Alterations in Individual Molecular Species of Human Platelet Phospholipids during Thrombin Stimulation: Electrospray Ionization Mass Spectrometry-Facilitated Identification of the Boundary Conditions for the Magnitude and Selectivity of Thrombin-Induced Platelet Phospholipid Hydrolysis†

Xianlin Han, Rose A. Gubitosi-Klug, Brendan J. Collins, and Richard W. Gross*

Division of Bioorganic Chemistry and Molecular Pharmacology, Departments of Medicine, Chemistry and Molecular Biology & Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Received December 12, 1995; Revised Manuscript Received February 26, 1996⊗

ABSTRACT: Although the rapid thrombin-induced release of arachidonic acid in human platelets has been known for over 20 years, the amount of arachidonic acid mass mobilized and the source of the released arachidonic acid has remained a subject of intense controversy. Herein, we exploit the analytic power and sensitivity of electrospray ionization mass spectrometry to identify plasmenylethanolamines as the largest source of arachidonic acid mass released during thrombin stimulation and to demonstrate the presence of multiple novel molecular species of plasmenylethanolamines in human platelets. Specifically, 90 s after thrombin stimulation a total of 60.1 nmol of arachidonic acid-containing phospholipids/10^9 platelets was hydrolyzed which included the loss of 31.8 nmol/10^9 platelets from ethanolamine glycerophospholipids (hydrolysis of plasmenylethanolamines represented 63% of the mass lost from the ethanolamine glycerophospholipid pool) but only 10.9 nmol/10^9 platelets from choline glycerophospholipids. Human platelet phosphatidylserine and phosphatidylinositol pools contained similar amounts of arachidonic acid mass in resting platelets (≈20 nmol/10^9 platelets), and each pool contributed 8.7 nmol/10^9 platelets after thrombin stimulation. From these results, a lower boundary for the rate of thrombin-induced arachidonic acid mobilization in human platelets can be set at >60 nmol/10^9 platelets, thereby identifying specific kinetic characteristics and substrate selectivities of the phospholipase(s) activated during platelet stimulation. Collectively, these results underscore the importance of plasmenylethanolamines as the major storage depot of arachidonic acid in resting platelets and as the major source of arachidonic acid mobilized after thrombin stimulation of human platelets.

Platelet activation is accompanied by the production of oxygenated metabolites of arachidonic acid which are potent modulators of platelet function (e.g., Hamberg et al., 1974; Hamberg & Samuelsson, 1974; Amezcua et al., 1978; Rittenhouse-Simmons & Deykin, 1981). Since the rate-determining step in the production of biologically active eicosanoid metabolites in human platelets is the availability of nonesterified arachidonic acid (e.g., Bills et al., 1976; Jesse & Cohen, 1976; Marcus et al., 1980; Siess et al., 1984), substantial attention has focused on the identification of the molecular targets of the phospholipases activated during platelet stimulation. During the last two decades, numerous groups have measured alterations in platelet phospholipid mass after thrombin or collagen stimulation in an attempt to identify the class and type of the phospholipases activated during platelet stimulation (e.g., Lewis & Majerus, 1969; Bills et al., 1977; Rittenhouse-Simmons & Deykin, 1977; Broekman et al., 1981; Walenga et al., 198l; Sutherland & Amin, 1982; Neufeld & Majerus, 1983; Mahadevappa & Holub, 1984; Smith et al., 1985; Silk et al., 1989; Colard et al., 1989). However, these studies each came to substantially different conclusions regarding (1) the major phospholipid classes responsible for arachidonic acid mass released during platelet stimulation, (2) the importance of plasmalogen molecular species during thrombin stimulation, (3) the relative contributions of different anionic phospholipid pools (e.g., phosphatidylinositol and phosphatidylserine), (4) the magnitude of phospholipid hydrolysis during maximal activation of platelets; and (5) the regioselectivity, *sn*-2 aliphatic specificity, and phospholipid subclass specificity of the participating phospholipase(s).

More recently, further discrimination of thrombin-induced lipid turnover was attempted through analyses of alterations in the mass of individual molecular species of platelet phospholipids (Takamura et al., 1987, 1989) utilizing analytic techniques dependent on reverse-phase HPLC[1] separation of individual molecular species. However, those studies were confounded by (1) the lability of the vinyl ether linkage on

† This research was supported by NIH Grant R01HL34839 and the Mass Spectrometry Resource Grant R01HL95418.

* Correspondence should be addressed to this author at Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, 660 South Euclid, Box 8020, St. Louis, MO 63110. Telephone: 314-362-2690. Fax: 314-362-1402.

⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

[1] Abbreviations: FAB, fast atom bombardment; ESI, electrospray ionization; MS, mass spectrometry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 18:3−18:3 PC, 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine; 18:3−18:3 PE, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; SAPI, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography; GC, gas chromatography.

S0006-2960(95)02927-8 CCC: $12.00

silica matrices, (2) the incomplete resolution of phospholipid molecular species by the reverse-phase chromatographic techniques employed, and (3) the intrinsic difficulties inherent in quantitative analyses employing multiple sequential analytic steps each utilized at the limits of their sensitivity leading to geometrically accumulating errors.

Mass spectroscopy of individual phospholipid molecular species potentially represents the most sensitive, discriminating, and direct method to assess alterations in phospholipid molecular species in biologic tissues. However, prior mass spectrometric analyses of phospholipids have previously required relatively high energy ionization (e.g., fast atom bombardment desorption of phospholipids from target matrices) which resulted in extensive and differential fragmentation of the quasimolecular ions of individual phospholipid classes and subclasses, thereby precluding quantitative analyses by this technique (e.g., Jensen et al., 1986; Münster et al., 1986; Chen et al., 1990; Pramanik et al., 1990). Further confounding the utilization of FAB ionization for quantitative analyses of phospholipids is the extensive dependence of the ionization efficiency on the surface properties of individual phospholipids in the target matrix (Gross, 1984). Collectively, these factors have effectively precluded the use of FAB in quantitative mass spectrometric analyses of cellular phospholipids.

Recently, we demonstrated that electrospray ionization (ESI) results in the efficient production of low energy molecular ions of phospholipids without fragmentation which was largely independent of the surface properties of individual phospholipid subclasses and molecular species (Han & Gross, 1994). Through exploiting the inherent advantages of ESI-MS for the quantitative analysis of alterations in individual molecular species of human platelet phospholipids, we now report that (1) plasmenylethanolamine molecular species represent the largest endogenous storage depot of arachidonic acid in human platelets, (2) hydrolysis of plasmenylethanolamine molecular species is the largest source of arachidonic acid mass released during thrombin stimulation of human platelets, (3) hydrolysis of phosphatidylserine accounts for heretofore unrecognized amounts of arachidonic acid mass released during platelet thrombin activation, and (4) the flux of arachidonic acid during the first 90 s of thrombin activation exceeds 60 nmol/10^9 platelets. The mathematical constraints imposed by these analyses identify the boundary conditions for the minimal specific activities, mass abundance, and substrate specificities of the platelet phospholipases activated during thrombin stimulation.

MATERIALS AND METHODS

Materials. Commercially available phospholipids including 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (18:3−18:3 PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine (18:3−18:3 PE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS) used for internal standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Commercially unavailable phospholipids including plasmenylcholines and plasmenylethanolamines were prepared as described previously (Han & Gross, 1992; Han et al., 1992). The mass of purchased phospholipids was quantitated by capillary gas chromatography after acid methanolysis by comparisons with arachidic acid as previously described (Fink & Gross, 1984). Human thrombin (3000 NIH units/mg of protein) was obtained from Sigma (St. Louis, MO).

Preparation of Human Platelet Phospholipid Extract after Thrombin Activation. Human platelets were freshly prepared by platelet pheresis and quantified at the Washington University Platelet Pheresis Center (Washington University, St. Louis). Platelet rich plasma was placed in conical polyethylene tubes and centrifuged for 20 min at $750g_{max}$ (1600 rpm) to form platelet pellets. The pellets were subsequently resuspended in buffer (46 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 1 mM KH_2PO_4, and 1 mM glucose at pH 7.1, buffer A) prior to recentrifugation ($750g_{max}$), decantation of the supernatant and resuspension in buffer A. Next, the washed platelet suspension was divided into two parts. From each part, aliquots of samples were removed for subsequent quantification of protein mass by Bio-Rad analysis. Then, the platelet suspension was brought to 1 mM [Ca^{2+}] by the dropwise addition of $CaCl_2$ solution. To one aliquot of washed human platelets, thrombin (1 unit/mL) was added while an equal volume of buffer A was added to another sample (control). Both samples were subsequently incubated for 90 s at 22 °C. Experiments were terminated by the coaddition of $CHCl_3$/MeOH (1/1, v/v) containing internal standards DMPC (14:0−14:0 PC) and 18:3−18:3 PE (30 μg each/10^9 platelets; 10^8 platelets were used in a typical experiment) and DMPS (3 μg/10^9 platelets) for mass spectroscopic analyses or alternatively by addition of 18:3-18:3 PC and 18:3−18:3 PE (1 mg of each; 10^{10} platelets were used in a typical experiment) for HPLC analyses. Next, additional $CaCl_2$ was added (final concentration of 3 mM [Ca^{2+}]) to facilitate extraction of anionic phospholipids. Human platelet phospholipids (and included internal standards) were extracted by the Bligh and Dyer method (Bligh & Dyer, 1959), dried under a nitrogen stream, dissolved in $CHCl_3$, reextracted and dried under a nitrogen stream. The final lipid residue was immediately resuspended in 500 μL of 1:2 chloroform/methanol for electrospray ionization mass spectroscopic analyses.

