Supporting Information

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SI Materials and Methods

Generation of Liposomes. Liposomes were prepared immediately before use from distearoyl phosphatidylcholine (PC) (18:0a/18:0-PC) and varying amounts of aminophospholipids (APLs) (from 0 to 50%). Lipids were dried in a glass vial by evaporation under N_2 and suspended in 0.5 mL buffer (20 mM Hepes, 140 mM NaCl, pH 7.35) with vortexing. In all cases the total concentration of lipid was 0.5 mM, and the molar proportions of phosphatidylserine (PS) (27%) to phosphatidylethanolamine (PE) (73%) remained constant. Liposomes were then generated by 10 freeze thaw cycles with liquid nitrogen, followed by passing through Liposofast miniextruder with 100-nm pore membranes (Avestin) 19 times.

Extraction of Native Phospholipids from Platelets. Lipids were extracted by adding a solvent mixture (1 M acetic acid, propan-2-ol, hexane; 2:20:30, vol/vol/vol) to the sample at a ratio of 2.5 mL of solvent mixture per mL sample, vortexing, and then adding 2.5 mL hexane. After vortexing and centrifugation, lipids were recovered in the upper hexane layer. The lipids were then reextracted by the addition of 2.5 mL of hexane. The combined hexane layers were dried, dissolved in methanol, and stored at −80 °C.

Normal Phase HPLC-UV of Native Platelet Phospholipids. Fractionation of platelet lipid classes used a Spherisorb S5W 150 × 4.6-mm column (Waters) with a gradient of 50–100% B over 25 min (A, hexane:2-propanol, 3:2; B, solvent A:water, 94.5:5.5) at a flow rate of 1.5 mL/min. Absorbance was monitored at 205 nm and products identified by comparison with a mixture of standard PLs.

Precursor, Neutral Loss, and Multiple Reaction Monitoring of Platelet Cellular Phospholipids. All MS was performed on a 4000 Q-Trap hybrid instrument (Sciex). Crude lipid extracts from resting platelets were examined for PS by neutral loss of 87 atomic mass units (amu) in negative mode. Spectra were acquired scanning Q1 from 600 to 1,000 amu over 6 s, declustering potential (DP) −140, electron potential (EP) −10, collision energy (CE) −50, and cell exit potential (CXP) −6. Full mass spectra of purified platelet PE were acquired in positive mode scanning 600–900 amu over 2 s, DP 140 and EP 10. In addition, purified platelet PE was examined for alkyl-acyl- and diacyl-PE species by the neutral loss of 141.1 amu in positive mode. Spectra were acquired scanning Q1 from 600 to 1,000 amu over 12 s, DP 140, EP 10, CE 50, and CXP 10. Crude lipid extracts from platelets were analyzed by multiple reaction monitoring (MRM) after separation by reverse phase HPLC (Phenomenex Luna 3 μ C18 150 × 2 mm) with a gradient of 50–100% B over 10 min followed by 30 min at 100% B (A, methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; B, methanol, 1 mM ammonium acetate) with a flow rate of 200 μL/min.

Synthesis of Biotinylated Standards and Generation of Standard Curves. 1,2-Dimyristoyl-sn-glycero-3-PS (DMPS) [molecular weight (MW) 679.5], 1-stearoyl-2-oleoyl-sn-glycero-3-PS (18:0/18:1-PS; MW 789.6), 1-stearoyl-2-arachidonoyl-sn-glycero-PS (18:0a/20:4-PS, MW 811.6), 1,2-dimyristoyl-sn-glycero-3-PE (DMPE) (di-14:0-PE, MW 724.6), 1-(1Z-stearoyl)-2-arachidonoyl-sn-glycero-3-PE (18:0p/20:4-PE, MW 751.6), and 1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (18:0a/20:4-PE, MW 767.6) were biotinylated in organic solvent, as below. Each (1 mg) was evaporated to dryness before addition of chloroform (220 μL) and methanol (110 μL). NHS-biotin (NB) (6 mg) was added, followed by triethylamine (3.3 μL). The solution was left at room temperature for 30 min before purification by reverse phase on a Discovery C18 column (25 cm × 4.6 mm, 5 μm) at 1 mL/min, using 50–100% mobile phase B (A: water, 5 mM ammonium acetate, B: methanol, 5 mM ammonium acetate) over 15 min, then holding at 100% B for 20 min. Elution was monitored at 205 nm. Lipids were dried by evaporation and weighed. Standard curves were generated by varying the concentration of the representative analyte standards (1 pg–10 ng on column) with a fixed of amount of the internal standards (di-14:0-PS-B and di-14:0-PE-B; 100 pg on column). Where analyte standards were not commercially available, analytes were quantified using data for 18:0p/20:4-PE-B for PE-B plasmalogens, and 18:0a/20:4-PE-B for PE-B diacyls.

