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Evaluation of Fluorescent and Colored Phosphatidylcholine Analogs as Substrates for the Assay of Phospholipase A_2 [1]

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Two fluorescent phospholipid analogs, 1-*O*-{12-(2-naphthyl)-dodec-11-enyl}-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine and dansyl-PC [*rac* 1-*O*-(*N*-dansyl-11-amino-1-undecyl)-2-*O*-decanoyl-glycero-3-phosphocholine], and a red-colored analog, dabsyl-PC [*rac* 1-*O*-(*N*-dabsyl-11-amino-1-undecyl)-2-*O*-decanoyl-glycero-3-phosphocholine], were evaluated as substrates for the assay of phospholipase A_2 (PLA_2) (*Crotalus adamanteus*). The assay reaction was monitored by separation of the fluorescent or colored substrate and product by TLC and quantitation by fluorescence or absorption scanning. All three substrates gave similar (within an order of magnitude) activities with PLA_2. Dansyl-PC was best suited to this TLC assay. Dabsyl-PC was less sensitive to detection by absorbance, but had the advantage of being red colored and readily detected by the unaided eye. © 1990 Academic Press, Inc.

Phospholipase A_2 (PLA_2)[3] catalyzes hydrolysis of the 2-acyl ester of *sn*-3-glycerophospholipids (1). Recently we reported the synthesis of a naphthylvinyl-labeled glycerol–ether analog of phosphatidylcholine (NVPC, Fig. 1) as a substrate for a HPLC assay of PLA_2 (2) and a dansyl-labeled glycerol–ether analog of phosphatidylcholine (dansyl-PC, Fig. 1) as a substrate for a TLC assay of enzymes of the platelet-activating factor (PAF) cycle (lyso-PAF acetyltransferase, lyso-PAF acyltransferase, and PAF acetylhydrolase) (3). These phospholipids and their glycerol-containing products of enzyme action are fluorescent. Separation of substrate and product by HPLC or TLC and quantitation by fluorescence detection provide a simple and efficient method for assay of these enzymes. We report here a new red-colored dabsyl-labeled glycerol–ether analog of phosphatidylcholine (dabsyl-PC, Fig. 1) which is also useful as a substrate for a TLC assay of PLA_2. In order to evaluate the use of these phospholipids as substrates for PLA_2 assays, we compare here their activities with PLA_2 (*Crotalus adamanteus*) in an assay based on TLC separation of substrate and product followed by fluorescence or visual-absorption scanning of the TLC plate. This assay is an attractive alternative to assays using radiolabeled phospholipids.

EXPERIMENTAL PROCEDURES

Materials. NVPC was synthesized by the procedure of Hendrickson *et al.* (2). Dansyl-PC and dabsyl-PC were synthesized by the procedure of Schindler *et al.* (3) using dansyl chloride and dabsyl chloride, respectively, to N-acylate the amino lipid 1-*O*-(11-amino-1-undecyl)-2-*O*-decanoyl-glycero-3-phosphocholine. These phospholipids gave single spots on TLC and were completely hydrolyzed by phospholipase A_2 to a single lyso-phospholipid. PLA_2 was purified from *C. adamanteus* venom according to the method of Wells (4). Triton X-100 was obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were HPLC grade. High-performance thin-layer chromatography (HPTLC) plates (10 × 10 cm, cat. No. 60077) were obtained from Analtech, Inc. (Newark, DE).

Enzyme assays. A measured amount of phospholipid substrate solution in chloroform was dried under argon

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[3] Abbreviations used: PLA_2, phospholipase A_2; NVPC, 1-*O*-{12-(2-naphthyl)-dodec-11-enyl}-2-*O*-decanoyl-*sn*-glycero-3-phosphocholine; dansyl-PC, *rac* 1-*O*-(*N*-dansyl-11-amino-1-undecyl)-2-*O*-decanoyl-glycero-3-phosphocholine; dabsyl-PC, *rac* 1-*O*-(*N*-dabsyl-11-amino-1-undecyl)-2-*O*-decanoyl-glycero-3-phosphocholine; PAF, platelet-activating factor; HPTLC, high-performance thin-layer chromatography; PMN, polymorphonuclear neutrophil.