Electrospray Ionization Mass Spectrometry of Phospholipids. ESI mass spectral analyses were performed utilizing a Finnigan TSQ-700 Spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray interface (Analytica of Branford, Branford, CT) as described previously (Han & Gross, 1994). Typically, a 5-min period of signal averaging in the profile mode was employed for each spectrum of platelet extract, and the mass of all ions was rounded to the nearest integer. All samples of platelet extracts were diluted in 1:2 chloroform/methanol prior to direct infusion into the ESI chamber using a syringe pump (Harvard Apparatus model 22, South Natick, MA) at a flow rate of 1.5 μL/min. Anionic phospholipids in the diluted platelet extract were analyzed by ESI mass spectrometry in the negative-ion mode and quantitated by comparisons of the individual ion peak intensity with internal standard (i.e., DMPS) after correction for ^{13}C isotope effects. Similarly, choline glycerophospholipids in the diluted platelet extract were directly quantitated as their sodium adducts by comparisons with internal standard (i.e., sodiated DMPC) in the positive-ion mode after ESI. Ethanolamine glycerophospholipids in the platelet extract were rendered mildly basic by addition of 5 μmol of NaOH/mL of methanol and were directly quantitated by comparisons with internal standard (i.e., 18:3−18:3 PE)

in the negative-ion mode by ESI. Spectra of platelet ethanolamine glycerophospholipids which did not contain exogenously added internal standard did not display any demonstrable molecular ions in that region (Figure 1, insert). This selectivity of ESI-MS for direct analyses of distinct phospholipid classes was achieved by exploiting differential ionization propensities in the positive and negative ion modes of each phospholipid class (Han & Gross, 1994). Identification of ion peaks corresponding to individual molecular species was substantiated utilizing tandem mass spectrometry after ESI as described previously (Han & Gross, 1995) based on the assumption that the ratio of *sn*-2 carboxylate anion to *sn*-1 carboxylate anion is 3:1 for phospholipids containing 20 carbons and four double bonds or less, and that the ratio is one for phospholipids containing more than 20 carbons and/or four double bonds (Huang et al., 1992; Han & Gross, 1995). Plasmalogen molecular species were distinguished from alkyl-acyl phospholipid molecular species by treating human platelet phospholipid extracts with acidic vapors prior to mass spectroscopic analyses as described previously (Ford et al., 1992; Kayganich & Murphy, 1992). All molecular species of individual ion peaks of either phosphatidylserines or phosphatidylinositols reported herein were similarly identified by using negative-ion ESI tandem mass spectrometry (Han & Gross, 1995).

Quantitation of Phospholipids Using Electrospray Ionization Mass Spectrometry. To determine the utility of ESI-MS for quantitation of choline phospholipid mixtures using ESI-MS, the quasimolecular ion intensities of equimolar mixtures of individual phospholipid molecular species were examined by positive-ion ESI-MS. Positive-ion ESI mass spectra of an equimolar mixture of four individual molecular species (2.5 pmol/μL of each infused for 1 min at 1 μL/min) of choline glycerophospholipids (i.e., DLPC, DMPC, DPPC, and DSPC) contained four sodiated ion peaks (i.e., *m*/*z* 645, 701, 757, and 813, respectively) which were of nearly identical intensity (within 5% which is within current limits of standard quantitation) after correction for ^{13}C-isotope effects [the ^{13}C-isotope peaks of these choline glycerophospholipids contained precisely the theoretical amounts of isotopic ions (i.e., 35, 40, 44, and 48% for DLPC, DMPC, DPPC, and DSPC, respectively)]. The effects of unsaturation and differential surface characteristics were extensively examined, and saturated vs unsaturated phospholipids from different subclasses gave identical ion intensities. Quantitative analyses of ethanolamine glycerophospholipid mixtures using ESI-MS were substantiated by examining ion intensities resulting from the coaddition of known amounts of DMPE with 18:3−18:3 PE as internal standard in $CHCl_3$/MeOH (1/1, v/v) to the platelet suspension prior to the Bligh and Dyer extraction and ESI-MS analyses. The increase in the ion intensity of the DMPE molecular ion ($[M-H]^-$) as a function of the amount of exogenous DMPE mass added was linear ($y = 1.02x - 0.08$) with a correlation coefficient (γ^2) = 0.995.

The quantitative accuracy of human platelet anionic phospholipid determinations was substantiated by coaddition of known amounts of either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) or bovine liver phosphatidylinositol with DMPS as internal standard in $CHCl_3$/MeOH (1/1, v/v) to the platelet suspension prior to the Bligh−Dyer extraction and ESI-MS analyses. The increase in the peak intensity of the POPS molecular ion ($[M-H]^-$) as a function of the amount of exogenous POPS mass added was linear ($y = 1.04x - 0.13$) with a correlation coefficient (γ^2) = 0.997. Similar experiments correlating the mass increase obtained from the intensity of 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol (SAPI) molecular ion ($[M-H]^-$) to the amount of exogenous SAPI (from bovine liver phosphatidylinositol) mass added also demonstrated a linear correlation ($y = 1.053x + 0.053$) with a correlation coefficient (γ^2) = 0.998.

Quantification of Human Platelet Choline and Ethanolamine Glycerophospholipid Molecular Species after Stimulation by Thrombin Using Reverse-Phase HPLC. Bligh and Dyer extracts (Bligh & Dyer, 1959) from control and thrombin-treated platelets containing exogenously added internal standards were loaded onto Supelclean (Supelco, PA) and washed with $CHCl_3$ (30 mL). Phospholipids were subsequently eluted with $CHCl_3$/MeOH (1/2,v/v), evaporated under N_2, and applied to Silica gel G plates (1000 μm, Analtech). Phospholipid classes were resolved utilizing a mobile phase comprised of chloroform/acetone/methanol/acetic acid/ water (6/8/2/2/1,v/v/v/v/v). Individual phospholipid classes (ascertained by the migration of standards) were extracted with three washes of chloroform/methanol (1/2, v/v). Individual molecular species of choline and ethanolamine glycerophospholipids were resolved after preparation of their monobenzoate derivatives as previously described (Blank et al., 1983). Briefly, samples were evaporated under N_2 prior to their resuspension in ferrous sulfate-washed diethyl ether and subsequent incubation with *Bacillus cereus* phospholipase C (20 units). Sealed tubes, wrapped in aluminum foil, were incubated for 60 min at 22 °C. The resultant diradyl glycerols were directly benzoylated with benzoic anhydride utilizing *N*,*N*-dimethyl-4-aminopyridine as catalyst (Blank et al., 1983). Derivatives of choline and ethanolamine glycerophospholipid molecular species were resolved by reverse-phase HPLC utilizing an octadecyl silica stationary phase (4.6 mm × 25 cm; 5 μm silica particles) and acetonitrile/2-propanol (85/15,v/v) as the mobile phase. The mass of major molecular species of benzoylated diradyl glycerols was quantitated by comparisons of the integrated areas from absorbance profiles to those of internal standards at 230 nm. Molecular species which comprised only diminutive amounts of lipid mass (less than 3% of lipid in each subclass) were excluded from the tabulated comparisons.

RESULTS

Thrombin-Induced Alterations in the Mass of Ethanolamine Glycerophospholipid Individual Molecular Species. Electrospray ionization mass spectrometric investigation of control human platelet phospholipids in the negative-ion mode demonstrated that (1) plasmenylethanolamine was the predominant ethanolamine glycerophospholipid subclass in human platelets [peaks at *m*/*z* 723 (16:0−20:4 and 18:2−18:2 plasmenylethanolamines), 749 (18:1−20:4 and 16:0−22:5 plasmenylethanolamines), 751 (18:0−20:4 and 16:0−22:4 plasmenylethanolamines), and 779 (18:0−22:4 and 20:0−20:4 plasmenylethanolamines)], (2) plasmenylethanolamines were highly enriched in arachidonic acid (peaks at *m*/*z* 723, 749, 751, 779) with a total of 38.0 nmol of arachidonic acid/10^9 platelets (58% of the 65.8 nmol of arachidonic acid in the ethanolamine glycerophospholipid pool in 10^9 platelets were comprised of plasmalogen molecular species; Table 1),