Annexin V Binding to Activated Platelets. Platelets were activated using thrombin (0.2 U/mL) for up to 120 min. Each sample was analyzed by biotinylation and LC/MC/MC or by flow cytometry for annexin V binding using a commercially available kit (Annexin V-FITC; Beckman Coulter), in the presence of Tyrodes and 3 mM calcium.

SI Results

Characterization of the Most Abundant Native PS and PE Species in Platelets. The predominant native PS and PE species in platelets were first determined using mass spectrometry as (see below for full details): 18:0a/18:1-PS (*m/z* 788.8), 18:0a/20:4-PS (*m/z* 810.7), 16:0p/20:4-PE (*m/z* 722.6), 18:0a/18:1-PE (*m/z* 744.6), 18:1p/20:4-PE (*m/z* 748.6), 18:0p/20:4-PE (*m/z* 750.6), and 18:0a/20:4-PE (*m/z* 766.6), all $[M-H]^-$ (Fig. S1, shown in positive ion mode). Determination of the major APL species should be undertaken with any new cell type being analyzed for APL externalization to ensure all relevant species are being monitored, because those externalized may differ between tissues.

PS molecular species. Lipid extracts from platelets were analyzed using direct infusion electrospray ionization (ESI)-MS/MS for ions showing neutral loss of 87 amu in negative ion mode. The $[M-H]^-$ ions with the greatest intensity (*m/z* 788.8 and 810.7) correspond to 36:1a- and 38:4-PS (Fig. S1*A*) (1, 2). For each, several isobaric lipids are possible, thus an LC/MS/MS method was devised for each of the possible molecular species, using as daughter ion the carboxylate anion corresponding to the unsaturated (sn2) fatty acid (*Materials and Methods*). For both *m/z* values, one species alone accounted for the majority (>95% intensity) of the signal. Thus, the two most abundant PS species in platelets were identified as 18:0a/18:1-PS (*m/z* 788.8) and 18:0a/20:4-PS (*m/z* 810.7).

PE molecular species. Before MS analysis, platelet PE was first purified using normal phase chromatography, as described in *SI Materials and Methods* (3). Samples were analyzed using direct infusion and the $[M+H]^+$ ions with the greatest intensity selected for further analysis (Fig. S1*B*). These ions were suggested as PEs with the following structures $[M+H]^+$: 36:4p-PE (*m/z* 724.5), 36:1a-PE (*m/z* 746.6), 38:5p-PE or 38e:6-PE (*m/z* 750.6), 38:4p-PE or 38e:5-PE (*m/z* 752.6), 38:4a-PE (*m/z* 768.6), 38:2a-PE (*m/z* 772.6), 38:1a-PE (*m/z* 774.7), 40:0p-PE or 40e:6-PE (*m/z* 788.7), 40:0e-PE (*m/z* 790.6), and 40:1a-PE (*m/z* 802.7) (1, 2). To determine the fatty acid composition of these lipids, LC/MS/MS analysis was carried out in negative ion mode, monitoring parent ions $[M-H]^-$ fragmenting to daughters of all potential sn2 fatty acid carboxylate anions, as described below.

i) For several ions only one single lipid was observed, and the structures of these were determined as $[M-H]^-$ *m/z* 722.6 (16:0p/20:4-PE), *m/z* 744.6 (18:0a/18:1-PE), *m/z* 748.6 (18:1p:20:4-PE), *m/z* 750.6 (18:0p/20:4-PE), and *m/z* 766.6 (18:0a/20:4-PE).

ii) The prominent ion at *m/z* 790.6 ($[M+H]^+$) could represent 40:0e-PE, although ether-PLs are not thought to be abundant in platelets. To investigate this, negative ion LC/MS/MS monitored transitions of *m/z* 788.6 ($[M-H]^-$) to carboxylate anions of daughter fragments derived from all possible sn2 fatty acids. Additionally, a negative MS/MS spectrum for *m/z* 788.6 was generated during LC/MS/MS elution, with data-dependent acquisition in ion trap mode. Neither approach could identify the ion as a known PE lipid. Similarly, *m/z* 774.7, 788.7, and 802.7 could not be confirmed as PE, or gave very weak signals. Thus, these ions are not considered to be PEs and were not monitored in subsequent studies.

iii) For the *m/z* 772.6 ion, two weak signals were observed, corresponding to 18:1a/20:1-PE and 20:0a/18:2-PE. However, when monitoring externalization in platelets, biotinylated forms of these were frequently below the limit of detection and so they were not included in the assay.

iv) For *m/z* 770, weak signals corresponding to both 18:1a/20:1-PE and 20:0a/18:2-PE were both observed, but these were too weak for routine monitoring in samples.

