC (linear regression,  $y = 0.79x + 65$ ;  $r^2 = 0.994$ ), indicating that the TLC method can be substituted by the LSC method in the determination of NAPE-PLD activity.

Figure 5 shows profiles of radiolabeled lipids in assay samples extracted with chloroform–methanol (total lipid) compared with the methanolic supernatant from samples precipitated with zirconium. This verifies that the radioactivity detected by LSC is indeed NAE, while NAPE has been completely precipitated.

#### Specificity of the zirconium assay

In microsomal membranes, NAE can be further degraded to ethanolamine and free fatty acid by a fatty acid amidohydrolase that hydrolyzes acylamides and monoacyl esters (30). Hydrolysis of the newly generated radio-

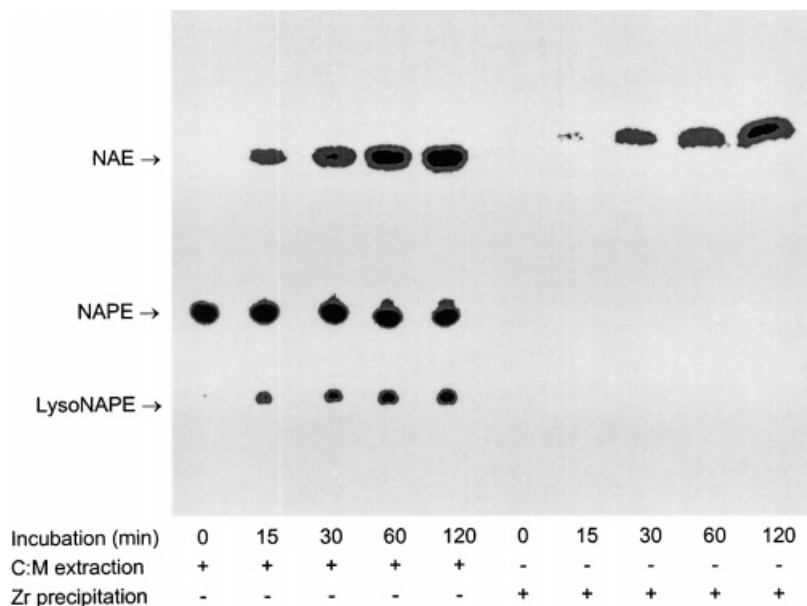


**Fig. 4.** Correlation of NAPE-PLD activity detected by TLC and LSC. Rat heart microsomal fraction (200  $\mu$ g) was incubated for 0, 15, 30, and 60 min at 37°C with 2 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1'-<sup>14</sup>C]palmitoyl)ethanolamine (NAPE; 5,000 dpm/nmol) in a total volume of 200  $\mu$ l of 60 mM BTP, 2 mM DTT, Triton X-100 (0.4 mg/ml) pH 8.0, 10 mM PMSF, and 5  $\mu$ l of DMSO. Either the reactions were terminated by the addition of 1.5 ml of chloroform–methanol 2:1 (v/v) followed by extraction of the organic layer for detection by TLC, or the reactions were terminated by the addition of 1.0 ml of methanol, and 100  $\mu$ l of 0.2 M  $ZrOCl_2 \cdot 0.1$  M NaOH and 150  $\mu$ l of 0.1 M  $NaH_2PO_4$  were added for precipitation of the anionic phospholipids followed by LSC of 700  $\mu$ l of the supernatant. Data represent means  $\pm$  SEM of one experiment performed in triplicate. The results shown are representative of three independent experiments.

**TABLE 1.** Comparison of NAPE-PLD activity detected by TLC and LSC among microsomes prepared from different bovine organs

Bovine Organ (Microsomes)	TLC Results	LSC Results
	<i>pmol NAE/min/mg protein</i>	
Brain	12.3	14.6
Kidney	7.0	6.0
Spleen	6.3	7.1
Lung	4.6	7.2
Heart	3.0	2.1
Liver	2.7	2.2