Table 1: Alterations in Ethanolamine Glycerophospholipid Mass during Thrombin Stimulation of Human Platelets[a]

		resting (nmol/10^9)		stimulated (nmol/10^9)		change (%)	
PE[b]	*m/z*[c]	ESI-MS	HPLC	ESI-MS	HPLC	ESI-MS	HPLC
P16:0−18:2	699	2.2 ± 0.2		1.9 ± 0.2		−13.7	
P16:0−18:1	701	1.5 ± 0.2		0.9 ± 0.2		−40.0	
P18:1−16:0	701	0.7 ± 0.2		0.4 ± 0.1		−42.9	
D16:0−18:2	715	2.3 ± 0.4		2.0 ± 0.3		−13.1	
D16:0−18:1	717	2.3 ± 0.2	4.3 ± 0.7	1.3 ± 0.2	1.9 ± 0.1	−43.5	−55.9
P16:0−20:4	723	7.7 ± 0.7	19.2±2.0	3.5 ± 0.3	10.2 ± 0.2	−54.6	−46.9
P18:2−18:2	723	3.9 ± 0.7		1.8 ± 0.3		−53.9	
D16:1−20:4	737	2.1 ± 0.6		1.8 ± 0.3		−14.3	
D16:0−20:4	739	5.3 ± 0.4	9.5 ± 0.8	3.1 ± 0.6	4.3 ± 0.5	−41.6	−54.8
D18:1−18:2	741	3.8 ± 0.4		2.6 ± 0.4		−31.6	
D18:1−18:1	743	5.9 ± 0.4		3.3 ± 0.5		−44.1	
D18:0−18:1	745	2.9 ± 0.4		1.7 ± 0.3		−41.4	
P18:1−20:4	749	10.4 ± 0.8	8.0 ± 1.0	5.3 ± 0.3	4.8 ± 0.1	−49.1	−40.0
P16:0−22:5	749	2.1 ± 0.8		1.1 ± 0.2		−47.7	
P18:0−20:4	751	17.6 ± 1.2	26.0 ± 3.1	7.6 ± 0.9	11.8 ± 0.5	−56.9	−54.7
P16:0−22:4	751	5.9 ± 1.2		2.5 ± 0.9		−57.7	
D16:0−22:6	763	3.6 ± 0.4		2.9 ± 0.5		−19.5	
D18:1−20:4	765	5.8 ± 0.4	4.2 ± 0.3	3.3 ± 0.3	2.3 ± 0.6	−43.2	−45.3
D16:0−22:5	765	2.9 ± 0.4		1.6 ± 0.3		−44.9	
D18:0−20:4	767	14.6 ± 1.1	28.7 ± 3.1	7.9 ± 0.8	18.2 ± 3.8	−45.9	−36.6
D16:0−22:4	767	7.3 ± 1.1		3.9 ± 0.8		−46.6	
P18:0−22:6	775	2.0 ± 0.2		1.1 ± 0.2		−45.0	
P18:1−22:5	775	2.0 ± 0.2		1.1 ± 0.2		−45.0	
P18:1−22:4	777	3.5 ± 0.4		2.1 ± 0.3		−40.0	
P18:0−22:5	777	3.4 ± 0.4		2.1 ± 0.3		−38.3	
P18:0−22:4	779	6.7 ± 0.5		4.6 ± 0.4		−31.4	
P20:0−20:4	779	2.3 ± 0.5		1.5 ± 0.4		−34.8	
D18:1−22:5	791	2.3 ± 0.2		1.8 ± 0.3		−21.8	
D18:1−22:4	793	2.1 ± 0.3		1.4 ± 0.3		−33.4	
D18:0−22:4	795	2.6 ± 0.5		2.2 ± 0.5		−15.4	
total		137.7	99.9	78.3	53.5	−43.2	−46.5
total AA[d] in pool		65.8	95.6	34.0	51.6	−48.4	−46.1
total AA[d] in plasmenyl-PE		38.0	53.2	17.9	26.8	−52.9	−49.7
total AA[d] in phosphatidyl-PE		27.8	42.4	16.1	24.8	−42.1	−41.6
total plasmenyl-PE		71.9	53.2	37.5	26.8	−47.9	−49.7
total phosphatidyl-PE		65.8	46.7	40.8	26.7	−38.0	−42.9

[a] Washed human platelets were incubated in the absence or presence of 1 unit/mL thrombin for 90 s. Incubations were terminated by addition of chloroform/methanol containing internal standards. Membrane phospholipids were extracted using Bligh and Dyer method and either directly analyzed by negative-ion ESI-MS under mildly basic conditions (ESI-MS) or separated by TLC and converted into their monobenzoate derivatives prior to quantitation of the major molecular species by reverse-phase HPLC (HPLC) as described in Materials and Methods. The results are expressed in nmol/10^9 platelets and represent X ± S.E. of six separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylethanolamine and plasmenylethanolamine molecular species, respectively. Percentage change represents the ratio of the decrease in mass of the phospholipid molecular species after thrombin stimulation relative to the mass of this species in resting platelets. [b] Several minor species corresponding to the alkyl ether species (*m/z* 703, 721, 731, 751, and 753) were also found which persisted even after the samples were treated with acidic vapor. Since these species were of insufficient mass (<0.5% each) to perform tandem mass spectrometric analyses to confirm their identity, they have not been included in the table. [c] The mass of all ions was rounded to the nearest integer. [d] Arachidonic acid-containing phospholipids.

and (3) human platelet ethanolamine glycerophospholipids contained several heretofore undescribed molecular species of plasmenylethanolamine and phosphatidylethanolamine (Figure 1A and Table 1).

Comparisons of ESI mass spectra of control and thrombin-stimulated human platelet phospholipid extracts in the negative-ion mode (Figure 1 and Table 1) demonstrated that plasmenylethanolamines underwent a 53% decrease in mass during 90 s of thrombin stimulation resulting in the liberation of 20.1 nmol of arachidonic acid/10^9 platelets from the plasmenylethanolamine pool. Thrombin-induced hydrolysis of human platelet phosphatidylethanolamine molecular species resulted in a 42% decrease in mass which accounted for a total of only 11.7 nmol of arachidonic acid/10^9 platelets from the phosphatidylethanolamine pool (Table 1). Thus, thrombin induced a loss of almost twice as much arachidonic acid mass from the plasmenylethanolamine compartment in comparison to the phosphatidylethanolamine compartment. The most abundant plasmenylethanolamine molecular species, 1-*O*-(*Z*)-Octadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphoethanolamine underwent a 57% decrease in mass accounting for a loss of 10 nmol of arachidonic acid/10^9 platelets (Table 1).

Thrombin-Induced Alterations in the Mass of Choline Glycerophospholipid Individual Molecular Species. Examination of human platelet phospholipids by ESI mass spectroscopy in the positive-ion mode demonstrated the predominance of phosphatidylcholine molecular species containing either palmitic or stearic acids at the *sn*-1 position and (typically) either oleic or linoleic acids at the *sn*-2 position [(*m/z* 781 (sodiated 16:0−18:2 phosphatidylcholine), 783 (sodiated 16:0−18:1 phosphatidylcholine), and 809 (sodiated 18:0−18:2 or 18:1−18:1 phosphatidylcholines); Table 2 and Figure 2A]. Choline glycerophospholipids contained 41.4 nmol of arachidonic acid/10^9 platelets (Table 2).

Comparisons of ESI mass spectra of chloroform extracts of control and thrombin-stimulated human platelet phospholipids in the positive-ion mode demonstrated that thrombin induced the hydrolysis of approximately 26% of phosphatidylcholine molecular species reflecting a loss of 10.9 nmol

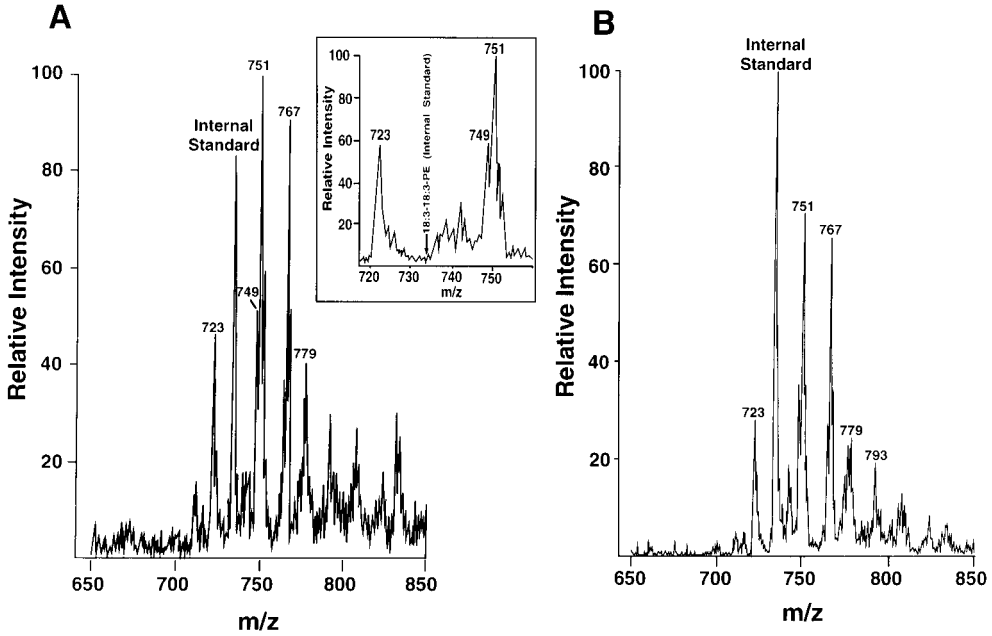

FIGURE 1: Negative-ion electrospray ionization mass spectra of ethanolamine glycerophospholipids in resting and thrombin-stimulated human platelets. (A) A negative-ion ESI mass spectrum of membrane extract from resting human platelets was obtained in the presence of NaOH in 1:2 chloroform/methanol. Briefly, human platelet lipids were extracted, diluted with 1:2 chloroform/methanol in the presence of excess NaOH (i.e., NaOH/lipid molar ratio >1), and infused directly into the ESI chamber with a syringe pump at a flow rate of 1.5 μL/min for mass analyses as described in Materials and Methods. Individual ethanolamine glycerophospholipid molecular species were identified utilizing tandem ESI mass spectrometry and are listed in Table 1. The internal standard is 18:3−18:3 phosphatidylethanolamine. (B) A negative-ion ESI mass spectrum of membrane extract from thrombin-stimulated human platelets was acquired under identical conditions. The insert is an expanded mass spectrum of the region around *m*/*z* 735 obtained from platelet extracts which did not contain exogenously added 18:3−18:3 phosphatidylethanolamine which demonstrates that no endogenous molecular ions are present in this region.

