Analytical Biochemistry **276,** 27–35 (1999)
Article ID abio.1999.4280, available online at http://www.idealibrary.com on IDEAL®

Intramolecularly Quenched BODIPY-Labeled Phospholipid Analogs in Phospholipase A_2 and Platelet-Activating Factor Acetylhydrolase Assays and *in Vivo* Fluorescence Imaging

H. Stewart Hendrickson,*[,1] Elizabeth K. Hendrickson,* Iain D. Johnson,† and Steven A. Farber‡
**Department of Chemistry, University of Washington, Seattle, Washington 98133-1700; †Molecular Probes, Inc., Eugene, Oregon 97402-0469; and ‡Department of Embryology, Carnegie Institute of Washington, Baltimore, Maryland 21210*

Received May 17, 1999

Phospholipase substrate analogs containing both a fluorescent BODIPY group and a quenching 2,4-dinitrophenyl (DNP) group were synthesized. They showed little fluorescence, but upon hydrolysis became fluorescent as the quenching group was removed. Two substrates were phosphatidylethanolamine analogs with a BODIPY-pentanoyl group at the *sn*-2 position and DNP linked to the amino head group. The third was a phosphatidylcholine analog with a BODIPY-labeled alkyl ether at the *sn*-1 position and a *N*-(DNP)-8-amino-octanoyl group at the *sn*-2 position. These compounds were evaluated as substrates for cytosolic (85 kDa) phospholipase A_2 ($cPLA_2$) and plasma platelet-activating factor acetylhydrolase (rPAF-AH). Two were good substrates for $cPLA_2$ (specific activities: 18 and 5 nmol min^{-1} mg^{-1}) and all were good for rPAF-AH (specific activities: 17, 11, and 6 μmol min^{-1} mg^{-1}). The minimal amount of enzyme detectable was 50 ng for $cPLA_2$ and 0.1 ng for rPAF-AH. These substrates were active in assays of PLA_2 in zebrafish embryo extracts and one was well suited for imaging of PLA_2 activity in living zebrafish embryos. Embryos were injected with substrate at the one- to four-cell stage and allowed to develop until early somitogenesis when endogenous PLA_2 activity increases dramatically; substrate persisted (12 h) and specifically labeled cells of the developing notochord. © 1999 Academic Press

Key Words: **BODIPY; cytosolic (85 kDa) phospholipase A_2; plasma platelet-activating factor acetylhydrolase; fluorescence; quenching; zebrafish; embryo; imaging; assay.**

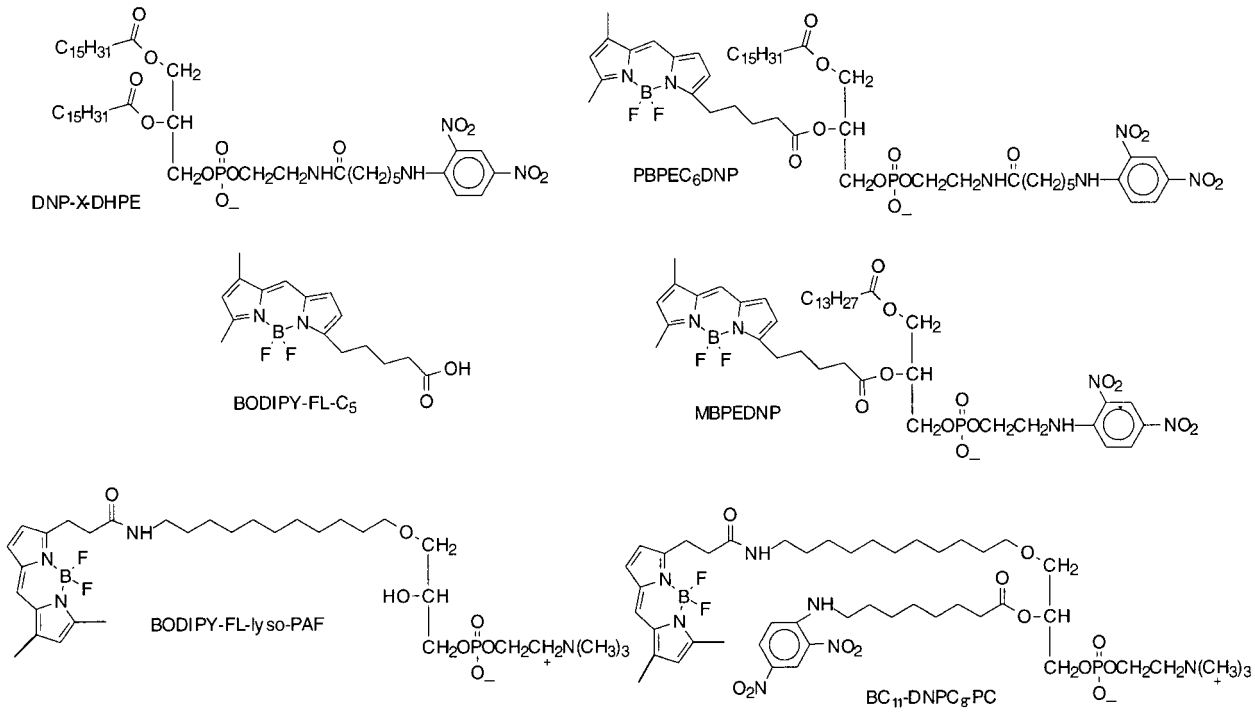
The 85-kDa cytosolic phospholipase A_2 ($cPLA_2$)[2] (a group IV phospholipase A_2) plays an important role in mediating the release of arachidonic acid from the *sn*-2 position of phospholipids for eicosanoid biosynthesis (1). The human plasma platelet-activating factor acetylhydrolase (rPAF-AH) is a 45-kDa secreted enzyme that catalyzes the hydrolysis of the *sn*-2 acetyl group of platelet-activating factor (2). It has been widely studied for its association with inflammatory diseases, and may also play an important protective role in oxidative processes that are thought to be associated with early vascular disease since it will also hydrolyze phospholipids with an oxidatively fragmented acyl group at the *sn*-2 position, but not phospholipids with long-chain *sn*-2 fatty acids (3).

