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Platelet Lipidomic Profiling: Novel Insight into Cytosolic Phospholipase $A_2\alpha$ Activity and Its Role in Human Platelet Activation

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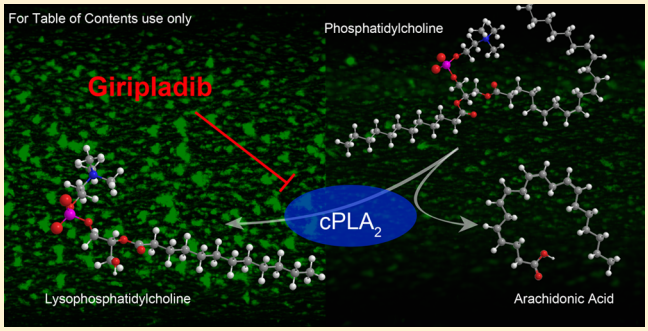
Supporting Information

ABSTRACT: With a newer, more selective and efficacious cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) inhibitor available, we revisited the role of cPLA$_2\alpha$ activity in platelet activation and discovered that a component of platelet signaling, even larger than previously appreciated, relies on this enzyme. In a whole blood shear-based flow chamber assay, giripladib, a cPLA$_2\alpha$ inhibitor, reduced platelet adhesion and accumulation on collagen. Moreover, giripladib differentially affected P-selectin expression and GPIIbIIIa activation depending on the agonist employed. While protease-activated receptor 1 (PAR1)-mediated platelet activation was unaffected by giripladib, the levels of PAR4- and GPVI-mediated platelet activation were significantly reduced. Meanwhile, the thromboxane A_2 receptor antagonist SQ29548 had no effect on PAR-, GPVI-, or puriniergic receptor-mediated platelet activation, suggesting that another eicosanoid produced downstream of arachidonic acid liberation by cPLA$_2\alpha$ was responsible for this large component of PAR4- and GPVI-mediated platelet activation. In parallel, we profiled PAR-mediated changes in glycerophospholipid (GPL) mass with and without giripladib to better understand cPLA$_2\alpha$-mediated lipid metabolism. Phosphatidylcholine and phosphatidylethanolamine (PE) demonstrated the largest consumption of mass during thrombin stimulation. Additionally, we confirm phosphatidylinositol as a major substrate of cPLA$_2\alpha$. A comparison of PAR1- and PAR4-induced metabolism revealed the consumption of more putative arachidonyl-PE species downstream of PAR1 activation. Instead of enhanced cPLA$_2\alpha$ activity and therefore more arachidonic acid liberation downstream of PAR4, these results indicate the major role that cPLA$_2\alpha$ activity plays in platelet function and suggest that a novel eicosanoid is produced in response to platelet activation that represents a large component of PAR4- and GPVI-mediated responses.



Platelet stimulation with thrombin results in the production of thromboxane A_2 (TXA_2) through both platelet–thrombin receptors protease-activated receptor 1 (PAR1) and PAR4.[1,2] Circulating TXA_2 activates the TXA_2 receptor (TP), a G_q-coupled receptor that enhances or amplifies other platelet stimuli.[3,4] As a result, the pathway regulating its generation has been an area of pharmacological interest for the prevention of platelet-dependent vascular occlusion for many decades. TXA_2 is produced by the action of cyclooxygenase-1 (COX-1) and TXA_2 synthase on arachidonic acid liberated from glycerophospholipids (GPLs). Platelet COX-1 is the target of aspirin, which is widely used for the prevention of myocardial infarction, stoke, and occlusive vascular events.[5,6]