200 micrograms of microsomal fraction from various cow tissues was incubated in duplicate (TLC samples) or triplicate (LSC samples) for 0, 15, 30, 60, and 120 min at 37°C with 2 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1'-<sup>14</sup>C]palmitoyl)ethanolamine (NAPE; 5,000 dpm/nmol) in a total volume of 200  $\mu$ l of 60 mM BTP, 2 mM DTT, Triton X-100 (0.4 mg/ml), pH 8.0, 10 mM PMSF, and 5  $\mu$ l of DMSO. In one experiment the reactions were terminated by the addition of 1.5 ml of chloroform–methanol 2:1 (v/v), and the organic layer was extracted and evaporated under  $N_2$ , and the samples were applied to TLC and developed in chloroform–methanol–ammonium hydroxide 80:20:2 (v/v/v). In another experiment reactions were terminated by the addition of 1.0 ml of methanol, and 100  $\mu$ l of 0.2 M  $ZrOCl_2 \cdot 0.1$  M NaOH and 125  $\mu$ l of 0.1 M  $NaH_2PO_4$  were added for precipitation of the anionic phospholipids followed by LSC of 700  $\mu$ l of the supernatant.



**Fig. 5.** Comparison of NAPE-PLD activity between samples extracted with chloroform–methanol 2:1 (v/v) and samples precipitated by zirconium depicted as a radioactivity scan of a TLC plate. Rat heart microsomal fraction (200  $\mu$ g) was incubated as described in the legend to Fig. 4. Either the reactions were terminated by the addition of 1.5 ml of chloroform–methanol 2:1 (v/v) and the organic layer was extracted and evaporated under  $N_2$  (C:M extraction), or the reactions were terminated by the addition of 1.0 ml of methanol and 100  $\mu$ l of 0.2 M  $ZrOCl_2$ –0.1 M NaOH and 150  $\mu$ l of 0.1 M  $NaH_2PO_4$  were added for precipitation of the anionic phospholipids followed by evaporation under  $N_2$  of 700  $\mu$ l of the supernatant (Zr precipitation). All samples were applied to TLC and developed in chloroform–methanol–ammonium hydroxide 80:20:2 (v/v/v). The results shown are representative of three independent experiments, each performed in triplicate.

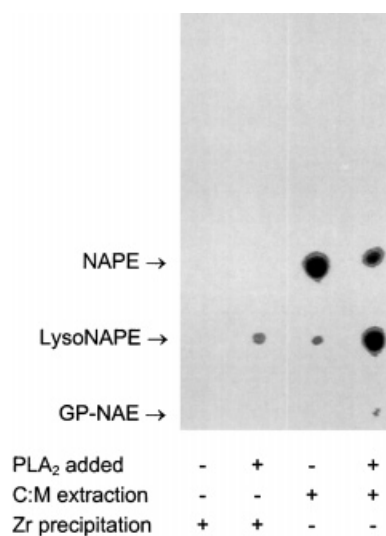
labeled NAPE by a fatty acid amidohydrolase will result in ethanolamine and radiolabeled free fatty acid. This will not influence the detection of NAPE-PLD activity in the zirconium assay because free fatty acids will not be precipitated by zirconium. However, this reaction can be prevented if desired by addition of PMSF or methyl arachidonyl fluorophosphate to the reaction mixture (18), which are routinely used in the TLC-based assay system.

Theoretically, the compound phosphoryl-NAE could be formed by a phospholipase C-type catalyzed reaction using NAPE as substrate. Phosphoryl-NAE has, however, never been found in vivo or in cell culture systems. In addition, Schmid et al. (11) have shown that NAPE is not degraded by a phospholipase C-type enzyme in tissue homogenates. If such a phospholipase C enzyme should exist in some special tissues the generated phosphoryl-NAE would probably be hydrolyzed quickly (31) and thus result in overestimation of NAPE-PLD activity independent of whether phosphoryl-NAE is precipitated by zirconium. This compound has therefore not been tested in the zirconium assay.

LysoNAPE and GP-NAE can be formed if radiolabeled NAPE is hydrolyzed by PLA<sub>2</sub> in the microsomal fraction. It was therefore tested whether these metabolites could be precipitated by zirconium or whether they would cause a false-positive result for NAPE-PLD activity by being detected by LSC after the precipitation procedure. LysoNAPE was generated by PLA<sub>2</sub> treatment of radiolabeled NAPE. The precipitation procedure was carried out with the resulting mixture of NAPE and lysoNAPE. In addition to complete precipitation of NAPE, more than 95% lysoNAPE was precipitated by zirconium (Fig. 6). Detection by LSC confirmed the results of TLC detection. In another set of experiments precipitation of GP-NAE was found to be precipitated more than 95% when 2 nmol of NAPE was replaced by 2 nmol of radiolabeled GP-NAE and precipitated without incubation (data not shown).