We initiated this study with the aim of developing a substrate analog for fluorescent imaging of $cPLA_2$ in cells. We were looking for a nonfluorescent analog that would become highly fluorescent upon hydrolysis by PLA_2. Bis-BODIPY-C_{11}-PC (4) has been used for this purpose, but it gives excimer fluorescence that is

[2] Abbreviations used: BODIPY-FL-C_5, 4,4-difluor-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid; BODIPY-FL-C_5-HPC, 1-hexadecanoyl-2-(BODIPY-FL-pentanoyl)-*sn*-glycero-3-phosphocholine; Bis-BODIPY-C_{11}-PC, 1,2-bis-(BODIPY-FL-undecanoyl)-*sn*-glycero-3-phosphocholine; BODIPY-FL-lyso-PAF, 1-(*N*-(BODIPY-FL-pentanoyl)-11-aminoundecyl)-*sn*-glycero-3-phosphocholine; BC_{11}-$DNPC_8$-PC, 1-(*N*-(BODIPY-FL-pentanoyl)-11-aminoundecyl)-2-((*N*-(2,4-dinitrophenyl)amino)-8-amino-octanoyl)-*sn*-glycero-3-phosphocholine; DNP-X DHPE, N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1,2-dipalmitoyl)-*sn*-glycero-3-phosphoethanolamine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; EM, embryo medium (see "zebrafish *in vitro* assay"); MBPEDNP, *N*-(2,4-dinitrophenyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-*sn*-glycero-3-phosphoethanolamine; $PBPEC_6DNP$, *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-*sn*-glycero-3-phosphoethanolamine; rPAF-AH, recombinant platelet-activating factor acetylhydrolase; $cPLA_2$, cytosolic phospholipase A_2; $ppPLA_2$, porcine pancreatic phospholipase A_2.

[1] To whom correspondence should be addressed. Fax: (206) 685-8665. E-mail: hend@u.washington.edu.

0003-2697/99 $30.00



SCHEME 1. Synthetic precursors (left) and phospholipid substrate analogs (right).

highly concentration dependent. Thuren *et al.* (5) developed an intramolecularly quenched pyrene-labeled phospholipid analog, with a fluorescent pyrenehexanoyl group at the *sn*-2 position and a quenching *N*-trinitrophenyl group on phosphatidylethanolamine, as a substrate for the assay of PLA_2. We sought to modify this type of analog with a BODIPY group, suitable for fluorescence microscopy with an argon–ion laser.

Given the availability of BODIPY-labeled PAF analogs that we had developed and other BODIPY-labeled phospholipids available from Molecular Probes, Inc. (Eugene, OR) (see left side of Scheme 1), we synthesized the three substrate analogs shown on the right in Scheme 1. Since plasma rPAF-AH is also specific for the hydrolysis of phospholipids with bulky and polar acyl groups at the *sn*-2 position, we also looked at these analogs as possible substrates for the assay of rPAF-AH. Here we present fluorescence-based continuous assays of $cPLA_2$ and rPAF-AH, and the use of one of these substrates for *in vivo* fluorescent imaging of $cPLA_2$ in zebrafish embryos.

MATERIALS AND METHODS

Materials

Source of lipids and enzymes. $cPLA_2$, a human recombinant 85-kDa cytosolic enzyme (6), was obtained from Professor M. Gelb, University of Washington. rPAF-AH, a human recombinant plasma enzyme (7), was obtained from ICOS (Bothell, WA). $ppPLA_2$ was purified from porcine pancreas as described by Nieuwenhuizen *et al.* (8). These enzymes were greater than 95% pure on the basis of SDS–PAGE. Concentrations were determined from their extinction coefficients at 280 nm: $ppPLA_2$, 1.23 mL mg^{-1} cm^{-1}; $cPLA_2$, 0.827 mL mg^{-1} cm^{-1}; PAF-AH, 1.4 mL mg^{-1} cm^{-1} (L. Paul, ICOS, personal communication). *Naja naja* PLA_2 and dimyristoyl phosphatidylethanolamine were obtained from Sigma (St. Louis, MO). DTPM was prepared as described by Jain *et al.* (9). DNP-X DHPE, BODIPY-FL-C_5, BODIPY-FL-C_5-HPC, and BODIPY-FL-lyso-PAF were obtained from Molecular Probes (Eugene, OR).

Synthesis of Lipid Substrates

Synthesis of PBPEC$_6$DNP. DNP-X DHPE was deacylated at the *sn*-2 position with *N. naja* PLA_2 as described by Ali and Bittman (10). The lysophospholipid was acylated with BODIPY-FL-C_5 in the presence of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (11) to give PBPEC$_6$DNP. The product was purified by DEAE-cellulose chromatography (12). TLC: single spot, R_f = 0.66, $CHCl_3$:CH_3OH 4:1, detected by UV fluorescence and phosphorus-specific spray. Vis absorption spectrum: 505 nm (BODIPY, $\epsilon = 78 \times 10^3$ M^{-1} cm^{-1}); 350 nm (DNP, $\epsilon = 24 \times 10^3$ M^{-1} cm^{-1}).

Synthesis of MBPEDNP. Dimyristoylphosphatidylethanolamine was converted to the *N*-DNP derivative with 2,4-dinitrofluorobenzene as described (13). This was then deacylated using PLA_2 and reacylated with BODIPY-FL-C_5 as described above for PBPEC_6DNP. The product was purified by DEAE-cellulose chromatography. TLC: single spot, R_f = 0.70, $CHCl_3$:CH_3OH 4:1, detected by UV fluorescence and phosphorus-specific spray. Vis absorption spectrum: 505 nm (BODIPY, $\epsilon = 78 \times 10^3\ M^{-1}\ cm^{-1}$); 350 nm (DNP, $\epsilon = 24 \times 10^3\ M^{-1}\ cm^{-1}$).

Synthesis of BC_{11}-$DNPC_8$-PC. 8-Amino-octanoic acid was converted to the *N*-DNP derivative as described above for phosphatidylethanolamine. BODIPY-FL-lyso-PAF was deacylated using PLA_2 and reacylated with BODIPY-FL-C_5 as described above for PBPEC_6DNP. The product was purified by DEAE-cellulose chromatography. TLC: single spot, R_f = 0.42, $CHCl_3$:CH_3OH:conc NH_3:H_2O 90:54:5.5:2, detected by UV fluorescence and phosphorus-specific spray. Vis absorption spectrum: 505 nm (BODIPY, $\epsilon = 80 \times 10^3\ M^{-1}\ cm^{-1}$); 350 nm (DNP, $\epsilon = 22 \times 10^3\ M^{-1}\ cm^{-1}$).