The liberation of arachidonic acid from GPL sources occurs through the phospholipase A_2 family of enzymes. Platelets contain the 85 kDa cytosolic phospholipase A2 α (cPLA$_2\alpha$), which requires micromolar Ca^{2+} for activity,[7] and the 14 kDa secretory PLA_2 (sPLA$_2$),[8] which requires millimolar concentrations of Ca^{2+} for activity. However, there is no evidence of the activity of sPLA$_2$ within the platelet cytosol. Additionally, evidence of activity of a calcium-independent PLA_2 (iPLA$_2$) in the platelet has been suggested on the basis of the effectiveness of the iPLA$_2$ inhibitor bromoenol lactone (BEL) against TXA_2 production.[9,10] Indeed, arachidonic acid release has been observed in platelets isolated from cPLA$_2\alpha^{-/-}$/sPLA$_2$-IIA$^{-/-}$ mice, suggesting the involvement of another PLA_2 isoform.[11] However, genetic evidence in human platelets demonstrates that the majority of platelet TXA_2 generation occurs as a result of cPLA$_2\alpha$ activity.[12]

The lack of an efficacious cPLA$_2\alpha$ inhibitor has prevented a detailed investigation into arachidonic acid mobilization in the platelet or its influence on platelet activation. However, pharmaceutical companies have recently bolstered their efforts to target PLA_2 enzymes because of their relationship to the progression of a number of inflammatory diseases. The cPLA$_2\alpha$ inhibitor Wyeth-2, also known as giripladib and PLA-695, inhibits cPLA$_2\alpha$-mediated release of arachidonic acid from GPL sources and was originally developed and tested for efficacy

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against arthritis. Clinical trials were halted because of unforeseen gastrointestinal pain (clinical trials.gov); however, the compound remains the most specific and potent inhibitor of cPLA$_2\alpha$ activity available. In 2008, Wyeth reported that oral treatment with 3 or 100 mg/kg twice daily significantly reduced clinical and microscopic disease scores in a murine, collagen-induced arthritis model. Similarly, oral treatment with 3 or 10 mg of giripladib/kg in a K/BxN model of arthritis significantly reduced ankle swelling and microscopic disease scores [Drug Data Report 30(7), 611 (2008)]. Given its efficacy, it has been repurposed and is being studied as a possible radiosensitizing agent in the treatment of radioinsensitive tumors.[13,14] We utilized giripladib to improve our understanding of cPLA$_2\alpha$ activity during GPL remodeling after thrombin stimulation and the role of cPLA$_2\alpha$ in platelet activation.

We used a lipidomic approach developed by the Brown lab[15] to profile changes in the mass of GPLs following stimulation with thrombin. The application of electrospray ionization mass spectrometry to the field of lipidomics has accelerated our understanding of lipid metabolism by allowing the detection of lipid molecular species quickly from a small amount of source material. With an initial phase of separation by liquid chromatography prior to direct injection, lipid species from different classes can be identified from a complex mixture. Furthermore, the injection of internal standards has facilitated reliable quantitation of these lipid species. We profiled changes in GPLs at the species level and challenged these patterns with the cPLA$_2\alpha$ inhibitor giripladib.

EXPERIMENTAL PROCEDURES

Blood Collection and Platelet Isolation. Human platelets were obtained from healthy volunteers. The studies were approved by the Vanderbilt University Internal Review Board. Informed consent was obtained from all individuals prior to the blood draw. Blood was collected into sodium citrate anticoagulant (final concentration of 0.32%) through a 19 gauge needle. Washed platelets were prepared as previously described[16] and suspended in Tyrodes buffer (10 mM HEPES, 11.9 mM sodium bicarbonate, 127.2 mM sodium chloride, 5 mM potassium chloride, 0.4 mM sodium phosphate monobasic, 1 mM magnesium chloride hexahydrate, and 5 mM D-glucose) to a density of 3.0×10^8 cells/mL.