Thus, the predominant PE species detected in platelets were 16:0p/20:4-PE, 18:0a/18:1-PE, 18:1p:20:4-PE,18:0p/20:4-PE, and 18:0a/20:4-PE. Table S1 shows the species with specific daughter ions monitored in subsequent studies.

Establishing a Quantitative Assay for Surface Exposed Aminophospholipids. Biotinylated 18:0a/18:1-PS, 18:a/20:4-PS, 18:0p/20:4-PE, 18:0a/20:4-PE, di-14:0-PS, and di-14:0-PE were synthesized by treatment of commercially available lipids with NB, as described in *SI Materials and Methods*. Daughter ions for the 18:0a/20:4-PS-B species include *m/z* 283.4 and 303.4, corresponding to carboxylate anions for 18:0 (stearic acid) and 20:4 (arachidonic acid), respectively (Fig. S1*C*). *m/z* 723.6 is from the neutral loss of 313 amu (the biotinylated PS headgroup) (Scheme S1). The *m/z* 312.2 ion is formed by rearrangement and cyclization of the biotinylated PS headgroup ($[M-H-C_{13}H_{18}N_3O_4S]^-$) (Scheme S1). The *m/z* 419.4 corresponds to loss of both the sn2 fatty acid and the biotinylated PS headgroup. Daughter ions for the 18:0a/20:4-PE-B species include $[M-H]^-$ fragments derived from the acyl chains *m/z* 283.3 and 303.4, and *m/z* 366.3 corresponding to the biotinylated PE headgroup (Fig. S1*D*). MS/MS fragmentation patterns for 18:a/20:4-PE and -PS are shown in Scheme S1 and Fig. S1 *C* and *D*.

The assay was first optimized using liposomes with APL concentrations that mimic the amount of surface exposed APLs seen in activated platelets (at typical plasma concentration of 2×10^8 platelets/mL). Total lipid concentration was 4 μM, composed of 2.5 μM PC (18:0a/18:0-PC) and 1.5 μM APL (18:0a/20:4-PS and 18:0a/20:4-PE at a ratio of 27–73%). Biotinylation is undertaken by incubating liposomes for 10 min in the presence of 1 mg/mL Sulfo-NHS-Biotin (SNB), followed by addition of 50 mM lysine for a further 10 min at room temperature. Next, Di-14:0-PS-B and di-14:0-PE-B are added as internal standards (10 ng per sample). A previous version of this assay was used to show externalization of oxidized phospholipids on the platelet surface.

Removing unreacted biotin. A saturating concentration of lysine is added immediately before lipid extraction to remove unreacted biotinylation reagent, rather than centrifuging the cells, which could alter activation status. A final concentration of 50 mM lysine was chosen according to titrating levels (Fig. S2 *A* and *B*).

Optimizing biotinylation conditions. Liposomes were treated with varying amounts of SNB, to ensure that the reaction had proceeded to completion within 10 min, with 1.5 mg/mL SNB selected for assay development (Fig. S2 *C* and *D*).

Extraction method. To ensure maximal lipid recovery, different extractions were compared. Using a hexane/isopropanol/acetic acid liquid extraction, native PLs extract efficiently (4). In contrast, owing to the presence of a more polar headgroup, biotinylated APLs were poorly recovered but more efficiently extracted using chloroform/methanol extraction as described by Bligh and Dyer (Table S2) (5). Thus, hexane/isopropanol/acetic acid was used for lipid profiling of native APL in platelets, whereas chloroform/methanol was used for all studies with biotinylated lipids, as described in *Materials and Methods*.

Chromatography and MS/MS detection. A series of reverse phase columns were tested and best results obtained with an Ascentis C18, 5-μm particle size, 150 × 2.1-mm column (Sigma-Aldrich), with an isocratic separation (*Materials and Methods*) (Fig. S2 *E*–*K*). For detection of PS-B, the daughter ion corresponding to loss of headgroup (NL 313) was used (Fig. S2 *E* and *F*), whereas for PE-B, daughter ions corresponding to either sn2 fatty acid (*m/z* 303 or 283) or headgroup (*m/z* 366) are shown (Fig. S2 *G*–*K*). Because the headgroup ion for PE-B is small, we used the sn2 fatty acid for quantitation. All MS conditions used are given in Table S1.

Standard Curves, Sensitivity, Linearity, and Use for Detection of Externalized APL by Cells. Standard curves were constructed, varying concentrations of the representative analyte standards (18:0a/18:1-PS-B, 18:0a/20:4-PS-B, 18:0p/20:4-PE-B, and 18:0a/20:4-PE-B), with a fixed of amount of internal standards (di-14:0-PS-B and di-14:0-PE-B), as described in *Materials and Methods*. Quantitation was achieved using 18:0a/20:4-PE-B for 18:0a/18:1-PE-B; and 18:0p/20:4-PE-B for both plasmalogen PE-Bs (16:0p/20:4-PE, 18:1p/20:4-PE-B). All standard curves were linear for amounts of analyte between 1 pg and 10 ng on column (Fig. S2 *L*–*O* and Table S2). Intersample coefficient of variation was 5–8%, with intrasample approximately 10–12%.