Table 2: Alterations in Choline Glycerophospholipid Mass during Thrombin Stimulation of Human Platelets[a]

PC[b]	*m*/*z* ([M+Na]$^+$)[c]	resting (nmol/10^9)		stimulated (nmol/10^9)		change (%)	
		ESI-MS	HPLC	ESI-MS	HPLC	ESI-MS	HPLC
P16:0−16:0	741	5.1 ± 0.2		4.1 ± 0.2		−19.7	
D16:0−16:1	755	6.5 ± 0.1		4.8 ± 0.2		−26.2	
D16:0−16:0	757	5.3 ± 0.1		4.2 ± 0.2		−20.8	
P16:0−18:1	767	4.9 ± 0.4		2.8 ± 0.1		−42.9	
D16:0−18:2	781	15.3 ± 0.2	29.9 ± 1.5	10.8 ± 0.3	25.0 ± 4.1	−29.5	−16.4
D16:0−18:1	783	26.3 ± 0.7	73.1 ± 2.2	18.0 ± 0.3	63.6 ± 5.3	−31.6	−13.0
D16:0−18:0	785	4.7 ± 0.1		3.3 ± 0.1		−29.8	
P16:0−20:4	789	4.1 ± 0.2		2.3 ± 0.1		−44.0	
P18:0−18:1	795	5.1 ± 0.1		2.8 ± 0.1		−45.1	
D16:1−20:4	803	2.2 ± 0.1		1.9 ± 0.1		−13.7	
D16:0−20:4	805	10.1 ± 0.2	20.2 ± 1.3	7.5 ± 0.3	15.5 ± 3.9	−25.8	−23.3
D18:0−18:2	809	18.3 ± 0.1		13.0 ± 0.2		−29.0	
D18:1−18:1	809	6.1 ± 0.1		4.4 ± 0.2		−27.9	
D18:0−18:1	811	12.5 ± 0.1		9.5 ± 0.2		−24.0	
P18:0−20:4	817	2.6 ± 0.1		2.0 ± 0.1		−23.1	
D18:1−20:4	831	9.5 ± 0.4		7.0 ± 0.2		−26.4	
D18:0−20:4	833	12.9 ± 0.4	30.5 ± 0.8	9.8 ± 0.5	22.0 ± 3.8	−24.1	−27.9
total		151.5	153.7	108.2	126.1	−28.6	−18.0
total AA[d] in pool		41.4	50.7	30.5	37.5	−26.4	−26.1
total AA[d] in plasmenyl-PC		6.7		4.3		−35.9	
total AA[d] in phosphatidyl-PC		34.7	50.7	26.2	37.5	−24.5	−26.1

[a] Washed human platelets were incubated in the absence or presence of 1 unit/mL of thrombin for 90 s. Incubations were terminated by addition of chloroform/methanol containing internal standards. Membrane phospholipids were extracted using Bligh and Dyer method and either directly analyzed by positive-ion ESI-MS (ESI-MS) or separated by TLC and converted into their monobenzoate derivatives prior to quantitation of the major molecular species by reverse phase HPLC (HPLC) as described in Materials and Methods. The results are expressed in nmol/10^9 platelets and represent X ± SE of six separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylcholine and plasmenylcholine molecular species, respectively. Percentage change represents the ratio of the decrease in mass of the phospholipid molecular species after thrombin stimulation relative to the mass of this species in resting platelets. [b] Several minor species corresponding to the alkyl ether species (*m*/*z* 743, 791, 797, and 819) were also found which persisted even after the samples were treated with acidic vapor. Since these species were of insufficient mass (<1% each) to perform tandem mass spectrometric analyses to confirm their identity, they have not been included in the table. [c] The mass of all ions was rounded to the nearest integer. [d] Arachidonic acid-containing phospholipids.

of arachidonic acid/10^9 platelets from this compartment (Figure 2 and Table 2). Positive-ion ESI mass spectrometry demonstrated the selective hydrolysis of phosphatidylcholine molecular species containing arachidonic acid but, in contrast to other previous studies (Smith et al., 1985; Takamura et al., 1987), also demonstrated that substantial hydrolysis of

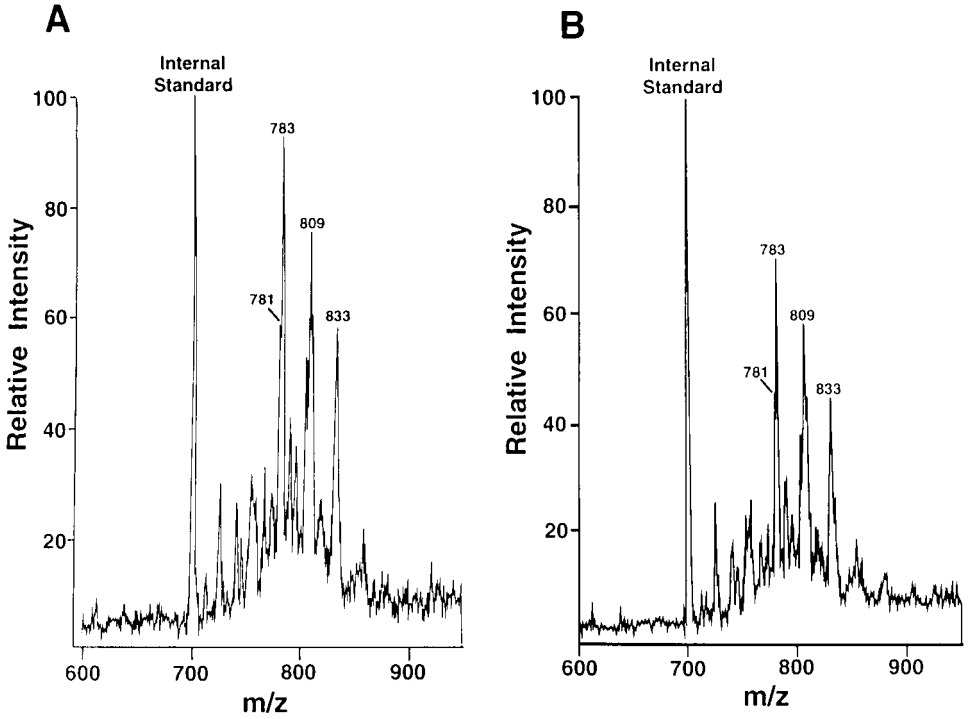

FIGURE 2: Positive-ion electrospray ionization mass spectra of choline glycerophospholipids in resting and thrombin-stimulated human platelets. (A) Membrane lipids in human platelets were extracted, diluted with 1:2 chloroform/methanol, and infused directly into the ESI chamber with a syringe pump at a flow rate of 1.5 μL/min for positive ion ESI mass spectroscopy as described in Materials and Methods. Individual choline glycerophospholipid molecular species were identified utilizing ESI tandem mass spectrometry and are listed in Table 2. The internal standard is 14:0−14:0 phosphatidylcholine. (B) A corresponding positive-ion ESI mass spectrum of membrane extract from thrombin-stimulated human platelets was acquired under the identical conditions.

phosphatidylcholine molecular species containing either oleic or linoleic acids at the *sn*-2 position was manifest (Figure 2 and Table 2). Prior studies have demonstrated the accumulation of diglyceride molecular species containing oleic or linoleic acids at the *sn*-2 position which are believed to be the result of phospholipases C and/or D acting on phosphatidylcholine (Siess et al., 1984; Rubin 1988).

Thrombin-Induced Alterations in the Mass of Human Platelet Serine and Inositol Glycerophospholipids. Electrospray ionization mass spectra of phospholipid extracts of human platelets in the negative-ion mode displayed multiple individual molecular species of phosphatidylserines [*m*/*z* 789 (18:0−18:1 phosphatidylserine), 809 (18:1−20:4 phosphatidylserine) and 811 (18:0−20:4 phosphatidylserine), etc.] in the indicated proportions (Figure 3A and Table 3). The predominant molecular species of human platelet phosphatidylserine contained arachidonic acid at the *sn*-2 position and stearic acid at the *sn*-1 position. Collectively, a total of 21.5 nmol of arachidonic acid/10^9 platelets was present in the phosphatidylserine pool.