Flow Cytometry. For detection of P-selectin or GPIIbIIIa activation, platelets at a density of 1.5×10^7 cells/mL were preincubated with Allophycocyanin (APC)-conjugated CD62P and Phycoerythrin (PE)-conjugated PAC1 (BD biosciences, San Jose, CA) before a 10 min preincubation with inhibitor or vehicle followed by stimulation with the appropriate agonist for 10 min. Giripladib was a gift provided by C. Lindsley and synthesized at the Vanderbilt Center for Neuroscience and Drug Discovery (Nashville, TN). SQ29584 and U46619 were from Cayman chemical (Ann Arbor, MI). PAR1-activating peptide (AP) (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). Adenosine diphosphate (ADP) was purchased from Sigma-Aldrich. Convulxin (CVX) was purchased from Santa Cruz (Dallas, TX). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Samples were fixed with 1% paraformaldehyde in PBS for 20 min before dilution of the samples with Tyrode's buffer. Data were analyzed using FACS DiVa acquisition software (BD biosciences) and Winlist software (Verity Software House) for analysis. The mean fluorescence intensity was determined by collecting 100000 events within the platelet gate.

Aggregation and ATP Release. Washed platelets were suspended in Tyrode's buffer at a density of 3.0×10^8 cells/mL and loaded into glass cuvettes with stir bars. Aggregation (optical density) and ATP release (luminescence) were assessed simultaneously in a Chrono-log (Havertown, PA) model 700 lumiaggregometer. Platelets were preincubated for 10 min with dimethyl sulfoxide (DMSO) or giripladib, and aggregation/ATP release was monitored for at least 10 min.

TXB2 Enzyme-Linked Immunosorbent Assay (ELISA). Platelets were suspended in Tyrode's buffer at a density of 3.0×10^8 cells/mL and aliquoted into 500 μL volumes. Each sample was treated with either DMSO or giripladib before being stimulated with the indicated agonist. Platelets were immediately pelleted at 13000*g* for 3 min. Supernatants were collected carefully avoiding platelet pellets and immediately frozen for future analysis. Samples were analyzed for TXB$_2$ content using the Enzo TXB2 ELISA kit according to the manufacturer's instructions.

Flow Chamber Assay. Experiments were conducted as described by Gailani et al.[17] Glass capillary tubes (1 mm × 0.1 mm) were coated overnight with 100 μg/mL collagen I (Chrono-log, Havertown, PA) at pH 4 in acetate buffer. The day of the experiment, tubes were rinsed once with Tyrode's buffer and then blocked with 0.5% fatty acid free BSA (Sigma-Aldrich, St. Louis, MO) in Tyrode's buffer for 1 h at room temperature. Whole blood was collected into syringes filled with $^1/_{10}$ volume of 3.2% sodium citrate (final concentration of 0.32%). Blood was treated with DiOC$_6$ (Sigma-Aldrich) for at least 20 min and inhibitor or vehicle for at least 10 min before perfusion through the capillary tube with a syringe pump. Both control and treated conditions were run in parallel to avoid unwanted effects of longer incubation periods in the vehicle or any time-dependent changes in the reactivity of the blood. Prior to entering the capillary tube, blood was mixed with $^1/_5$ volume of CaCl$_2$/MgCl$_2$ in HEPES-saline buffer (final concentrations of 2.5 mM CaCl$_2$ and 1.25 mM MgCl$_2$). After perfusion for 15 min, blood was replaced with Tyrode's buffer and then 4% paraformaldehyde each for 1 min at a consistent flow rate. Images were captured with a 4× objective on an LSM710 META inverted microscope (Zeiss, Oberkochen, Germany). Analysis was performed using ImageJ. The volume was assessed by calculating the total area for each stack and then multiplying this value by the height of the stack before adding them together. The percent coverage was calculated from a z-project (sum of slices) picture as depicted in Figure 3A.

Preparation of Samples, Mass Spectrometry, and Data Analysis. Platelets at a density of 3.0×10^8 cells/mL were preincubated with vehicle control or giripladib for 10 min followed by stimulation with PAR agonists. Reactions were stopped with an equal volume of acidified methanol [0.1 N HCl/MeOH (1:1)]. Lipids were extracted using a modified Bligh and Dyer procedure.[15] An equal volume of chloroform was added, and after being vortexed, samples were allowed to separate into two phases overnight at 4 °C. The methanol layer was collected and dried under vacuum (Labconoco Centrivap Concentrator, Kansas City, MO). Samples were reconstituted in a methanol/chloroform mixture and spiked with known concentrations of standards (Avanti Polat Lipids, Alabaster, AL) prior to analysis by electrospray ionization-assisted mass spectrometry as previously described.[18,19]