Confirming Detection of Externalized APL in Platelets. SNB has been extensively used by the proteomic community for labeling cell surface proteins, since it was first described as a membrane-impermeable biotinylation reagent (6). Its use in this way was also recently reviewed by Elia (7). To confirm detection of external APLs, a direct comparison was performed between biotinylation initiated by SNB, and its cell permeable analog, NB, with both incubated with platelets for 0–30 min. For NB, lipids were extracted in the absence of lysine to ensure that the reagent was able to fully biotinylate all cellular APL species. The data shows that at 10 min (the time used in our experiments), almost none of the PE or PS species are biotinylated (Fig. S3 *A* and *B*). At times longer than 10 min, some small biotinylation becomes evident. However, we cannot distinguish whether this biotinylation is SNB becoming internalized, or labeling of a small amount of APL that is cycling between the outer and inner membranes. However, the data conclusively show that SNB does not appreciably biotinylate intracellular APL during a 10-min incubation with live platelets. In contrast, Fig. 2 *E* and *F* show the detection of externalized APLs that occurs on thrombin activation at 180 min, and that is virtually absent in control platelets also incubated for 180 min (all with 10 min SNB). The difference between the control and thrombin-treated platelets represents the externalization that occurs on activation of the cells.

SI Discussion

To determine which APLs to measure in our assay, we first characterized the predominant PS and PE species and found them consistent with previous reports for platelets and neutrophils (3, 8). This exercise should be repeated if using the assay for

other cell types, to ensure that the most abundant APL species are being monitored. For biotinylation, a 10-min incubation with SNB was chosen because derivatization of purified standards or liposomes was essentially complete within this time period (Fig. S2). Additionally, longer incubation periods could adversely impact platelet activation status. We recognize that APLs may be recycled between the inner and outer leaflet of the platelets during the 10-min incubation. Thus, the assay specifically labels APLs that were expressed on the external membrane surface during this period.

The assay could also be adapted to HPLC-fluorescence or absorbance detection, by using an amine-reactive fluorophore or chromophore in place of SNB, several of which are commercially available for protein-labeling studies. The only disadvantages of the assay vs. annexin V are (*i*) inability to distinguish individual cells that externalize APLs vs. from those that do not (e.g., positive and negative cells) and (*ii*) inability to study cells suspended in lipid-containing solutions (e.g., plasma, or serum-containing tissue culture medium) without prior centrifugation.