Formation of GP-NAE and lysoNAPE could therefore

potentially cause a small false-positive result when using zirconium precipitation and LSC for detection of NAPE-PLD activity. In many cases, however, formation of these by-products will contribute only insignificantly to the



**Fig. 6.** Efficiency of zirconium precipitation of lysoNAPE. TLC detection of lysoNAPE formed by 24 h of PLA<sub>2</sub> treatment (2 units) of 2 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1'-<sup>14</sup>C]palmitoyl)ethanolamine (NAPE; 5,000 dpm/nmol) in a total volume of 200  $\mu$ l of 60 mM BTP, 2 mM DTT, and Triton X-100 (0.4 mg/ml) pH 8.0, incubated at 37°C. Either the reactions were terminated by the addition of 1.0 ml of methanol and 100  $\mu$ l of 0.2 M  $ZrOCl_2$ –0.1 M NaOH and 150  $\mu$ l of 0.1 M  $NaH_2PO_4$  were added for precipitation of the anionic phospholipids followed by evaporation under  $N_2$  of 700  $\mu$ l of the supernatant (Zr precipitation), or the reactions were terminated by the addition of 1.5 ml of chloroform–methanol 2:1 (v/v) and the organic layer was extracted and evaporated under  $N_2$  (C:M extraction). All samples were applied to TLC, developed in chloroform–methanol–ammonium hydroxide 80:20:2 (v/v/v). Data are depicted as a radioactivity scan of a TLC plate. The results shown are representative of two independent experiments, each performed in duplicate.

amount of radioactivity detected by LSC. For example, in Fig. 5, 6.2% lysoNAPE was formed after 1 h. This contributed less than 1% to the amount of radioactivity detected in these samples by LSC. The incomplete precipitation of these *N*-acylphospholipids is probably due to the hydroxyl groups. Therefore, when the enzyme source is impure or not well characterized, selected samples should be run on a TLC plate in order to verify detection of NAPE-PLD activity by LSC. Even with this limit to its use, the zirconium assay is a valuable tool for screening inhibitors of NAPE-PLD activity as well as for purification purposes. The procedure has been applied successfully to screening of NAPE-PLD activity in various bovine tissues (Table 1).

#### General applicability of the zirconium assay

Phospholipids other than NAPE were tested for their ability to be precipitated by zirconium. Radiolabeled PE, PG, and PS could be precipitated 84, 97, and 99%, respectively, by the same procedure as described above. Addition of 1,500  $\mu$ l of 1 M HCl to samples containing PE improved the percentage of precipitation to 98%, as did addition of 100–750  $\mu$ l of 1 M HCl to samples with PG. PS was optimally precipitated under the same conditions as NAPE. Alkalinization of the sample solutions by addition of NaOH or sodium acetate decreased precipitation to 0–77%. PC, on the other hand, could not be effectively precipitated even if the pH was altered. This is probably due to the zwitterionic form of this phospholipid.

#### CONCLUSIONS

A rapid assay for detection of NAPE-PLD activity was developed by precipitation of NAPE along with lysoNAPE and GP-NAE, followed by measurements of the amount of radiolabeled NAE released into a methanolic supernatant. The precipitation is based on a chemical reaction of the zirconium ion with the phosphate group in the minus one state. This is to our knowledge the first report of quantitative zirconium precipitation of phospholipids. The assay is also applicable to precipitation of other anionic phospholipids such as PE, PG, and PS; however, the zwitterionic phospholipid PC cannot be effectively precipitated. Measurements of released NAE, ethanolamine, glycerol, or serine after zirconium precipitation of phospholipids are specific for PLD activity because of the chemical interaction with the phosphate group of the lipids that is required for precipitation. The detection method is therefore generally applicable to detection of anionic phospholipid-hydrolyzing phospholipase D activities, and may be useful for detection of any phospholipase activity with the substrate in the minus one state along with a neutral or positively charged product.

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