Phospholipid Dispersions

Phospholipids were stored as chloroform–methanol solutions at −20°C. Phospholipid concentrations were determined by phosphorus analysis (14). Aliquots of phospholipid analogs and DTPM were mixed and dried under a stream of nitrogen and then under high vacuum. Buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM $CaCl_2$) was added, and the solution was vortexed and sonicated to give an optically clear dispersion.

Fluorescence Spectroscopy

Fluorescence emission (excitation, 500 nm; emission, 512 nm; 3 nm bandwidths) was measured on a Perkin–Elmer MPF-44B spectrofluorometer equipped with stirring and temperature controls.

Enzyme Assays

ppPLA_2 and rPAF-AH. An aliquot of phospholipid was placed in a 1 × 1-cm fluorescence cuvette and buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM $CaCl_2$) was added to a total volume of 1.3 mL. The temperature was allowed to equilibrate at 35°C with stirring for several minutes and the background emission was recorded (the background rate was undetectable). Enzyme (1–10 μL) was added and the emission was recorded.

cPLA_2. An aliquot of phospholipid (78 μL of 0.05 mM substrate/0.5 mM DTPM) was placed in a 1 × 1-cm fluorescence cuvette and buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM $CaCl_2$, 30% glycerol) was added to a total volume of 1.3 mL. The temperature was allowed to equilibrate at 35°C with stirring for several minutes and the background emission was recorded (the background rate was undetectable). Enzyme (1–10 μL) was added with extra mixing of the viscous solution, and the emission was recorded.

Calibration. A measured amount of BODIPY-FL-C_5 (in the case of PBPEC_6DNP and MBPEDNP) or BODIPY-FL-C_5-lyso-PAF (in the case of BC_{11}-$DNPC_8$-PC) was added to the phospholipid substrate solutions under the same conditions as the assays above, and the increase in emission was recorded. The factor, picomoles of BODIPY product/intensity unit increase, was then used to convert emission increase/sec to picomoles of product/second in the assays.

Zebrafish in vitro assay. Single embryos were assayed as previously described (15). Briefly, embryos were placed in 0.15 mL of embryo medium (EM: 13.7 mM NaCl, 0.537 mM KCl, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 1.30 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$, pH 7.2) (16) containing 150–200 ng of a fluorescent phospholipid substrate, and sonicated for 2–5 s. After 1 h at 37°C, reactions were stopped by the addition of 0.45 mL of chloroform:methanol (2:1) (17), mixed, and centrifuged (30 s, 16,000*g*). The aqueous fraction was discarded and an aliquot of organic fraction was loaded on thin-layer chromatography plates (Si250 PA, J.T. Baker, Phillipsburg, NJ). Plates were developed in toluene: diethyl ether: ethanol: acetic acid (50:40:2:0.2) and quantified using a laser scanner (Molecular Dynamics, Sunnyvale, CA).

Fluorescence Imaging

PBPEC_6DNP was purified by TLC to remove low-abundance BODIPY-labeled degradation products immediately prior to injection into zebrafish embryos. Purified PBPEC_6DNP (5 μg) was dried under N_2 and resuspended in ethanol (10 μL) followed by the addition of EM (10 μL). The solution was again dried under N_2 until the volume was approximately 5 μL to ensure the evaporation of most of the ethanol. Phenol red (0.5 μL of a 2% stock) was added to allow easy identification of injected embryos. Embryos (one- to four-cell stage) were injected (2 nL of the solution described above) through the yolk cell to a region directly adjacent to the developing blastoderm. Embryos were incubated in EM (2–6 h at 28°C; 1 mL/embryo) and visualized using a Leica TCSNT confocal microscope.

RESULTS

Synthesis of BODIPY-DNP-Labeled Phospholipids

BODIPY-DNP-labeled phospholipids were synthesized by standard methods. The products were pure as judged by a single spot on TLC, and Vis absorption

TABLE 1

Activities of Enzymes with BODIPY-Labeled Substrates

Enzyme	Substrate[a]	Specific activity
pp-PLA_2[b]	BC_{11}-$DNPC_8$-PC	20 nmol min^{-1} mg^{-1}
	$PBPEC_6DNP$	15 μmol min^{-1} mg^{-1}
	MBPEDNP	3.1 μmol min^{-1} mg^{-1}
$cPLA_2$[c]	BC_{11}-$DNPC_8$-PC	5.0 nmol min^{-1} mg^{-1}
	$PBPEC_6DNP$	18 nmol min^{-1} mg^{-1}
	MBPEDNP	0.02 nmol min^{-1} mg^{-1}
rPAF-AH[b]	BC_{11}-$DNPC_8$-PC	17 μmol min^{-1} mg^{-1}
	$PBPEC_6DNP$	5.9 μmol min^{-1} mg^{-1}
	MBPEDNP	11 μmol min^{-1} mg^{-1}

[a] 3 μM substrate, 30 μM DTPM.
[b] Buffer: 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM Ca^{2+}.
[c] Buffer: 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM Ca^{2+}, 28% glycerol.

spectra indicated stoichiometric amounts of BODIPY and DNP chromophores as judged by their extinction coefficients (18). Fluorescence emission of the lipid dispersions in the enzyme assays prior to the addition of enzyme was about 10% or less of full scale at the highest signal gain used (30×).

Enzyme Assays, General Remarks

The specific activities of $ppPLA_2$, $cPLA_2$, and rPAF-AH with different substrates are shown in Table 1. All substrates were incorporated into anionic DTPM vesicles to enhance enzyme binding at the interface. $PBPEC_6DNP$ was the best substrate for $ppPLA_2$, while BC_{11}-$DNPC_8$-PC was a very poor substrate. BC_{11}-$DNPC_8$-PC was the best substrate for $cPLA_2$; MBPEDNP was a very poor substrate, while the introduction of a C_6 spacer in the head group of $PBPEC_6DNP$ greatly increased its activity. $cPLA_2$ was best determined in the presence of 28% glycerol; in the absence of glycerol, enzyme activity was erratic (19), often dying within the first minute. BC_{11}-$DNPC_8$-PC was the best substrate for rPAF-AH, although the other two substrates also gave good activities.

Assay of rPAF-AH

rPAF-AH was best assayed with 10 mol% BC_{11}-$DNPC_8$-PC in the presence of DTPM. Figure 1A shows a typical progress curve for the assay of 0.8 ng of enzyme. Activity was proportional to the amount of enzyme (Fig. 1B) and the minimal amount of enzyme detectable is estimated to be about 0.1 ng. The specific activity of the enzyme was about 17 μmol min^{-1} mg^{-1}. Activity as a function of bulk concentration of lipid is shown in Fig. 1C. An apparent $K_m = 9.8$ μM was observed. Activity as a function of surface concentration of substrate is shown in Fig. 1D. An apparent interfacial $K_m^* = 2.16$ mol% was observed.