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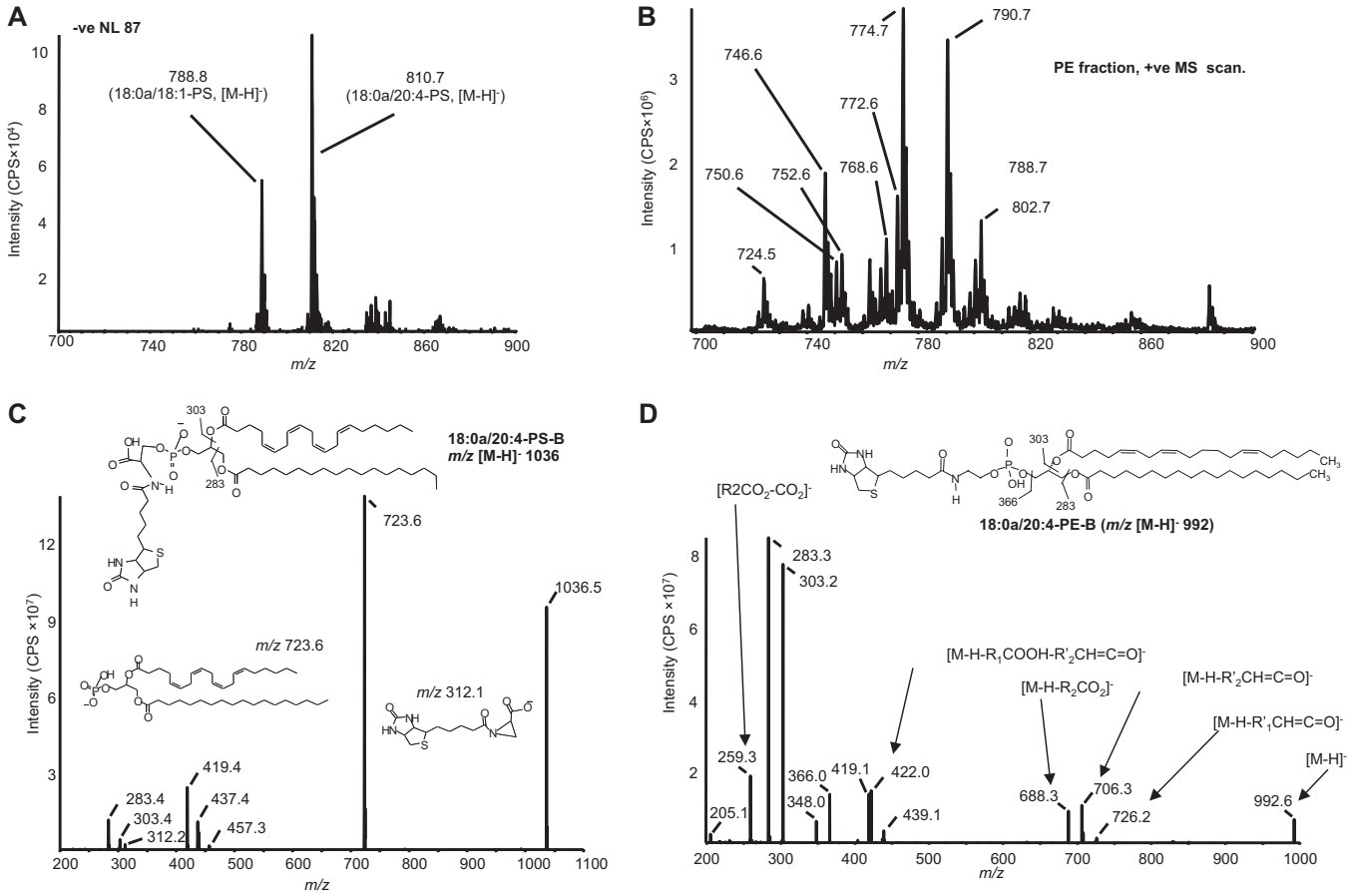


Fig. S1. Identification of PE and PS molecular species in platelets, and MS/MS analysis of biotinylated standards. (*A*) Neutral loss scan of PS from platelet lipids. Negative neutral loss scan of 87 amu in negative ion mode was carried out on lipid extracts from human platelets. (*B*) Positive ion scan of normal phase purified platelet PE lipids. PE was purified from platelet lipid extracts using normal phase chromatography, as described in *Materials and Methods*, then scanned in negative ion mode. (*C*) Negative MS/MS spectrum of biotinylated PS standard. 18:0a/20:4-PS-B (*m/z* 1036, $[M\text{-}H]^-$) was analyzed using the 4000 Q-Trap in ion trap mode. (*D*) Negative MS/MS spectrum of biotinylated PE standard. 18:0a/20:4-PE-B (*m/z* 992, $[M\text{-}H]^-$) was analyzed using the 4000 Q-Trap in ion trap mode.