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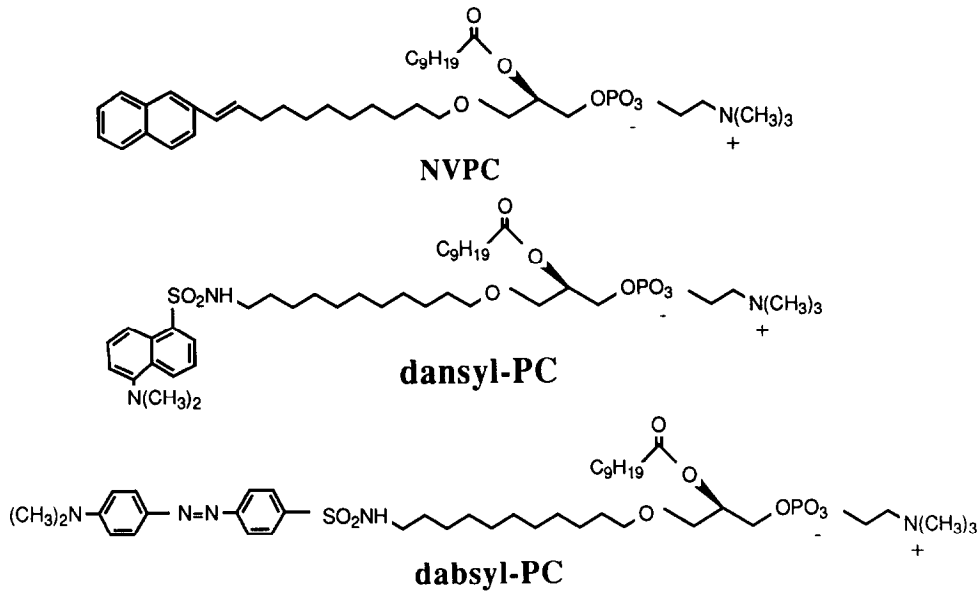

FIG. 1. Structures of fluorescent and colored phospholipid analogs.

and then under a high vacuum. Triton X-100 and buffer were added and the lipid was dispersed by vortexing and sonicating (bath sonicator) as necessary. All assays were performed in a total volume of 100 μl in a buffer containing 0.5 mM substrate, 1 mM Triton X-100, 0.15 M NaCl, 5 mM $CaCl_2$, 25 mM Tris, pH 8.0, at 25°C. At specific time intervals after the addition of enzyme, 10-μl aliquots of the reaction mixture were spotted onto TLC plates. Substrate and products were separated by developing the plates with the solvent system chloroform–methanol–concd ammonia–water (90:54:5.5:2, v/v). The orange-red color of the dabsyl-phospholipids on the plates tended to fade in time, but could be enhanced (more red in color) and were quite stable after spraying with a dilute solution of HCl. The plates were then scanned in an absorbance/fluorescence densitometer (Shimadzu Model CS-9000). For NVPC, excitation was set at 250 nm and fluorescence emission measured above 340 nm (filter No. 1). For dansyl-PC, excitation was set at 256 nm and emission measured above 400 nm (filter No. 2). For dabsyl-PC, a dual-wavelength absorbance mode was used with sample absorbance at 540 nm and reference background at 610 nm. The amount of lyso-PC was calculated as