Assays of $cPLA_2$ and $ppPLA_2$

$cPLA_2$ was best assayed with $PBPEC_6DNP$, although BC_{11}-$DNPC_8$-PC also gave good activity. MBPEDNP, which lacks the C_6 spacer in the head group, was a very poor substrate. Figure 2A shows a typical progress curve for the assay of 0.2 μg of enzyme with $PBPEC_6DNP$. Activity was proportional to the amount of enzyme (Fig. 2B), and the minimal amount of enzyme detectable is estimated to be about 50 ng. The specific activity of the enzyme was about 18 nmol min^{-1} mg^{-1}. These assays required 28% glycerol in the buffer, presumably to stabilize the enzyme; without glycerol, reactions were erratic and the enzyme activity often died in the first minute. Glycerol significantly increased the viscosity of the solution, so extra care was required to insure adequate mixing; after addition of enzyme the solution was stirred with a small paddle for several seconds. $ppPLA_2$ was best assayed with $PBPEC_6DNP$, although MBPEDNP was also a fair substrate. Activity with BC_{11}-$DNPC_8$-PC, however, was very poor.

Calibration of Assays

Enzyme assays were calibrated by determining the emission intensity increase as a function of the amount of fluorescent product. Product was added to phospholipid substrate solutions under the same conditions as the assay. Figure 3A shows the addition of BODIPY-FL-C_5 to buffer or $PBPEC_6DNP$/DTPM. Addition of BODIPY-FL-C_5 to buffer showed a linear increase in emission; however, addition to lipid showed a smaller nonlinear increase, presumably due to limited quenching in the presence of substrate DNP. The initial slope of emission increase in the presence of lipid (up to 40 pmol of BODIPY-FL-C_5) was nearly linear and was used to calibrate assays using $PBPEC_6DNP$ or MBPEDNP as substrate.

The addition of BODIPY-FL-C_5-lyso-PAF to buffer or BC_{11}-$DNPC_8$-PC/DTPM is shown in Fig. 3B. No significant quenching was observed in the presence of substrate. The slope of this emission increase in the presence of lipid was used to calibrate assays using BC_{11}-$DNPC_8$-PC as the substrate.

In Vitro Assays with Zebrafish Embryo Extract

To compare the fluorophores in a more physiologic context, the specific activity of zebrafish extract PLA_2 was determined using various BODIPY-PC analogs. Each assay contained the extract of a single embryo (1000-cell stage) in the presence of 150–200 ng of fluorescent substrate. These assays were performed under saturating conditions because extracts also contain endogenous zebrafish lipids (data not shown). The specific activity of a single BODIPY-labeled PC (BODIPY-