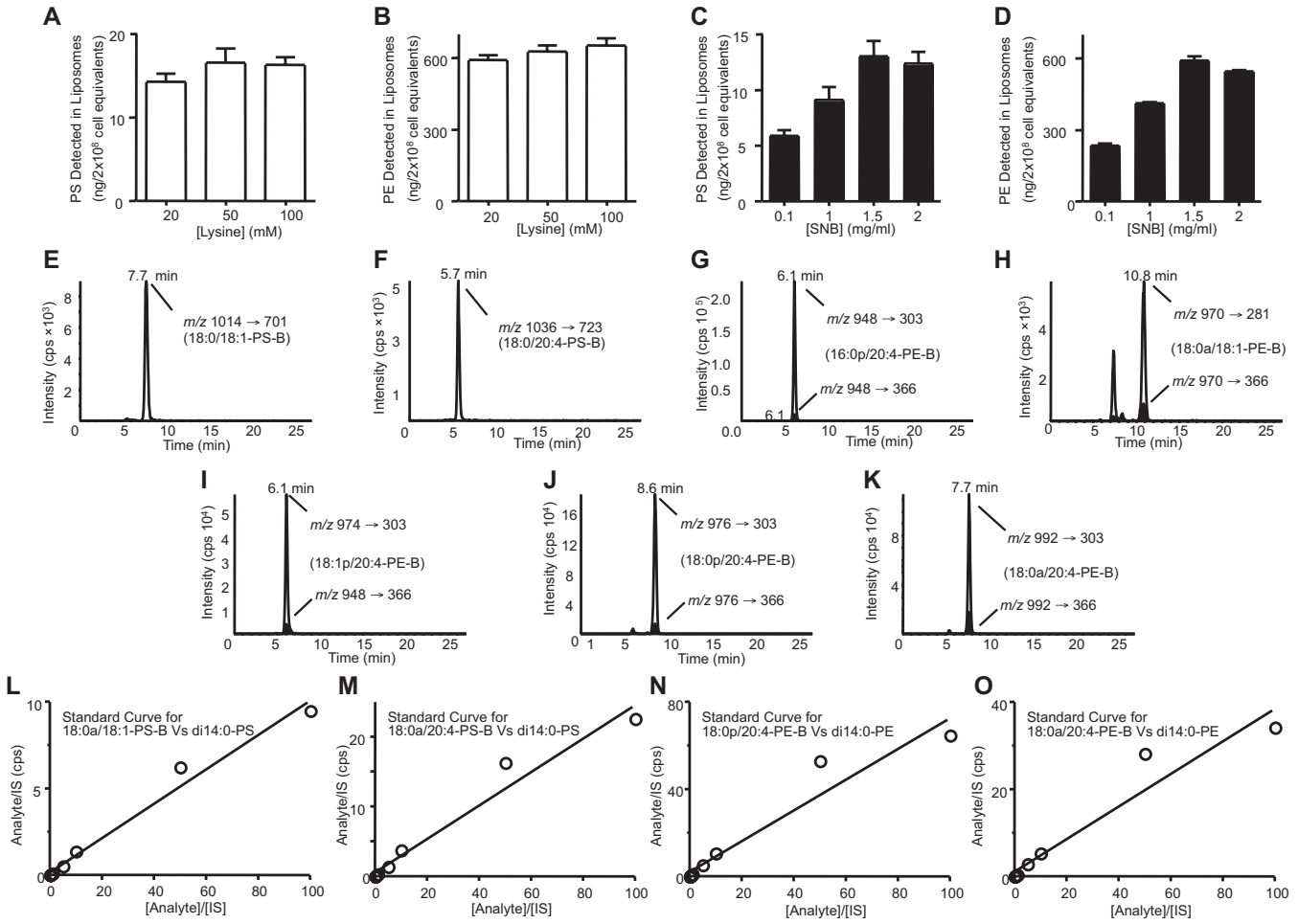


Fig. S2. Establishing a quantitative assay for surface exposed aminophospholipids. (*A* and *B*) Optimization of added lysine concentration. Liposomes were biotinylated using SNB and treated with increasing concentrations of lysine as described in *Materials and Methods*, then lipids extracted and analyzed using LC/MS/MS. (*C* and *D*) Optimization of SNB concentration for effective biotinylation. Liposomes were treated with increasing concentrations of SNB as described in *Materials and Methods*, then lysine added and lipids extracted and analyzed using LC/MS/MS as described in *Materials and Methods*. (*E* and *F*) Negative LC/MS/MS of 18:0/18:1-PS-B and 18:0/20:4-PS-B from human platelets. Platelets were thrombin activated and treated with SNB and lysine, then lipids were extracted and analyzed using LC/MS/MS, as described in *Materials and Methods*. Biotinylated PS is monitored based on neutral loss of the headgroup (313 amu). (*G–K*) Negative LC/MS/MS of 16:0p/20:4-PE, 18:0a/18:1-PE, 18:1p/20:4-PE, 18:0p/20:4-PE, and 18:0a/20:4-PE in human platelets. Platelets were activated and treated with SNB, then lipids were extracted and analyzed using LC/MS/MS, as described in *Materials and Methods*. Biotinylated PE is monitored either by formation of the Sn2 fatty acid carboxylate anion (*m/z* 303 for 20:4, *m/z* 281 for 18:1) or the headgroup ion *m/z* 366 on collision-induced dissociation. (*L–O*) Standard curves constructed for analytes vs. internal standards (IS) used for quantitation of externalized APLs. Standard curves were created by analyzing 100 pg internal standard (IS) (DMPS-B or DMPE-B) with varying amounts of the analyte standard (10 ng–1 pg) (18:0a/18:1-PS-B, 18:0a/20:4-PS-B, 18:0a/20:4-PE-B or 18:0p/20:4-PE-B), using LC/MS/MS as described in *Materials and Methods*.