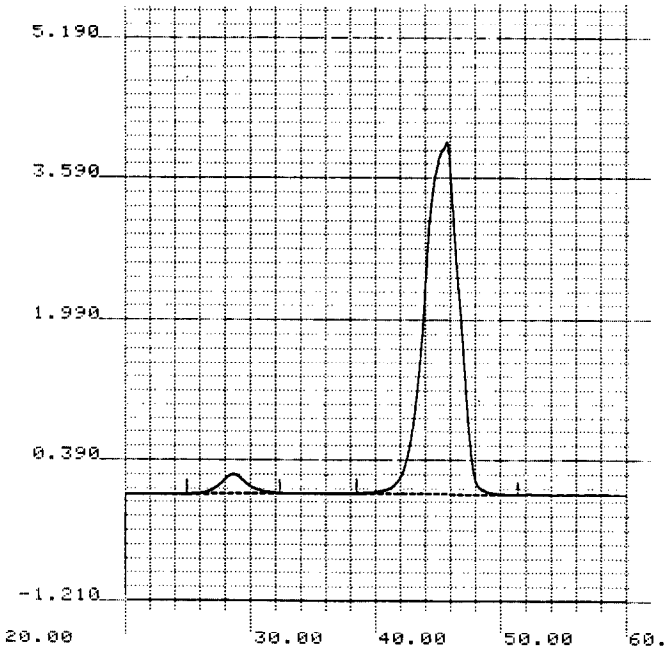

FIG. 2. Scan of TLC separation of dabsyl-PC and lyso-dabsyl-PC. Dual-wavelength absorption mode: sample at 540 nm, reference background at 610 nm. Ordinate: absorbance (arbitrary units); abcissa: distance on TLC plate (40 mm full scale, origin at left). Dabsyl-PC: large peak (95.9% area) to the right; lyso-dabsyl-PC: small peak (4.1% area) to the left. Origin: left side. HPTLC, silica gel; solvent: chloroform–methanol–concd ammonia–water (90:54:5.5:2, v/v).

$$\text{nmol lyso-PC} = \{(\text{area lyso-PC})/(\text{area lyso-PC} + \text{area PC})\}50 \text{ nmol}.$$

Initial rates were determined by fitting the data, nanomoles of lyso-PC versus time, to a second-order polynomial by a nonlinear regression analysis using the program Kinfit (5).

RESULTS

C. adamanteus PLA_2-catalyzed hydrolysis of NVPC, dansyl-PC, and dabsyl-PC was followed by TLC separa-

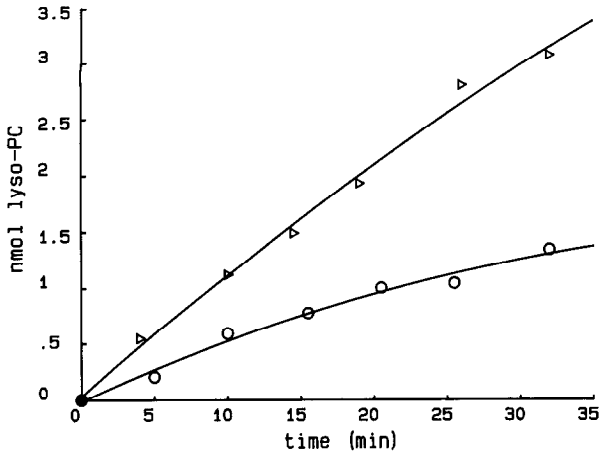

FIG. 3. Time course of PLA_2 (*C. adamanteus*) assay with dabsyl-PC. Enzyme: 12 ng (triangles), 7.2 ng (circles); 0.5 mM dabsyl-PC; 1 mM Triton X-100.