Electrospray ionization mass spectroscopic analyses of chloroform extracts of human platelets in the negative-ion mode also demonstrated the presence of multiple individual molecular species of inositol glycerophospholipids [*m*/*z* 836 (16:0−18:1 phosphatidylinositol), 858 (16:0−20:4 phosphatidylinositol), 864 (18:0−18:1 phosphatidylinositol) and 886 (18:0−20:4 phosphatidylinositol), etc.] which were similar to those previously described by Prescott and Majerus, (1981), revealing a total of 17.0 nmol of arachidonic acid/10^9 platelets (Figure 3A and Table 3). The predominant inositol glycerophospholipid contained arachidonic acid at the *sn*-2 position and stearic acid at the *sn*-1 position. Human platelets also contained phosphatidylinositol which possessed oleic acid at the *sn*-2 position (20% of total phosphatidylinositol pool).

Both phosphatidylserine and phosphatidylinositol molecular species containing arachidonic acid at the *sn*-2 position underwent dramatic declines after thrombin stimulation (Figure 3). A total of 8.7 nmol of arachidonic acid/10^9 platelets was lost from the phosphatidylserine pool, and 8.7 nmol of arachidonic acid/10^9 platelets was lost from the phosphatidylinositol pool (Table 3). Phosphatidylserine molecular species and phosphatidylinositol molecular species containing oleic acid at the *sn*-2 position were hydrolyzed nearly as rapidly as those containing arachidonic acid at the *sn*-2 position (Table 3).

Identification of Individual Phospholipid Molecular Species Utilizing ESI Tandem Mass Spectrometry. To analyze individual molecular species of human platelet phospholipids, tandem mass spectrometry was performed to discriminate between isobaric molecular species and individual regioisomers. Selected examples of ESI tandem mass spectra of human platelet phospholipids are shown in Figure 4. Negative-ion ESI tandem mass spectroscopy of the ion at *m*/*z* 723 displayed two carboxylate product ions at *m*/*z* 279 and 303 corresponding to linoleic acid and arachidonic acid, respectively. In addition, a cluster of product ions ($\approx$*m*/*z* 435) corresponding to the neutral loss of *sn*-2 fatty acid was also present. A product ion at *m*/*z* 196 in conjunction with the complete disappearance of *m*/*z* 723 after acidic vapor treatment were used to identify two plasmenylethanolamine molecular species corresponding to 18:2−18:2 plasmenylethanolamine and 16:0−20:4 plasmenylethanolamine with a ratio = 1:2. In ethanolamine glycerophospholipids many isobaric peaks were present reflecting the abundant diversity of individual molecular species in this pool (Table 1). In

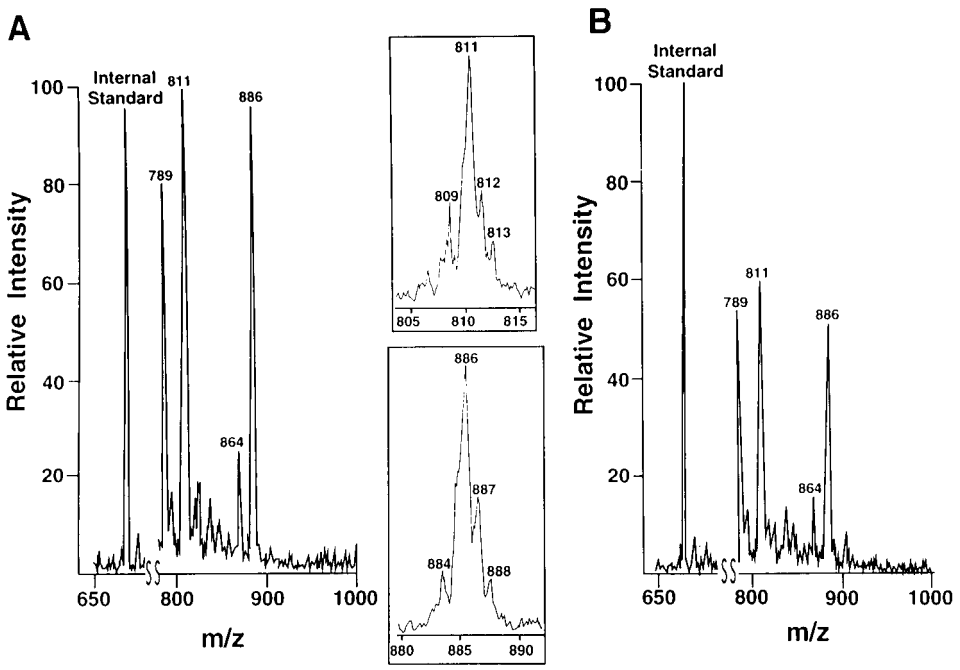

FIGURE 3: Negative-ion electrospray ionization mass spectra of anionic phospholipids in resting and thrombin-stimulated human platelets. (A) A negative-ion ESI mass spectrum of a membrane extract from resting human platelets displayed prominent peaks corresponding to phosphatidylserine (*m*/*z* 789 and 811) and phosphatidylinositol (*m*/*z* 864 and 886) molecular species which have been identified using ESI tandem mass spectrometry (refer to Table 3 for molecular species identification of isobaric peaks). (B) A corresponding negative-ion ESI mass spectrum of membrane extract from thrombin-stimulated human platelets was acquired under the identical conditions. The enlarged insets corresponding to peaks at *m*/*z* 811 and 886 demonstrate resolution of minor molecular species [e.g., *m*/*z* 809 (18:1−20:4 phosphatidylserine) and 884 (18:1−20:4 phosphatidylinositol)] as well as isotopic peaks. The membrane extracts of human platelets were prepared, diluted with 1:2 chloroform/methanol, and infused directly into the ESI chamber with a syringe pump at a flow rate of 1.5 μL/min for mass analyses as described in Materials and Methods. The internal standard is 14:0−14:0 phosphatidylserine.

Table 3: Alterations in Phosphatidylserine and Phosphatidylinositol Mass during Thrombin Stimulation of Human Platelets[a]

PS	MW[b]	resting (nmol/10^9)	stimulated (nmol/10^9)	change (%)
D18:0−18:2	788	4.7 ± 0.3	2.5 ± 0.1	−46.9
D18:0−18:1	790	13.0 ± 0.3	6.8 ± 0.4	−47.7
D18:1−20:4	810	6.4 ± 0.5	5.0 ± 0.5	−21.9
D18:0−20:4	812	15.1 ± 0.3	7.8 ± 0.5	−48.4
D18:0−22:6	836	2.2 ± 0.1	2.2 ± 0.1	0
total		41.4	24.3	−41.4
total AA[c] in pool		21.5	12.8	−40.5
PI	**MW[b]**	**resting (nmol/10^9)**	**stimulated (nmol/10^9)**	**change (%)**
D16:0−18:1	837	2.0±0.2	1.2±0.1	−40.0
D16:0−20:4	859	1.4±0.1	0.9±0.1	−35.8
D18:0−18:1	865	2.3±0.1	1.3±0.1	−43.5
D18:1−20:4	885	2.7±0.2	1.2±0.1	−55.6
D18:0−20:4	887	12.9±0.3	6.2±0.1	−52.0
total		21.3	10.8	−49.3
total AA[c] in pool		17.0	8.3	−51.2

[a] Washed human platelets were incubated in the absence or presence of 1 unit/mL thrombin for 90 s. Incubations were terminated by addition of chloroform/methanol containing internal standards and membrane phospholipids were extracted by the Bligh and Dyer method and directly analyzed by negative-ion ESI-MS as described in Materials and Methods. The results are expressed in nmol/10^9 platelets and represent X ± SE of six separate experiments. Percentage change represents the ratio of the decrease in mass of the phospholipid molecular species after thrombin stimulation relative to the mass of this species in resting platelets. [b] The mass of all ions was rounded to the nearest integer. [c] Arachidonic acid-containing phospholipids

contrast, in choline and anionic phospholipids only diminutive amounts of isobaric molecular species were present (Tables 2 and 3).

Alterations in the Mass of the Major Choline and Ethanolamine Glycerophospholipid Molecular Species after Thrombin Stimulation of Human Platelets Assessed by Reverse-Phase HPLC. To compare the results of thrombin-stimulated platelet phospholipid alterations assessed by ESI-MS with traditionally utilized analytic methodology, additional experiments were performed. Platelet phospholipids from control or thrombin-stimulated platelets were extracted, phospholipid classes were separated by TLC, hydrolyzed by phospholipase C, and benzoylated, and individual molecular species were separated by reverse-phase HPLC as described in Materials and Methods.

Quantification of ethanolamine glycerophospholipid molecular species by integration of the UV absorption profiles of reverse-phase HPLC resolved derivatized molecular species demonstrated that ethanolamine glycerophospholipids were predominantly comprised of plasmalogen molecular species (55%) containing arachidonic acid at the *sn*-2 position (Figure 5A and Table 1). These conclusions are qualitatively similar to conclusions reached utilizing ESI-MS. Analysis of choline glycerophospholipid molecular species by reverse-phase HPLC demonstrated a preponderance of 16:0−18:1 phosphatidylcholine (Figure 6A and Table 2) also in agreement with the ESI-MS studies. Approximately 30% of choline glycerophospholipids contained arachidonic acid at the *sn*-2 position (Table 2, Figure 6A).