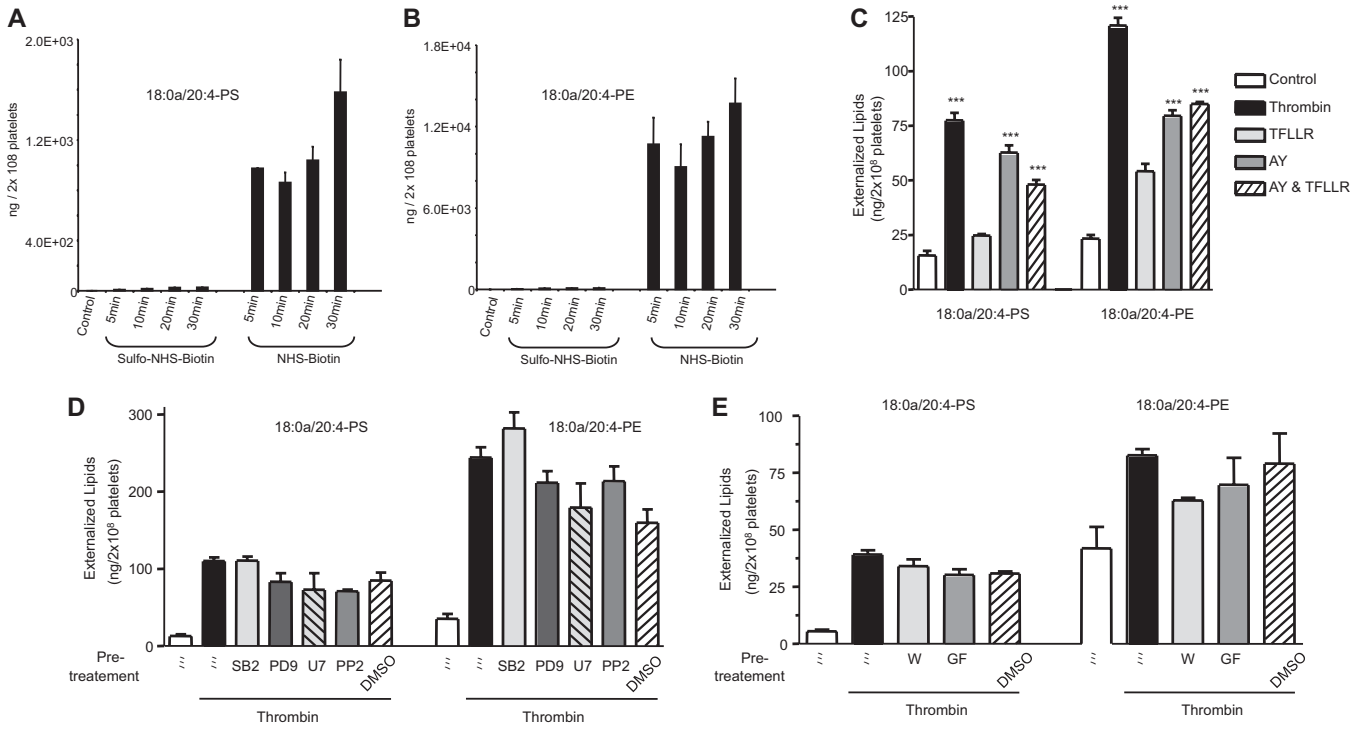
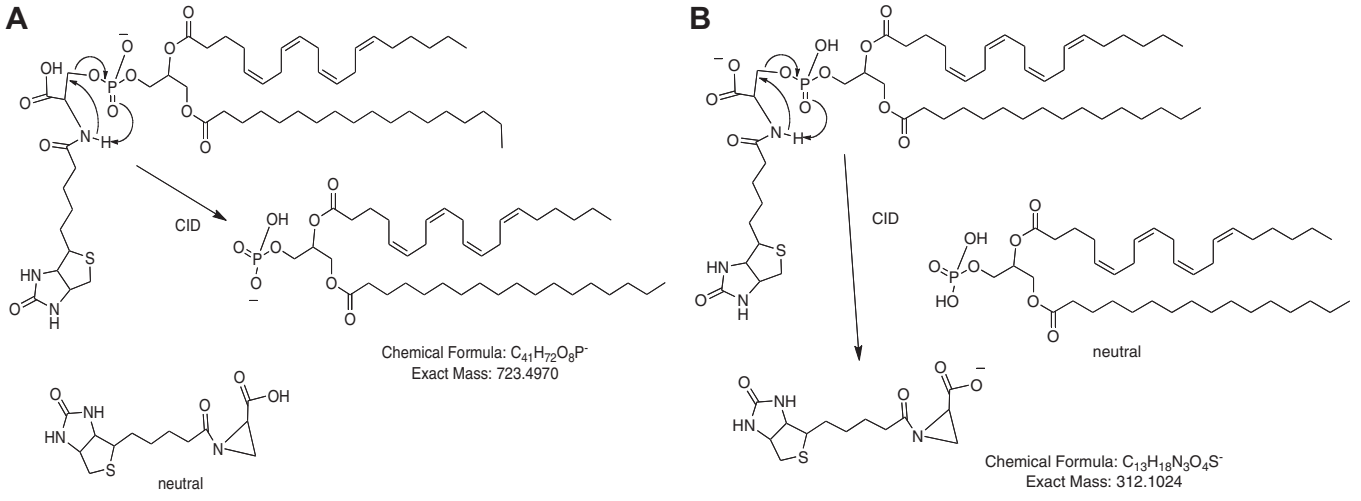