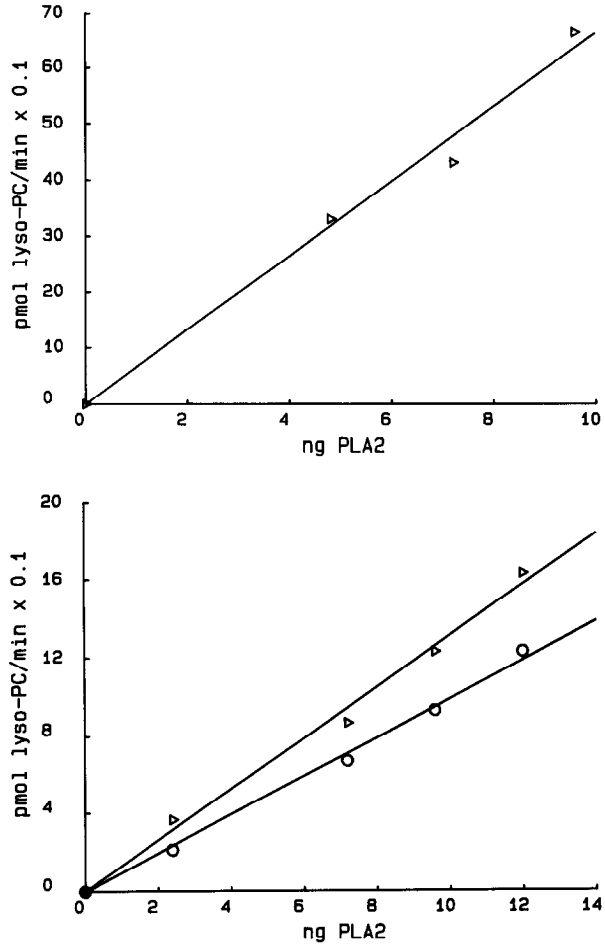

FIG. 4. Activities of PLA_2 (*C. adamanteus*) with NVPC, dansyl-PC, and dabsyl-PC versus amount of enzyme. Upper plot: activity with NVPC; lower plot: activities with dansyl-PC (triangles) and dabsyl-PC (circles).

tion of the substrate and lyso-PC product and quantitative analysis by fluorescence or absorption scanning of the TLC plate. These lipids migrate very similarly to natural phosphatidylcholine on TLC. A typical scan of dabsyl-PC and lyso-dabsyl-PC is shown in Fig. 2. With the same amount of total lipid applied, the total fluorescent intensity (or absorbance) was the same regardless of the extent of hydrolysis, indicating the same quantum yields (or extinction coefficients) for substrate and product. Scans of TLC separations of assay aliquots taken at time intervals were used to obtain initial rates as shown in Fig. 3. Plots of initial rates versus the amount of enzyme were used to calculate the specific activity of PLA_2 with each substrate as shown in Fig. 4. Table 1 lists activities with the three substrates.

The fluorescence analyses of NVPC and dansyl-PC were quite sensitive and could easily be performed on assays containing 0.1 mM substrate (1 nmol of phospholipid applied to the plate). Absorbance analysis of dabsyl-PC, however, required at least 0.5 mM substrate in the assay (5 nmol of phospholipid applied to the plate). The lower wavelength of emission from NVPC (>340 nm) resulted in more background noise from the TLC plate. Approximately 2 pmol of lyso-dansyl-PC per spot could accurately be determined, while lyso-dabsyl-PC required about 20 pmol. The sensitivity for lyso-NVPC was about 5 pmol.

DISCUSSION

The detection of fluorescent and colored phospholipid analogs by quantitative TLC analysis permits a rapid and sensitive assay of PLA_2. An assay volume of 100 μl provides sufficient aliquots for a detailed time-course analysis. Of the three phospholipid analogs, dansyl-PC is best suited to the TLC assay due to its high sensitivity for detection. NVPC was less well suited to the TLC assay since its emission at lower wavelengths (>340 nm) leads to more background noise from the plate. NVPC is much better suited to HPLC assays (2) where its sensitivity for detection in a HPLC fluorescence detector is quite high. Dabsyl-PC, because of its red color, has the advantage of being readily visible to the eye. Its disadvantage, however, is that it is less sensitive to detection

TABLE 1

Activity of PLA_2 with Phosphatidylcholine Analogs

PC analog	Activity (μmol min^{-1} mg^{-1})
NVPC (chiral)	66.3 ± 2.0
Dansyl-PC (racemic)	13.1 ± 0.3
Dabsyl-PC (racemic)	10.0 ± 0.2

Note. Substrate (0.5 mM), Triton X-100 (1 mM), NaCl (0.15 M), $CaCl_2$ (5 mM), Tris (25 mM), pH 8.0, 25°C. Activities include the standard error of fit to a plot of activity versus ng of enzyme.

by absorption and requires at least 0.5 mM substrate in the assay.

