[8] Phospholipase A_2 Assays with Fluorophore-Labeled Lipid Substrates

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Introduction

Fluorophore-labeled lipid substrates have been used in two different ways to assay phospholipase A_2 (PLA_2). Continuous assays utilize changes in the fluorescence properties of the fluorophore on hydrolysis. Pyrene-labeled phospholipids have been used in such assays. The assays involve changes in excimer to monomer fluorescence on hydrolysis.[1-5] In discontinuous assays, the fluorophore is used to aid in detection of the substrate and/or products. Fluorophores such as the NBD (7-nitrobenzo-2-oxa-1,3-diazol-4-yl) group have been used to determine PLA_2 activity in intact cells[6] and on biomembrane phospholipids.[7] Trinitrophenylaminolauric acid-labeled phospholipids were used by Gatt *et al.*[8] to assay various lipases. These methods involve separation of the fluorescent product followed by measurement of its fluorescence. Simpler, sensitive methods involve chromatographic separation of fluorescent substrate and product by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) and direct determination of the ratio of substrate to product by fluorescence detection. Two such methods involving HPLC with fluorescence detection[9] and fluorescence scanning of TLC plates[10] are described here.

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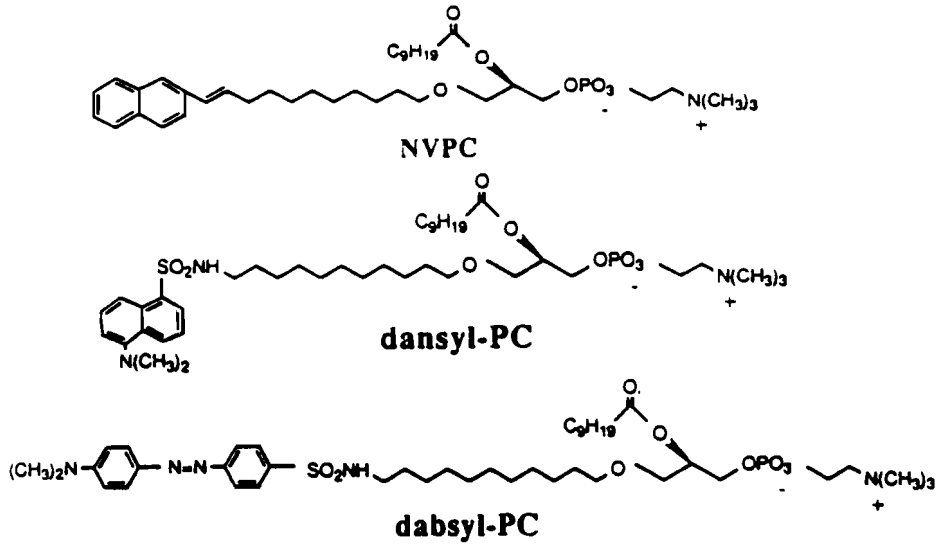

FIG. 1. Structures of phospholipid substrates.

Assay

Fluorescent phospholipid → fluorescent lysophospholipid + fatty acid

Principles. The fluorophore-labeled lipid substrates used (Fig. 1) have a fluorophore attached to the end of a hydrocarbon chain linked to the glycerol backbone by an ether linkage at the *sn*-1 position. Normal fatty acids are esterified at the *sn*-2 position. These substrates are resistant to phospholipase A_1 action, and the products are resistant to lysophospholipase action, making the assay quite specific for PLA_2. The phospholipid substrates and lysophospholipid products, both fluorescent, are separated by HPLC or TLC. The ratio of these two, determined by fluorescence detection, is used to calculate the amount of product released. Phospholipases C and D produce fluorescent hydrolysis products which are differentiated from that of PLA_2 by their chromatographic behavior.