Stimulation of platelets with 1 unit/mL thrombin resulted in the rapid hydrolysis of both choline and ethanolamine glycerophospholipid molecular classes as assessed by reverse-phase HPLC analyses (Figures 5 and 6 and Tables 1 and 2). After thrombin stimulation, human platelets lost nearly a fifth of their choline glycerophospholipid content (Table 2) and

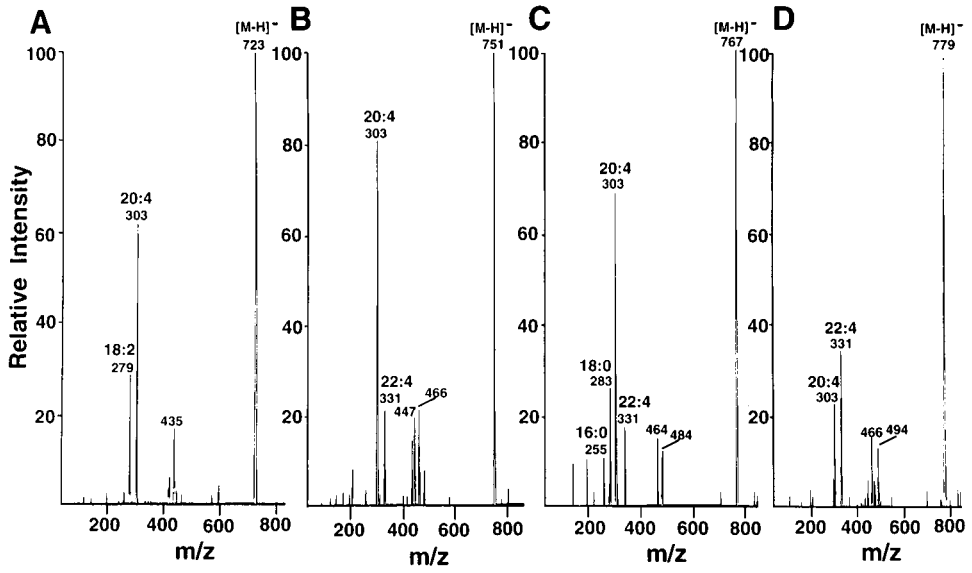

FIGURE 4: Negative-ion ESI tandem mass spectra of selected ethanolamine glycerophospholipids. Precursor ions at *m*/*z* 723 (A), *m*/*z* 751 (B), *m*/*z* 767 (C), and *m*/*z* 779 (D) in Figure 1 were mass selected and collisionally dissociated, and the resultant product ions were analyzed with unit mass resolution as described in Materials and Methods. Collisional dissociation of the precursor ion at *m*/*z* 723 (panel A) after negative-ion ESI demonstrated two carboxylate ions (*m*/*z* 279 and 303), corresponding to 18:2 and 20:4 fatty acids (respectively) facilitating its assignment as 16:0−20:4 and 18:2−18:2 plasmenylethanolamines in a 2:1 molar ratio. Collisional dissociation of the precursor ion *m*/*z* 751 (panel B) demonstrated two carboxylate ions (*m*/*z* 303 and 331) corresponding to 20:4 and 22:4 fatty acids (respectively) facilitating its assignment as 18:0−20:4 and 16:0−22:4 plasmenylethanolamines in a 3:1 ratio. Collisional dissociation of precursor ion *m*/*z* 767 (panel C) demonstrated four carboxylate ions (*m*/*z* 255, 283, 303, and 331), corresponding to 16:0, 18:0, 20:4, and 22:4 (respectively) facilitating its assignment as 18:0−20:4 phosphatidylethanolamine and 16:0−22:4 phosphatidylethanolamine in a 2:1 ratio. Collisional dissociation of precursor ion *m*/*z* 779 (panel D) demonstrated two carboxylate ions (*m*/*z* 303 and 331), corresponding to 20:4 and 22:4 (respectively) facilitating its assignment as 18:0−22:4 plasmenylethanolamine and 20:0−20:4 plasmenylethanolamines in a 3:1 ratio. For quantitative analyses of phospholipids, a ratio of product ions resulting from the *sn*-2 to *sn*-1 carboxylic constituents of 3:1 for chain lengths up to 20 carbons containing up to four double bonds and of 1:1 for constituents containing in excess of 20 carbons and four double bonds was used as described in previous studies (Huang et al., 1992; Han & Gross, 1995) which was also confirmed herein. Plasmenylethanolamine molecular species were distinguished from plasmanylethanolamine molecular species by demonstration of the disappearance of specific ion peaks from ESI mass spectra after platelet extracts were treated with acid vapor as described previously (Ford et al., 1992; Kayganich & Murphy, 1992).

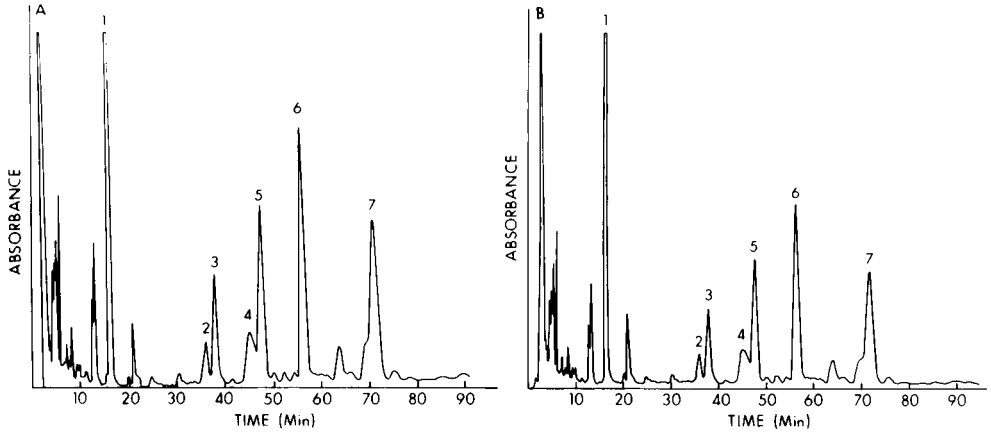

FIGURE 5: Reverse-phase HPLC of monobenzoate derivatives of ethanolamine glycerophospholipids in resting and thrombin-stimulated human platelets. Human platelet ethanolamine glycerophospholipids from resting (A) and thrombin-stimulated (B) human platelets were purified, hydrolyzed by *B. cereus* phospholipase C, and derivatized as described in Materials and Methods. Individual molecular species were separated by reverse-phase HPLC utilizing an octadecyl silica column employing acetonitrile/2-propanol (85/15, v/v) as the mobile phase. Major peaks include the monobenzoate derivatives of 18:3−18:3 phosphatidylethanolamine (1) (internal standard), 18:1−20:4 phosphatidylethanolamine (2), 16:0−20:4 phosphatidylethanolamine (3), 18:1−20:4 plasmenylethanolamine (4), 16:0−20:4 plasmenylethanolamine (5), 18:0−20:4 phosphatidylethanolamine (6), and 18:0−20:4 plasmenylethanolamine (7).

nearly half of their ethanolamine glycerophospholipid mass (Table 1). Specifically, plasmenylethanolamine molecular species lost 26.4 nmol of arachidonate/10^9 platelets while phosphatidylethanolamine molecular species lost 17.6 nmol of arachidonate/10^9 platelets as assessed by reverse-phase HPLC analyses (Table 1). These results are also qualitatively similar to results utilizing ESI-MS. The quantitative differences in these techniques are largely due to the fact that reverse-phase HPLC cannot be utilized to accurately quantitate minor constituents (i.e., 1−3% of total) and that peaks which were thought to represent single molecular species (by their symmetry) actually contain multiple different molecular species. Phosphatidylcholine lost a total of only 13.2 nmol of arachidonate/10^9 platelets as assessed by reverse-phase HPLC (Table 2). Large errors in molecular species analysis of choline glycerophospholipids by reverse-phase

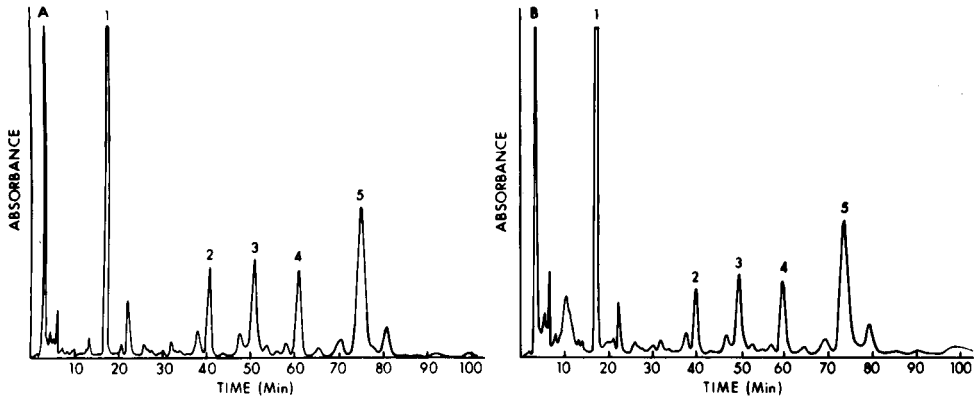

FIGURE 6: Reverse-phase HPLC of monobenzoate derivatives of choline glycerophospholipids in resting and thrombin-stimulated human platelets. Human platelet choline glycerophospholipids from resting (A) and thrombin-stimulated (B) human platelets were purified, hydrolyzed by *B. cereus* phospholipase C, and derivatized as described in Materials and Methods. Individual molecular species were separated by reverse-phase HPLC utilizing an octadecyl silica column employing acetonitrile/2-propanol (85/15, v/v) as the mobile phase. Major peaks identified include the monobenzoate derivatives of 18:3−18:3 phosphatidylcholine (1) (internal standard), 16:0−20:4 phosphatidylcholine (2), 16:0−18:2 phosphatidylcholine (3), 18:0−20:4 phosphatidylcholine (4), and 16:0−18:1 phosphatidylcholine (5).