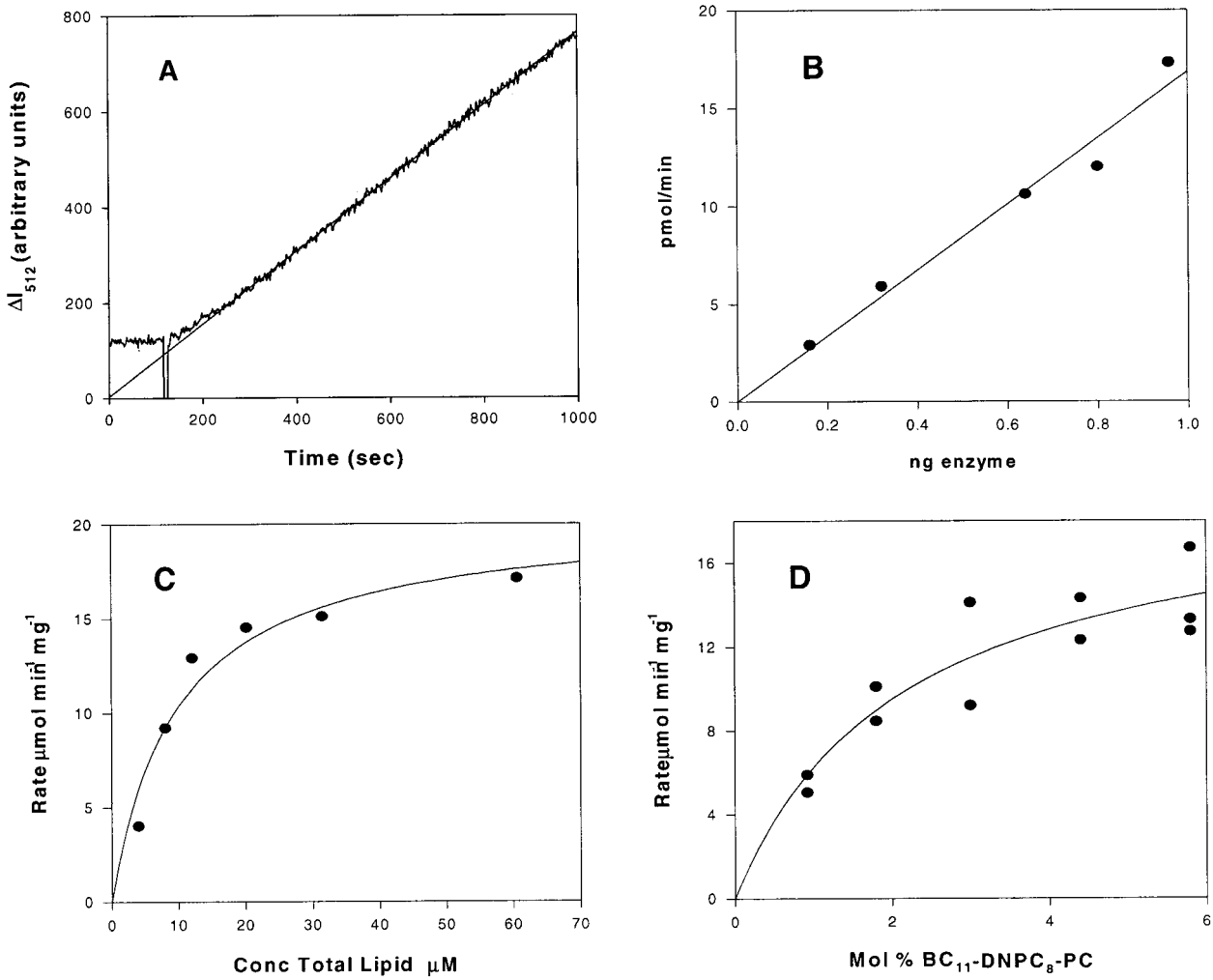


FIG. 1. Assay of rPAF-AH with BC_{11}-$DNPC_8$-PC. (A) Reaction progress curve: 0.8 ng of rPAF-AH with 10 mol% BC_{11}-$DNPC_8$-PC in 30 μM DTPM. (B) Rate as a function of the amount of enzyme; 10 mol% BC_{11}-$DNPC_8$-PC in 30 μM DTPM; specific activity = 16.8 μmol min^{-1} mg^{-1}. (C) Rate as a function of the bulk concentration of lipid; 4.76 mol% BC_{11}-$DNPC_8$-PC in DTPM; V_{max} = 20.4 ± 1.9 μmol min^{-1} mg^{-1}, apparent K_m = 9.8 ± 2.8 μM. (D) Rate as a function of the surface concentration of substrate; [DTPM] = 30 μM; V_{max} = 19.7 ± 2.8 μmol min^{-1} mg^{-1}, apparent interfacial K^*_m = 2.16 ± 0.82 mol %.

FL-C_5-HPC) was also determined and compared with the quenched fluorophores. The quenched compounds, BC_{11}-$DNPC_8$-PC and $PBPEC_6DNP$, were clearly better substrates than BODIPY-FL-C_5-HPC, with BC_{11}-$DNPC_8$-PC being significantly better than either MBPEDNP or $PBPEC_6DNP$ (Fig. 4).

Fluorescence Imaging

Having established that quenched fluorophores were good PLA_2 substrates *in vitro,* we examined whether they could function in the living zebrafish embryo. Embryos were injected with approximately 2 ng of substrate at the 1- to 4-cell stage and allowed to develop for a number of hours until early somitogenesis stages when endogenous PLA_2 activity increases dramatically (15). While BC_{11}-$DNPC_8$-PC is an excellent substrate *in vitro,* it is metabolized too rapidly for use in the living embryo (data not shown). However, $PBPEC_6DNP$ injected into the early blastula persisted until somitogenesis (12 h after injection) and often (25 of 45 injections) specifically labeled cells of the developing notochord in living embryos (Fig. 5). In most cases where notochord labeling was not observed, fluorescence was weakly observed in the yolk near the site of injection.

DISCUSSION

Thuren *et al.* (5) introduced the use of intramolecularly quenched pyrene-labeled phospholipid analogs as substrates for the assay of PLA_2. In looking for a suitable fluorescence-labeled substrate for fluorescence im-

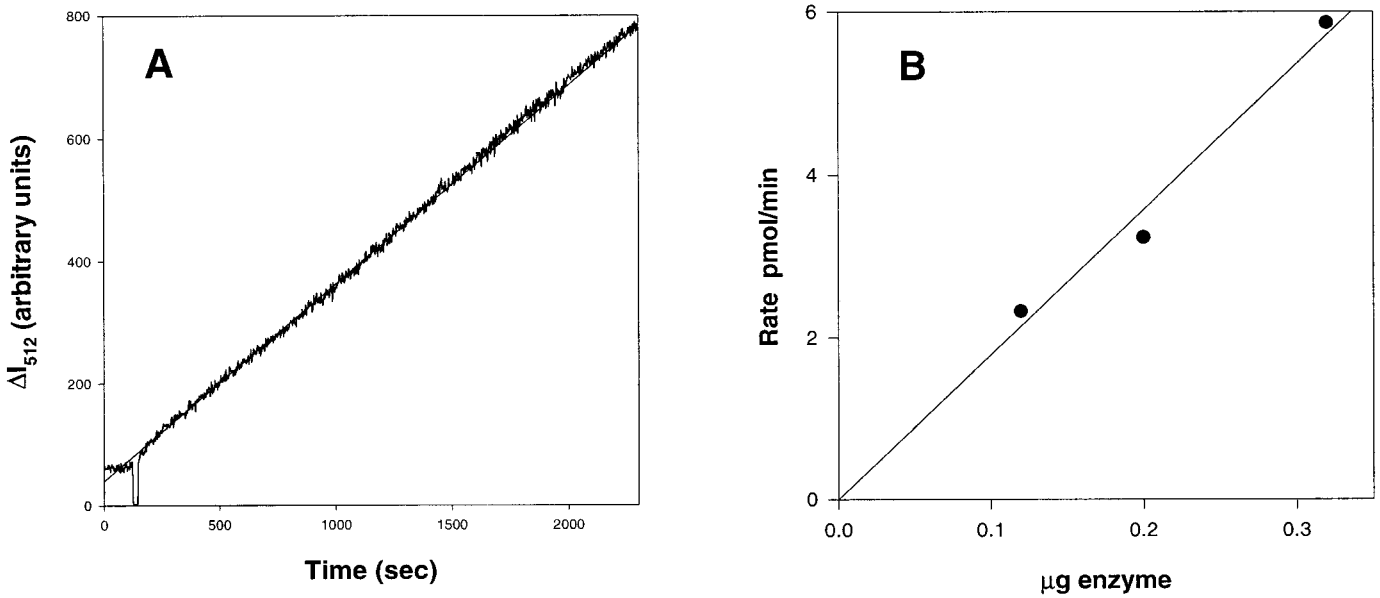


FIG. 2. Assay of $cPLA_2$ with 10 mol% $BPPEC_6DNP$ in 30 μM DTPM. (A) Reaction progress curve, 0.2 μg enzyme. (B) Rate as a function of the amount of enzyme; specific activity = 17.9 nmol min^{-1} mg^{-1}.

aging of PLA_2 activity, we considered an intramolecularly quenched BODIPY-labeled phospholipid analog; the reaction product would have spectral properties suitable for fluorescence microscopy using an argon–ion laser and be stable to photobleaching and insensitive to environmental factors, and the substrate would show little or no fluorescence emission. These analogs were easily synthesized from BODIPY-labeled PAF analogs that we have developed and other BODIPY-labeled phospholipids available from Molecular Probes. We used the 2,4-dinitrophenyl group as an intramolecular quencher since it can easily be added to amino groups by use of the Sanger reagent, 2,4-dinitrofluorobenzene.