The sensitivity of this assay for enzyme activity depends only on the ratio of product to substrate, provided enough phospholipid is applied to the plate for accurate detection. No internal standards are necessary since the total phospholipid (PC and lyso-PC) provides an internal standard. Activities as low as 10 pmol/min in an assay volume of 100 μl can easily be measured. Lower activities may be measured over time periods longer than the 30–40 min used in this study. Since these three phospholipid analogs have ether groups at the *sn*-1 position, they are not subject to hydrolysis by phospholipase A_1 or lysophospholipase. Activities of other phospholipases such as C or D are readily recognized and determined independent of PLA_2, since their products migrate differently on TLC and can be identified. This is illustrated by the simultaneous TLC assay of the enzymes of the PAF cycle (lyso-PAF acetyltransferase, lyso-PAF acyltransferase, and PAF acetylhydrolase) in PMN leukocytes using dansyl-PC, lyso-dansyl-PC, and dansyl-PAF as substrates and chromatographic identification of the products (3).

These phospholipid analogs were designed with the fluorophore or chromophore at the end of the *sn*-1 alkyl chain to minimize potential perturbing effects. NVPC gives an activity with PLA_2 similar to that observed with natural phosphatidylcholine (determined in a standard titrametric assay), and the PAF analog has activity for platelet aggregation similar to that of natural PAF (2). The dansyl and dabsyl groups are potentially more perturbing since they are bulkier and more polar than the naphthylvinyl group. Dansyl-PAF has very poor activity for platelet aggregation (Dr. Peter Schindler, personal communication), although it is a good substrate for PAF acetylhydrolase ((3), Dr. Ewa Ninio, personal communication). The activities of dansyl- and dabsyl-PC with PLA_2, however, are only moderately lower than that of NVPC, particularly when one considers that racemic mixtures of dansyl- and dabsyl-PC were used in this study compared to chiral NVPC, and the unnatural enantiomer acts as a competitive inhibitor (see Table 1). A variety of fatty acids can be esterified at the *sn*-2 position to facilitate studies of fatty acid specificity.

Fluorophore-labeled lipids have been used in continuous assays which depend on changes in fluorescent properties due to environmental factors upon hydrolysis. These substrates have a fluorescent group on the fatty acid which is hydrolyzed (6–8) or on the headgroup (9). Changes in fluorescent properties depend on partition of the labeled product away from the substrate interface and are quite sensitive to the detergent used in micellar systems and the nature of substrate aggregation. Proteins such as serum albumin can bind the fluorescent product and thus affect its fluorescence properties (8). Erroneous results may be obtained in crude enzyme preparations since other phospholipases may also give products with altered fluorescent properties and these assays do not identify the products. The substrates described here can be used under a variety of assay conditions since the fluorescent label is used only to enable sensitive detection of substrate and product. A variety of TLC solvent systems can be used which best separate the fluorescent products from the substrate (10).

The use of these fluorescent and colored phospholipid analogs for the TLC assay of PLA_2 represents an attractive alternative to assays using radiolabeled phospholipids. The former assays are less laborious and time-consuming, since they do not involve scraping of plates and lengthy periods of counting. Since the sensitivity of these assays, like the radioisotope assays, depends only on determination of the ratio of product to substrate, they should be equally sensitive. Dabsyl-PC has the advantage of providing an even simpler qualitative visual determination of activity, since the red spots can be readily detected by the unaided eye.

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