Table 4: Alterations in Arachidonic Acid Containing Phospholipid Mass in Different Phospholipid Pools during Thrombin Stimulation of Human Platelets Quantified by Electrospray Ionization Mass Spectrometry[a]

phospholipid pools	resting (nmol/10^9)	stimulated (nmol/10^9)	difference (nmol/10^9)	change (%)	AA-released (%)
PC	41.4	30.5	10.9	−26.4	18.1
plasmenyl-PC	6.7	4.3	2.4	−35.8	3.9
phosphatidyl-PC	34.7	26.2	8.5	−24.5	14.1
PE	65.8	34.0	31.8	−48.4	52.9
plasmenyl-PE	38	17.9	20.1	−52.9	33.4
phosphatidyl-PE	27.8	16.1	11.7	−42.1	19.5
PI	17.0	8.3	8.7	−51.2	14.5
PS	21.5	12.8	8.7	−40.5	14.5
Total	145.7	85.6	60.1	−41.2	100

[a] The cumulative amounts of arachidonic acid-containing phospholipids in each of the major phospholipid pools (by class and by subclass) from Tables 1−3 as quantitated by ESI-MS are expressed in nmol/10^9 platelets. "Difference" represents the decrease in the mass of arachidonic acid-containing phospholipids after thrombin stimulation (nmol/10^9 platelets). "Change" represents the percentage decrement of the mass of arachidonic acid-containing phospholipids after thrombin stimulation. "AA-released" represents the percentage contribution of each arachidonic acid-containing phospholipid pool to the total arachidonic acid mass released after thrombin stimulation of human platelets.

HPLC were due to the coelution of multiple molecular species (e.g., 18:0−18:2 and 18:1−18:1) with 16:0−18:1 which were subsequently independently documented by GC analysis of column eluents.

DISCUSSION

From the data accrued in this study, it is clear that the majority of arachidonic acid mass stored in human platelets is present in the ethanolamine glycerophospholipid pool, that plasmenylethanolamines represent the major pool from which arachidonic acid mass is mobilized during thrombin stimulation, and that other phospholipid pools, while hydrolyzed, contribute relatively smaller amounts of arachidonic acid mass after thrombin stimulation (Table 4). The rank order of contributors to arachidonic acid mass released from each phospholipid pool after 90s of thrombin stimulation is plasmenylethanolamine ≫ phosphatidylethanolamine > phosphatidylcholine > phosphatidylinositol ≈ phosphatidylserine (Table 4). Preliminary experiments have demonstrated a similar rank order of hydrolysis 30 s after thrombin stimulation. The combined total loss of arachidonylated choline and ethanolamine glycerophospholipids is over 2-fold more than the mass of the entire phosphatidylinositol pool in human platelets (Table 4), and the loss of arachidonic acid mass from ethanolamine and choline glycerophospholipids is similar to other recent measurements of the mass of nonesterified arachidonic acid accumulating in platelets after thrombin stimulation (Smith et al., 1985; Takamura et al., 1987) when contributions from other pools (e.g., phosphatidic acid, diglyceride, arachidonyl-CoA) are considered (*vide infra*).

The role of plasmalogens in the mobilization of arachidonic acid was first illustrated by Rittenhouse-Simmons et al. (1977) through the demonstration of a dramatic redistribution of radiolabeled arachidonic acid from endogenous phospholipid pools into plasmenylethanolamine molecular species after thrombin stimulation of platelets. Since the plasmenylethanolamine pool was radiolabeled with [^{3}H]-arachidonic acid to a relatively low specific activity compared to other arachidonic acid containing pools, and since the incorporation of concomitantly administered [^{14}C]glycerol was not changed after thrombin stimulation, these results were interpreted as being indicative of the activation of a phospholipase A_2 which led to the turnover of arachidonic acid in the plasmenylethanolamine pool. Chilton and Connell (1988) demonstrated that the majority of ionophore-mediated arachidonic acid release in the neutrophil originated from the plasmenylethanolamine compartment. Subsequently, Fonteh and Chilton (1992) utilized equilibrium labeling conditions to identify that the arachidonic acid mass released from mast cells after ionophore stimulation was due to hydrolysis of the plasmenylethanolamine pool. Similarly,

Wykle and co-workers showed substantial contributions of the plasmenylethanolamine pool to the [^{3}H]arachidonic acid mobilized during ionophore stimulation of neutrophils (Tessner et al., 1990). The current study identifies the alterations in the mass of individual molecular species of plasmenylethanolamines resulting from platelet activation elicited by a natural ligand to demonstrate that plasmenylethanolamine represents the largest source of arachidonic acid mass liberated in thrombin-stimulated human platelets.

Previous studies have reached widely disparate conclusions (over 5-fold differences) in both the amount of arachidonic acid mass released after thrombin stimulation (from 10−50 nmol of arachidonic acid/10^9 platelets) (Bills et al., 1977; Broekman et al., 1981; Neufeld & Majerus, 1983; Smith et al., 1985) and its source of origin (Bills et al., 1977; Broekman et al., 1980; Neufeld & Majerus, 1983; Broekman, 1986; Takamura et al., 1987). In part, the lower values result from the failure to account for arachidonic acid mass lost due to oxidation (e.g., the action of 12-lipoxygenase which is abundant in platelets), while the higher values reflect experiments done in the presence of lipoxygenase inhibitors (Smith et al., 1985). To circumvent potential complications from nonspecific effects of cyclooxygenase and lipoxygenase inhibitors, as well as difficulties resulting from other losses through arachidonic acid metabolism (e.g., arachidonoyl-CoA formation catalyzed by arachidonoyl-CoA synthetase), we employed the strategy of measuring the difference of phospholipids containing arachidonic acid in individual phospholipid classes, subclasses, and molecular species after thrombin stimulation of human platelets. The differences shown in Table 4 represent the lower boundary for the mass of arachidonic acid mobilized since other molecular species containing arachidonic acid (e.g., diglycerides, phosphatidic acid, arachidonoyl-CoA, etc.) are known to accumulate during platelet stimulation (total $\approx$ 10−20 nmol/10^9 platelets), and deacylation−reacylation cycling is rapid.

Prior studies have emphasized the importance of either the phosphatidylinositol pool or the phosphatidylcholine pool as major contributors to arachidonic acid mass released during thrombin stimulation (Bell & Majerus, 1980; McKean et al., 1981; Prescott & Majerus, 1981; Neufeld & Majerus, 1983; Mahadevappa & Holub, 1984; Vickers et al., 1984). The present study shows that although the measurements of the mass of arachidonic acid released from those pools was reasonably accurate, those contributions are dwarfed by the contribution of the ethanolamine glycerophospholipid pool (53%), which predominantly reflects hydrolysis of plasmenylethanolamine molecular species. In retrospect, it seems likely that the prior failure to recognize the contributions of plasmenylethanolamine molecular species to arachidonic acid mass is due to the lability of plasmenylethanolamine molecular species (resulting in part from the silicic acid catalyzed hydrolysis of the vinyl ether linkage) utilizing conventional chromatographic procedures without appropriate precautions.

Direct comparisons of our results with ESI-MS to prior methodologies which employed reverse-phase HPLC for assessment of the mass of individual molecular species were made. Several modifications of published procedures were necessary to facilitate preservation of the vinyl ether linkage during these multistep analyses. With these precautions, results with reverse-phase chromatographic analysis of peak areas under chromophores of individual molecular species are in reasonable qualitative agreement with the results of ESI-MS. However, the inability of reverse-phase HPLC analysis to quantitate the multiple low mass abundance molecular species present in ethanolamine glycerophospholipids (see Table 1) in conjunction with the fact that virtually every peak obtained during reverse-phase HPLC analyses contained more than a single molecular species (although the peaks appeared symmetric) both contribute to substantial errors in quantitative analyses and limit the utility of this approach. Accordingly, results of reverse-phase chromatographic analysis, while qualitatively accurate, need to be interpreted with appropriate caution.