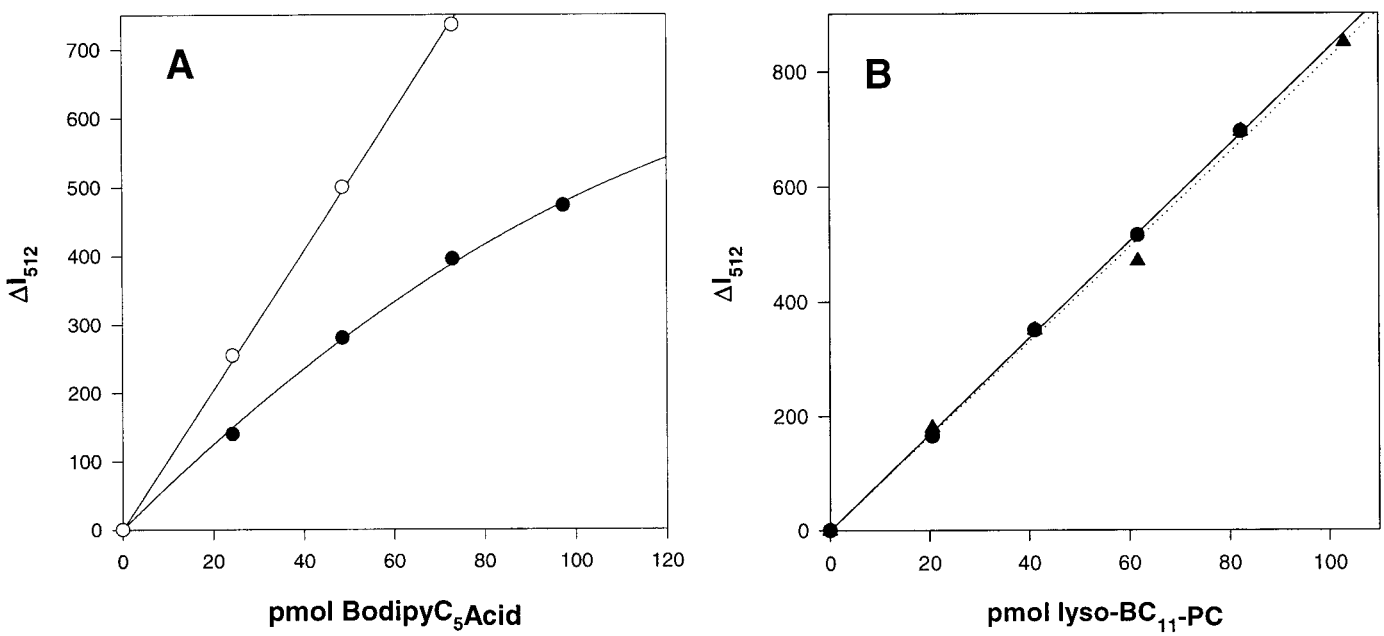


FIG. 3. Fluorescence emission as a function of added reaction products. (A) Addition of BODIPY-FL-C_5 to 1.3 mL of buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM $CaCl_2$) (○), or 10 mol% $PBPEC_6DNP$ in 30 μM DTPM (●). (B) Addition of BODIPY-FL-C_5-lyso-PAF into 1.3 mL of buffer (▲, dotted line), or 5.8 mol% BC_{11}-$DNPC_8$-PC in 30 μM DTPM (●, solid line).

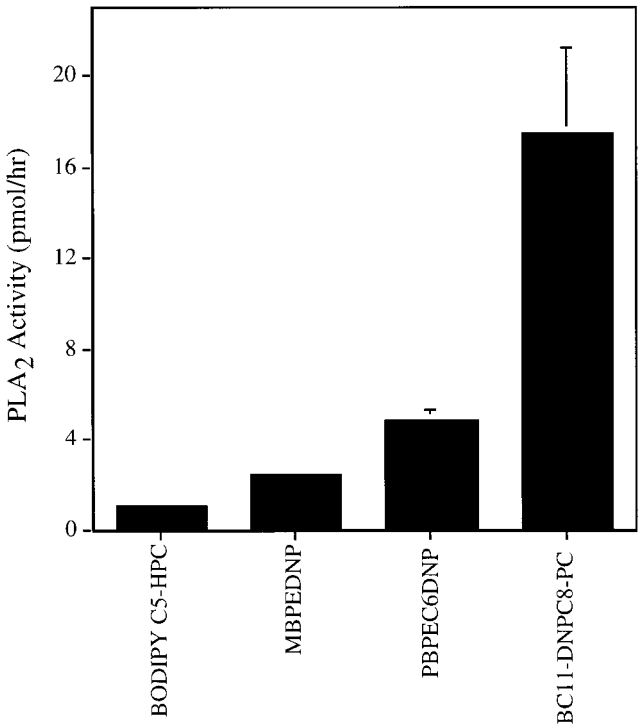


FIG 4. Zebrafish extract PLA_2 activity determined with BODIPY-labeled substrates. Activity was assayed as described under Materials and Methods. Individual embryo extracts were prepared from shield stage (approximately 6 h postfertilization) embryos and assayed for PLA_2 activity using various BODIPY-labeled PC analogs as described under Materials and Methods. Data represent the mean ($n = 4$) ± SE.

Several continuous fluorometric assays of the 85-kDa $cPLA_2$ were developed by Bayburt *et al.* (20). Assays using 1-pyrenedecanoyl-2-arachidonylphosphatidylcholine, adapted from that of Radvanyi *et al.* (21), are most sensitive (1.4 μmol min^{-1} mg^{-1}, 0.5 ng minimum detectable) but require 100% fluorescent substrate to achieve intermolecular excimer fluorescence. An assay using the nonfluorescent ester of γ-linolenic acid and 7-hydroxycoumarin to release a fluorescent product is less sensitive (36 nmol min^{-1} mg^{-1}, 10 ng minimum detectable) but convenient, and the substrate can be incorporated into vesicles of anionic lipid to give kinetics in the "scooting mode" (22). The assay of $cPLA_2$ described in this paper with $PBPEC_6DNP$ is almost as sensitive (18 nmol min^{-1} mg^{-1}, 50 ng minimum detectable) and the substrate can be incorporated into anionic lipid vesicles at a low mole fraction. With 10 mol% substrate, only about 3% of substrate was hydrolyzed in over 30 min of the assay shown in Fig. 2A.