Naphthylvinyl-PC {NVPC, 1-*O*-[12-(2-naphthyl)dodec-11-enyl]-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine} is used in the HPLC assay.[9] It absorbs strongly at 250 nm and emits above 340 nm. The naphthylvinyl group is rather nonperturbing; it gives about the same activity as natural phosphatidylcholine with snake venom and pancreatic PLA_2s, and the platelet-activating factor (PAF) analog of NVPC is nearly as active as the natural agonist. This lipid is better suited for the HPLC assay since its low-wavelength emission is more subject to background interference on

TLC plates. Dansyl-PC [1-*O*-(*N*-dansyl-11-amino-1-undecyl)-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine] and dabsyl-PC [1-*O*-(*N*-dabsyl-11-amino-1-undecyl)-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine] are best suited for the TLC assay.[10] Dansyl-PC absorbs at 256 nm and gives a strong emission above 400 nm. Dabsyl-PC is not fluorescent, but it has an intense red color (absorbs at 540 nm) which can readily be visualized by the unaided eye. The dansyl and dabsyl groups are more perturbing; the lipids are slightly poorer substrates for snake venom PLA_2, and the PAF analog of dansyl-PC is a very poor agonist.

Synthesis of Substrates. NVPC is synthesized according to the procedure of Hendrickson *et al.*[9] Dansyl-PC is synthesized according to the procedure described by Schindler *et al.*[11] by dansylation of the amino lipid 1-*O*-(11-amino-1-undecyl)-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine. Dabsyl-PC is synthesized by a similar procedure using dabsyl chloride instead of dansyl chloride to acylate the amino lipid.

Assay Procedure

Reagents

Buffer: 0.395 *M* NaCl, 66 m*M* Tris, 13.2 m*M* $CaCl_2$, pH 7
NVPC, 1 m*M* in $CHCl_3$
Dansyl-PC, 1 m*M* in $CHCl_3$
Dabsyl-PC, 1 m*M* in $CHCl_3$
Triton X-100, 10 m*M* in water
Quenching solvent: hexane/2-propanol/acetic acid (6:8:1.6, v/v)
HPLC solvent: hexane/2-propanol/water (6:8:1.6, v/v)
TLC solvent: $CHCl_3/CH_3OH$/concentrated ammonia/water (90:54:5.5:2, v/v)

Stock Substrate Solution. NVPC is used for assays involving HPLC. Dansyl-PC and dabsyl-PC are used for assays involving TLC. A measured amount (50 μl of a 1 m*M* stock solution) of substrate in chloroform is placed in a small test tube and dried under a stream of nitrogen and then under high vacuum for several minutes to remove the last traces of solvent. Triton X-100 (10 μl of 10 m*M*) and 190 μl of buffer are added, and the tube is repeatedly vortexed and sonicated in a bath sonicator until the lipid is completely dissolved and the solution is clear.

Enzyme Reaction. The stock substrate solution (40 μl) is mixed (vortex) with 60 μl of enzyme (about 2 ng of pure snake venom PLA_2) in a 6 × 50

[11] P. W. Schindler, R. Walter, and H. S. Hendrickson, *Anal. Biochem.* 174, 477 (1988). Dansyl-PC is available from Molecular Probes, Eugene, OR.

mm glass tube and incubated at room temperature. The final concentrations are as follows: 0.15 *M* NaCl, 0.1 m*M* substrate, 0.2 m*M* Triton X-100, 5 m*M* $CaCl_2$, 25 m*M* Tris.