The data from these experiments identify the minimal amount of phospholipase activity that must be expressed during 90 s of thrombin stimulation and specifically delineate the pools which serve as either the direct (e.g., direct hydrolysis of the *sn*-2 linkage of phospholipid substrate) or indirect (e.g., sequential hydrolysis of phospholipid and subsequent transacylation) targets of the participating phospholipases. Since ethanolamine glycerophospholipids account for the majority of mass lost from arachidonylated phospholipids, it is instructive to consider the potential mechanisms mediating alterations in ethanolamine glycerophospholipids during thrombin stimulation. Platelets either must contain a phospholipase which hydrolyzes ethanolamine glycerophospholipids directly or, in the alternative, must hydrolyze another phospholipid pool (e.g., phosphatidylcholine) which is coupled to the ethanolamine pool by formation of a lysophospholipid-acceptor which can serve as cosubstrate for transacylation. Whatever the case, since transacylation is stoichiometric, the boundary conditions for the lower limit for the mass of phospholipid hydrolyzed 90 s after thrombin stimulation is 60 nmol (i.e., $\approx$ 40 nmol/min). Platelets contain 60 ng/10^9 platelets of cPLA$_2$ (Kramer et al., 1993), and the maximal specific activity of cPLA$_2$ documented thus far under optimal conditions (i.e., with high concentrations of glycerol, diglyceride, substrate excess, appropriate phosphorylated isoform, etc.) is 20 units/mg of protein (Kramer et al., 1993). Several investigators have utilized inhibitors to suggest the importance of cPLA$_2$ in catalyzing the majority of arachidonic acid mass released in thrombin stimulated human platelets (e.g., Bartoli et al., 1994). However, recent results show that at least some of these inhibitors (e.g., trifluoromethyl ketones) also are potent inhibitors of calcium-independent phospholipase A$_2$, another major class of intracellular phospholipases (Ackermann et al., 1995). From the measured values of the specific activity of cPLA$_2$ and its mass in platelets, it can be calculated that cPLA$_2$ is responsible for hydrolyzing a maximum of 1−2 nmol of phospholipid (<3% of phospholipid hydrolyzed) within 60 s. Two possibilities exist to explain this paradox. Either the specific activity of cPLA$_2$ has been vastly underestimated and is two orders of magnitude higher *in vivo* than that currently measurable *in vitro* or, in the alternative, another phospholipase is a major contributor of arachidonic acid mass released during thrombin stimulation. We point out that the estimate of arachidonic acid mobilization at 40 nmol/min represents a lower boundary condition since it does not account for deacylation−reacylation cycling or other futile cycles which are known to rapidly occur. Although the results of these experiments cannot discriminate between the initial direct hydrolysis of plasmenylethanolamine or, alternatively, initial hydrolysis of phosphatidylcholine fol-

lowed by transacylation, the *in vitro* substrate selectivities of the transacylase previously identified may provide salient clues. Lysoplasmenylethanolamine is the preferred acyl acceptor of the transacylase (Wykle et al., 1972; Suga et al., 1990) rendering the possibility that initial hydrolysis of plasmenylethanolamine occurs with subsequent transacylation from a phosphatidylcholine donor likely. Whatever the case, the current experiments demonstrate that the loss of arachidonic acid mass is over two orders of magnitude higher than is accountable by current kinetic data utilizing $cPLA_2$ and that major mass losses are present in phospholipid pools which are putatively suboptimal substrates for the $cPLA_2$ (i.e., ethanolamine glycerophospholipids). In conclusion, the present results underscore the importance of plasmenylethanolamines as the major contributor to arachidonic acid mass released during thrombin stimulation, thereby identifying an important target for the phospholipase catalyzing thrombin-mediated arachidonic acid release in platelets.

REFERENCES

Ackermann, E. J., Conde-Frieboes, K., & Dennis, E. A. (1995) *J. Biol. Chem. 270*, 445−450.

Amezcua, J. L., Parsons, M., & Moncada, S. (1978) *Thrombosis Res. 13*, 477−488.

Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M. H., Jain, M. K., & Apitz-Castro, R. (1994) *J. Biol. Chem. 269*, 15625−15630.

Bell, R. L., & Majerus, P. W. (1980) *J. Biol. Chem. 255*, 1790−1792.

Bills, T. K., Smith, J. B., & Silver, M. J. (1976) *Biochim. Biophys. Acta 424*, 303−314.

Bills, T. K., Smith, J. B., & Silver, M. J. (1977) *J. Clin. Invest. 60*, 1−6.

Blank, M. L., Cress, E. A., Lee, T., Stephens, N., Piantadosi, C., & Snyder, F. (1983) *Anal. Biochem. 133*, 430−436.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.

Broekman, M. J. (1986) *J. Lipid Res. 27*, 884−891.

Broekman, M. J., Ward, J. W., & Marcus, A. J. (1980) *J. Clin. Invest. 66*, 275−283.

Broekman, M. J., Ward, J. W., & Marcus, A. J. (1981) *J. Biol. Chem. 256*, 8271−8274.

Chen, S., Kirschner, G., & Traldi, P. (1990) *Anal. Biochem. 191*, 100−105.

Chilton, F. H., & Connell, Th. R. (1988) *J. Biol. Chem. 263*, 5260−5265.

Colard, O., Breton, M., Pepin, D., Chevy, F., Bereziat, G., & Polonovski, J. (1989) *Biochem. J. 259*, 333−339.

Fink, K. L., & Gross, R. W. (1984) *Circ. Res. 55*, 585−594.

Fonteh, A. N., & Chilton, F. H. (1992) *J. Immunol. 148*, 1784−1791.

Ford, D. A., Rosenbloom, K. B., & Gross, R. W. (1992) *J. Biol. Chem. 267*, 11222−11228.

Gross, R. W. (1984) *Biochemistry 23*, 158−165.

Hamberg, M., & Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3400−3404.

Hamberg, M., Svensson, J., Wakabayashi, T., & Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 345−349.

Han, X., & Gross, R. W. (1992) *Biophys. J. 63*, 309−316.

Han, X., & Gross, R. W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10635−10639.

Han, X., & Gross, R. W. (1995) *J. Am. Soc. Mass Spectrom. 6*, 1202−1210.

Han, X., Zupan, L. A., Hazen, S. L., & Gross, R. W. (1992) *Anal. Biochem. 200*, 119−124.

Huang, Z.-H., Gage, D. A., & Sweeley, C. C. (1992) *J. Am. Soc. Mass Spectrom. 3*, 71−78.

Jensen, N. J., Tomer, K. B., & Gross, M. L. (1986) *Lipids 21*, 580−588.

Jesse, R. L., & Cohen, P. (1976) *Biochem. J. 158*, 283−287.

Kayganich, K. A., & Murphy, R. C. (1992) *Anal. Chem. 64*, 2965−2971.

Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., & Jakubowski, J. J. (1993) *J. Biol. Chem. 268*, 26796−26804.

Lewis, N., & Majerus, P. W. (1969) *J. Clin. Invest. 48*, 2114−2123.

Mahadevappa, V. G., & Holub, B. J. (1984) *J. Biol. Chem. 259*, 9369−9373.

Marcus, A. J., Weksler, B. B., Jaffe, E. A., & Broekman, M. J. (1980) *J. Clin. Invest. 66*, 979−986.

McKean, M. L., Smith, J. B., & Silver, M. J. (1981) *J. Biol. Chem. 256*, 1522−1524.

Münster, H., Stein, J., & Budzikiewicz, H. (1986) *Biomed. Environ. Mass Spectrom. 13*, 423−427.

Neufeld, E. J., & Majerus, P. W. (1983) *J. Biol. Chem. 258*, 2461−2467.

Pramanik, B. N., Zechman, J. M., Das, P. R., & Bartner, P. L. (1990) *Biomed. Environ. Mass Spectrom. 19*, 164−170.

Prescott, S. M., & Majerus, P. W. (1981) *J. Biol. Chem. 256*, 579−582.

Rittenhouse-Simmons, S., & Deykin, D. (1977) *J. Clin. Invest. 60*, 495−498.

Rittenhouse-Simmons, S., & Deykin, D. (1981) *Research Monographs in Cell & Tissue Physiology*, Elsevier/North-Holl & Biomedical Press, Amsterdam.

Rittenhouse-Simmons, S., Russell, F. A., & Deykin, D. (1977) *Biochim. Biophys. Acta 488*, 370−380.

Rubin, R. (1988) *Biochem. Biophys. Res. Commun. 156*, 1090−1096.

Siess, W., Weber, P. C., & Lapetina, E. G. (1984) *J. Biol. Chem. 259*, 8286−8292.

Silk, S. T., Clejan, S., & Witkom, K. (1989) *J. Biol. Chem. 264*, 21466−21469.

Smith, J. B., Dangelmaier, C., & Mauco, G. (1985) *Biochim. Biophys. Acta 835*, 344−351.

Suga, K., Kawasaki, T., Blank, M. L., & Snyder, F. (1990) *J. Biol. Chem. 265*, 12363−12371.

Sutherl, C. A., & Amin, D. (1982) *J. Biol. Chem. 257*, 14006−14010.

Takamura, H., Narita, H., Park, H. J., Tanaka, K., Matsuura, T., & Kito, M. (1987) *J. Biol. Chem. 262*, 2262−2269.

Takamura, H., Tanaka, K., Matsuura, T., & Kito, M. (1989) *J. Biochem. (Tokyo) 105*, 168−172.

Tessner, T. G., Greene, D. G., & Wykle, R. L. (1990) *J. Biol. Chem. 265*, 21032−21038.

Vickers, J. D., Kinlough-Rathbone, R. L., & Mustard, J. F. (1984) *Biochem. J. 219*, 25−31.

Walenga, R. W., Opas, E. E., & Feinstein, M. B. (1981) *J. Biol. Chem. 256*, 12523−12528.

Wykle, R. L., Blank, M. L., Malone, B., & Synder, F. (1972) *J. Biol. Chem. 247*, 5442−5447.

BI952927V