To compare $cPLA_2$ with a more readily available secretory enzyme, we looked at the assay of $ppPLA_2$. MBPEDNP and $PBPEC_6DNP$ showed low activity (3–15 μmol min^{-1} mg^{-1}) compared to an activity of over 1000 μmol min^{-1} mg^{-1} with the natural phosphatidylcholine substrate (23). BC_{11}-$DNPC_8$-PC showed even lower activity; the presence of two bulky and polar acyl groups at the *sn*-1 and -2 positions of the substrate appears to be unfavorable for this enzyme.

rPAF-AH activity is usually determined in a radiochemical assay using [^{3}H-acetyl]PAF (24, 25). Discontinuous fluorometric assays have been developed that give similar sensitivity (26, 27). The continuous assay reported here for plasma rPAF-AH with BC_{11}-$DNPC_8$-PC is just as sensitive and more convenient to use. It can detect as little as 0.1 ng of enzyme at rates of a few picomoles/minute. MBPEDNP is also a suitable substrate for this assay. This assay is restricted to

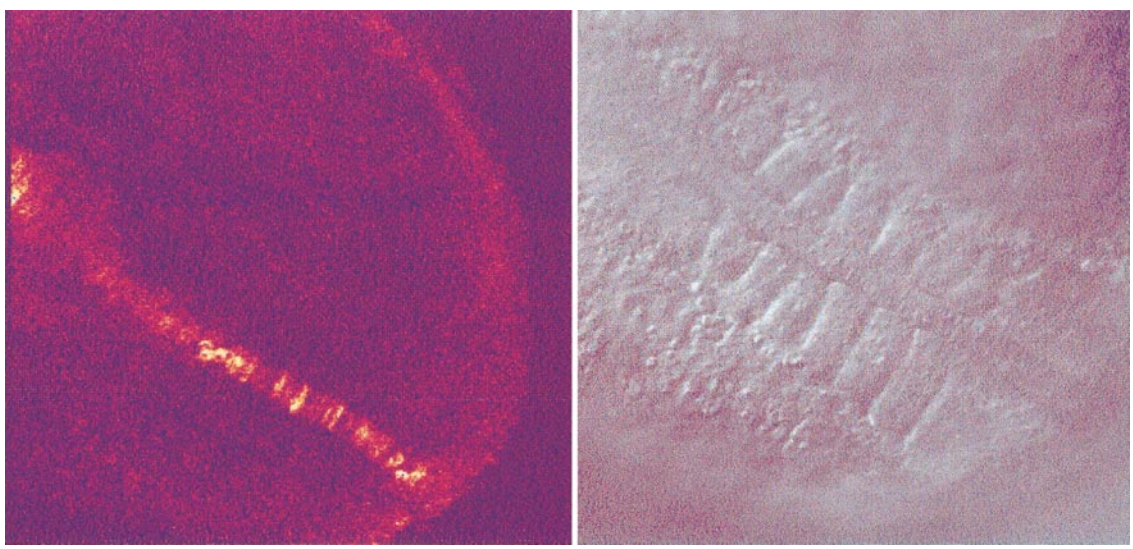


FIG 5. PLA_2 activity in living embryos. (Right) A dorsal DIC view of the somites and notochord of an embryo injected with $PBPEC_6DNP$. (Left) The same embryo visualized with confocal microscopy using only the argon laser (488 nm) for excitation.

the plasma enzyme since the cytosolic enzyme has strict specificity for an acetyl group at the *sn*-2 position of PAF (2). This assay is about 10-fold more sensitive than a continuous colorimetric assay using 2-thio PAF (Cayman Chem. Co., Ann Arbor, MI), and about 100-fold more sensitive than a pH-stat assay.

We utilized living zebrafish embryos to assess whether these quenched BODIPY-labeled phospholipids could be used for *in vivo* imaging. Bis-BODIPY-C_{11}-PC had previously been employed to measure PLA_2 activity in neutrophils (28) and specifically PLA_2 activity in zebrafish embryos (15); however, quenching is dependent on the use of exogenous lipids. We found that $PBPEC_6DNP$ could be injected into yolk of 1- to 4-cell-stage embryos and, in more than half of the cases, incorporated into the developing embryo. When blastoderm labeling was observed, the notochord, and cells adjacent to the notochord, showed increased fluorescence indicative of PLA_2 activity. This is interesting because of the well-established signaling role of the notochord and its precursors in patterning the nervous system and the somites (structures that give rise to muscle, skin, and bones of the back) of vertebrates (29, 30).

It is presumed that cleavage of Bis-BODIPY-C_{11}-PC by PLA_2 results in a rapidly reacylated fluorescent phospholipid and a more diffusable fluorescent fatty acid. To better label only cells that contained active PLA_2 in a tissue or embryo containing a mixture of cell types, we designed BC_{11}-$DNPC_8$-PC such that cleavage by PLA_2 results in a fluorescent lyso lipid. While BC_{11}-$DNPC_8$-PC was the best PLA_2 substrate using embryo extracts (Fig. 4), it was found to be rapidly metabolized by living embryos when injected early in development. However, this fluorophore might prove superior in living systems when it is delivered shortly before assaying activity (i.e., by soaking embryos a liposome solution as opposed to injecting at the 1-cell stage). Since signal transduction events *in vivo* involve communication between a variety of cell types in spatially and temporally dynamic fashion, the ability to monitor signaling events in real time is essential. These data indicate that for PLA_2-mediated signaling, BODIPY-labeled phospholipids are excellent substrates for imaging enzymatic activity in a continuous assay.

The substrates reported here may also be used to identify isozymes in *in vitro* assays. For example, BC_{11}-$DNPC_8$-PC is a very poor substrate for the secreted $ppPLA_2$, but a good substrate for $cPLA_2$ and plasma PAF-AH; however, it cannot serve as a substrate for cytosolic PAF-AH, which has strict specificity for an acetyl group at the *sn*-2 position. Differences in the Ca^{2+} dependency (plasma PAF-AH is Ca^{2+}-independent) and relative specificities of the three substrates may distinguish between $cPLA_2$ and plasma PAF-AH. In living cells it would be difficult to distinguish the activities of these three enzymes, or possibly others not tested, with these substrates. Other methods, such as the use of selective inhibitors or antibodies (15), may be required for positive identification.

ACKNOWLEDGMENTS

This work was supported by a grant (MCB-9619859 to H.S.H) from the National Science Foundation, and by a National Institutes of Health Postdoctoral Fellowship (F32 NS10326 to S.A.F.).