HPLC Analysis with NVPC. At various time intervals over a period of 30–60 min, 10-μl aliquots are removed from the reaction mixture, added to 90 μl of quenching solvent in a 0.5-ml microcentrifuge tube, and vortexed immediately. The tube is centrifuged at 15,600 *g* (microcentrifuge) for 2 min to remove any precipitated protein. Centrifugation is not necessary when only nanogram amounts of pure enzyme are used. Twenty microliters of the quenched, diluted sample is analyzed by HPLC on a 4.6 mm × 15 cm silica column [Waters Associates (Milford, MA), #85774] protected with a guard column. An autosampler (Spectra Physics, San Jose, CA) allows automated analysis of samples. A flow rate of 1 ml/min of HPLC solvent is maintained [Kratos (Ramsey, NJ) Spectroflow 400 pump], and the eluate is analyzed by a fluorescence detector (Kratos Spectroflow 980: excitation, 250 nm; emission, >320 nm; PMT, 775 volts; range, 0.1). The signal from the detector is analyzed using an integrator/plotter (Spectra Physics 4290). The integrator is programmed to calculate the fraction of the lyso-NVPC peak area (retention time 9.98 min) relative to the total peak areas for lyso-NVPC and NVPC (retention time 3.84 min). This value times the initial amount of NVPC present in the assay reaction (10 nmol) equals the amount of lyso-NVPC produced. A plot of nanomoles lyso-NVPC produced versus time (usually linear up to at least 5% hydrolysis) is used to determine the initial rate (activity, μmol/min). A control without enzyme shows no hydrolysis. Activities as low as 1 pmol/min in an assay volume of 100 μl can easily be measured.

TLC Analysis with Dansyl-PC. At various time intervals over a period of 30–60 min, 5-μl aliquots of the reaction mixture are removed and spotted directly onto TLC plates [HPTLC plates, 10 × 10 cm, Analtech (Newark, DE), #60077]. Substrate and products are separated by developing the plates in the TLC solvent. The plates are then scanned in a fluorescence densitometer [Shimadzu (Columbia, MD), CS-9000 with a fluorescence accessory]. Excitation is set at 256 nm, and emission is measured above 400 nm (filter #2). The amount of lysodansyl-PC produced is calculated from the fraction of lysodansyl-PC relative to total lysodansyl-PC and dansyl-PC in the same way as described for the HPLC analysis.

TLC Analysis with Dabsyl-PC. The procedure is similar to that with dansyl-PC with the following modifications. The assay requires at least 0.5 m*M* substrate and 1 m*M* detergent due to the less sensitive detection of the dabsyl group on TLC plates. After solvent development, the orange-red color tends to fade with time but can be enhanced (more red in color) and stabilized by spraying with a dilute solution of HCl. The plates are

scanned in the dual-wavelength absorbance mode: sample absorbance at 540 nm and reference background at 610 nm.

Choice of Enzyme and Conditions

Snake venom PLA_2s are quite active in the presence of Triton X-100, but other PLA_2s (particularly pancreatic PLA_2) may be much less active with this detergent. PLA_2 in human synovial fluid is best assayed with NVPC by the HPLC method using hexadecylphosphorylcholine instead of Triton X-100.[12] Pancreatic PLA_2 is best assayed using sodium cholate as the detergent. The concentration of substrate and the ratio of substrate to detergent may be varied as desired. High salt concentrations (>1 *M* NaCl) may cause the quenched sample for HPLC analysis to phase separate and give artifactual peaks on HPLC.

Applications

The HPLC and TLC assays are sensitive enough to detect low levels of PLA_2 in crude physiological sources such as synovial fluid (see previous section). Detailed time-course analyses are feasible because of the small amount of sample required and speed of analysis. The TLC assay is well suited for rapid and efficient screening of PLA_2 inhibitors since many assays can simultaneously be analyzed on a single TLC plate. Qualitative results may be obtained by simple viewing of the plate under UV light for dansyl-PC or ordinary light for dabsyl-PC.

Derivatives of NVPC, dansyl-PC, and dabsyl-PC are potentially useful in the assay of other enzymes of lipid metabolism. Lysodansyl-PC and the PAF analog of dansyl-PC were used as substrates in TLC assays of lyso-PAF acyltransferase, lyso-PAF acetyltransferase, and PAF acetylhydrolase in polymorphonuclear leukocytes.[11] Since the probe remains attached to the glycerol backbone, simultaneous assay of these enzymes of the PAF cycle is possible.

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