Fig. S3. SNB labels extracellular lipids on platelets; protease-activated receptor (PAR) agonists activate PE and PS externalization, whereas several intracellular signaling pathways are not required. (*A* and *B*) Washed platelets were incubated at room temperature for 5, 10, 20, and 30 min with the cell-impermeable SNB or cell-permeant NB before quenching of unreacted SNB with lysine for 10 min. Externalized APL were extracted and analyzed by LC/MS/MS as described in *Materials and Methods*. (*C*) Platelets were activated using thrombin (0.2 U/mL; PAR1 and PAR4), TFLLR-NH2 (TFLLR; 40 μM; PAR 1 agonist), AY-NH2 (AY; 150 μM; PAR4 agonist), or both of the selective agents (AY and TFLLR) for 30 min, before biotinylation, lipid extraction, and LC/MS/MS as described in *Materials and Methods*. (*D* and *E*) Platelets were activated with thrombin after preincubation with inhibitors, then surface APL analyzed as described in *Materials and Methods*. Inhibitors used are as follows: for p38 MAP kinase (SB203580, 10 μM; SB2), MAPK/ERK kinase 1 (PD98059, 50 μM; PD9), phospholipase C (U-73112, 5 μM, U7), Src-family tyrosine kinase (PP2, 50 μM), PI3 kinase (Wortmannin, 100 nM; W), protein kinase C (GF-109203X, 1 μM; GF), or vehicle (DMSO, 0.5%). For all experiments, $n = 3$; mean ± SEM; data representative of three independent donors. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. thrombin activated, using ANOVA and Bonferroni post hoc test.



Scheme S1. Proposed collision-induced-decomposition fragmentation pattern for biotinylated PS.

Table S1. Mass spectrometry parameters for the most abundant PS and PE species in platelets

Lipid name	Mass (amu)	B-Mass (amu)	Parent ion (*m/z*)	Daughter ion (*m/z*)	RT (min)	DP	CE	CXP
18:0a/18:1-PS	789	1,015	1,014.6	701.6	7.7	−140	−44	−23
18:0a/20:4-PS	811	1,037	1,036.6	723.6	5.7	−145	−42	−23
16:0p/20:4-PE	723	949	948.8	303.3	6.1	−170	−58	−5
18:0a/18:1-PE	745	971	970.8	281.2	10.8	−170	−58	−5
18:1p/20:4-PE	749	975	974.8	303.3	6.1	−160	−60	−5
18:0p/20:4-PE	751	977	976.8	303.3	8.6	−160	−60	−5
18:0a/20:4-PE	767	993	992.8	303.3	7.7	−170	−58	−5
14:0a/14:0-PS	679	905	904.7	591.6	3.3	−150	−42	−17
14:0a/14:0-PE	635	861	860.8	227.3	4.1	−135	−60	−13

Mass, nominal mass of the molecular species; B-Mass, nominal mass of the molecular species after biotinylation; Parent ion, *m/z* of the negative ion $[M\text{-}H]^-$ after biotinylation; Daughter ion, *m/z* of the daughter ion used to measure the species during MS/MS (MRM); either the parent minus the biotinylated PS head-group or a fragment derived from the Sn2 fatty acid. CE, collision energy, and CXP, cell exit potential used for analysis; DP, declustering potential; RT, retention time of the species on reverse phase HPLC.

Table S2. Validation parameters

Lipid name	Intrasample variation (%)	Intersample variation (%)	Recovery (%)	r^2
18:0a/18:1-PS-B	4.2	5.5	63	0.981
18:0a/20:4-PS-B	8.1	7.5	58	0.965
18:0p/20:4-PE-B	7.8	5.7	67	0.936
18:0a/20:4-PE-B	9.1	6.2	56	0.934
14:0a/14:0-PS-B	11.3	8.9	64	—
14:0a/14:0-PE-B	14.4	8.8	75	—

Intrasample variation was determined by analyzing one sample 10 times and calculating the coefficient of variation. Intersample variation was carried out by analyzing six separate samples. Percentage recovery was estimated by extracting samples and comparing peak area with unextracted standard ($n = 6$). r^2 (fit to linearity) was determined using Excel for standard curves with standards plotted as [A/IS] (integrated area), vs. [A/IS] (ng), as described in *SI Materials and Methods*. —, not applicable.

Table S3. Summary of pathways involved in APL exposure during different activation conditions

Pathway	Thrombin	Energy depletion	Apoptosis
Calcium	Y	Y	Y
Caspase	N	N	Y
TMEM-16F	Y	Y	N

N, no; Y, yes; TMEM-16F, transmembrane protein-16F.