REFERENCES

1. Leslie, C. C. (1997) Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.* **272,** 16709–16712.
2. Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1997) Platelet-activating factor acetylhydrolases. *J. Biol. Chem.* **272,** 17895–17898.
3. Stremler, I. E., Stafforini, D. M., Prescott, S. M., and McIntyre, T. M. (1991) Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates. *J. Biol. Chem.* **266,** 11095–11103.
4. Haugland, R. P. (1996) Handbook of Fluorescent Probes and Research Chemicals, pp. 472–473, Molecular Probes, Inc., Eugene, OR.
5. Thuren, T., Virtanen, J. A., Somerharju, P. J., and Kinnunen, P. K. J. (1988) Phospholipase A2 assay using an intramolecularly quenched pyrene-labeled phospholipid analog as substrate. *Anal. Biochem.* **170,** 248–255.
6. Bayburt, T., and Gelb, M. H. (1997) Continuous, vesicle-based fluorimetric assays of 14- and 85-kDa phospholipases A_2. *Biochemistry* **36,** 3216–3231.
7. Tjoelker, L. W., Wilder, C., Eberhardt, C., Stafforini, D. M., Dietsch, G., Schimpf, B., Hooper, S., Trong, H. L. Cousens, L. S., Zimmerman, G. A., Yamada, Y., McIntyre, T. M., Prescott, S. M., and Grey, P. W. (1995) Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* **374,** 549–552.
8. Nieuwenhuizen, W., Künze, H., and de Haas, G. H. (1974) Phospholipase A2 (phosphatide acylhydrolase, EC 3.1.1.4) from porcine pancreas. *Methods Enzymol.* **32,** 147–154.
9. Jain, M. K., Yu, B.-Z., Rogers, J., and Berg, O. (1991) Interfacial catalysis by phospholipase A2: Dissociation constants for calcium, substrate, products, and competitive inhibitors. *Biochemistry* **30,** 7306–7317.
10. Ali, S., and Bittman, R. (1989) Mixed-chain phosphatidylcholine analogues modified in the choline moiety: Preparation of isomerically pure phospholipids with bulky headgroups and one acyl chain twice as long as the other. *Chem. Phys. Lipids* **50,** 11–21.
11. Martin, S. F., Josey, J. A., Wong, Y.-L., and Dean, D. W. (1994) General method for the synthesis of phospholipid derivatives of 1,2-*O*-diacyl-*sn*-glycerols. *J. Org. Chem.* **59,** 4805–4820.
12. Kates, M. (1972) Techniques of Lipidology, pp. 408–411, North-Holland, Amsterdam.
13. Christie, W. W. (1982) Lipid Analysis, 2nd ed., p. 131, Pergamon, Oxford.
14. Eaton, B. R., and Dennis, E. A. (1976) Analysis of phospholipase C (*Bacillus cereus*) action toward mixed micelles of phospholipid and surfactant. *Arch. Biochem. Biophys.* **176,** 604–609.
15. Farber, S. A., Olson, E., Clark, J. D., and Halpern, M. E. (1999) Characterization of Ca^{2+}-dependent phospholipase A_2 activity during zebrafish embyrogenesis. *J. Biol. Chem.* **274,** 19338–19346.

16. Westerfield, M. (1995) The Zebrafish Book, 3rd ed., Univ. of Oregon, Eugene, OR.
17. Bligh, E., and Dyer, W. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–918.
18. Haugland, R. P. (1996) Handbook of Fluorescent Probes and Research Chemicals, Tables 13.2 (p. 292) and 13.4 (p. 304), Molecular Probes, Inc., Eugene, OR.
19. Burke, J. R., Witmer, M. R., and Tredup, J. A. (1999) The size and curvature of anionic covesicle substrate affects the catalytic action of cytosolic phospholipase A_2. *Arch. Biochem. Biophys.* 365, 239–247.
20. Bayburt, T., Yu, B.-Z., Street, I., Ghomashchi, F., Laliberté, F., Perrier, H., Wang, Z., Homan, R., Jain, M. K., and Gelb, M. H. (1995) Continuous, vesicle-based fluorimetric assays of 14- and 85-kDa phospholipases A_2. *Anal. Biochem.* **232,** 7–23.
21. Radvanyi, F., Jordan, L., Russo-Marie, F., and Bon, C. (1989) A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. *Anal. Biochem.* **177,** 103–109.
22. Bayburt, T., and Gelb, M. H. (1997) Interfacial catalysis by human 85 kDa cytosolic phospholipase A_2 on anionic vesicles in the scooting mode. *Biochemistry* **36,** 3216–3231.
23. Waite, M. (1987) The Phospholipases, p. 163, Plenum, New York.
24. Blank, M. L., Hall, M. N., Cress, A. E., and Snyder, F. (1983) Inactivation of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine by a plasma acetylhydrolase: Higher activities in hypertensive rats. *Biochem. Biophys. Res. Commun.* **113,** 666–671.
25. Stafforini, D. M., and Tjoelker, L. W. (1999) *in* Lipase and Phospholipase Protocols (Doolittle, M., and Rue, K., Eds.), pp. 49–58, Humana Press, Totowa, NJ.
26. Schindler, P. W., Walter, P., and Hendrickson, H. S. (1988) Fluorophore-labeled ether lipids: Substrates for enzymes of the platelet-activating factor cycle in peritoneal polymorphonuclear leukocytes. *Anal. Biochem.* **174,** 477–484.
27. Balestrieri, C., Camussi, G., Giovane, A., Iorio, E. L., Quagliuolo, L., and Servillo, L. (1996) Measurement of platelet-activating factor acetylhydrolase activity by quantitative high-performance liquid chromatography determination of coumarin-derivatized 1-*O*-alkyl-2-*sn*-lysoglyceryl-3-phosphorylcholine. *Anal. Biochem.* **233,** 145–150.
28. Meshulam, T., Herscovitz, H., Casavant, D., Bernardo, J., Roman, R., Haugland, R., Strohmeier, G., Diamond, R., and Simons, E. (1992) Flow cytometric kinetic measurements of neutrophil phospholipase A activation. *J. Biol. Chem.* **267,** 21465–21470.
29. Halpern, M. E., Ho, R. K., Walker, C., and Kimmel, C. B. (1993) Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell* **75,** 99–111.
30. Yamada, T., Pfaff, S. L., Edlund, T., and Jessell, T. M. (1993) Control of cell pattern in the neural tube: Motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73,** 673–686.