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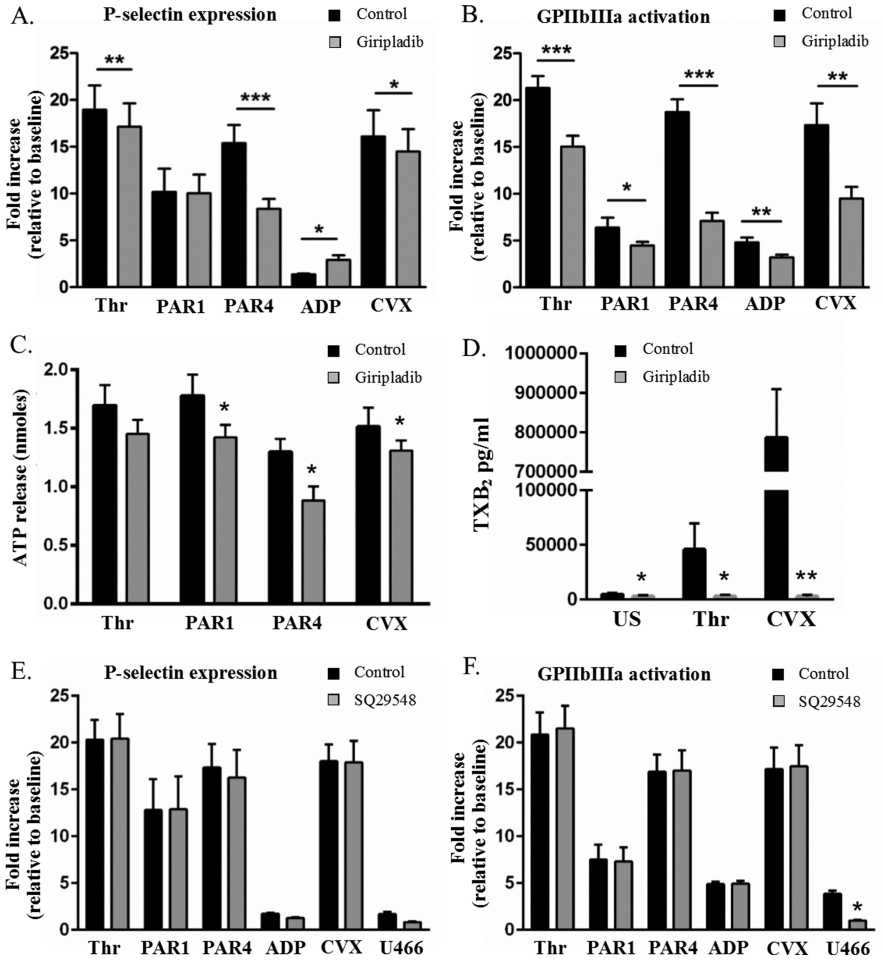

Figure 1. cPLA$_2\alpha$ inhibition reduces PAR4- and GPVI-mediated platelet activation. Effect of giripladib P-selectin expression (A) and GPIIbIIIa activation (B) on platelet activation by 10 nM thrombin (Thr), 20 μM protease-activated receptor (PAR) 1-AP, 200 μM PAR4-AP, 20 μM ADP, and 500 ng/mL convulxin (CVX) [SEM (n = 8), except for CVX (n = 4)]. (C) Effect of giripladib on ATP release [SEM (n = 4)]. (D) Effect of giripladib on TXB$_2$ release [SEM (n = 3)]. (E and F) Effect of SQ29584 on P-selectin expression (E) and GPIIbIIIa activation (F) by the aforementioned agonist and additionally 10 μM U46619 (U466), a thromboxane A2 receptor agonist [SEM (n = 4), except for U46619 (n = 3)]. Both giripladib and SQ29584 were used at a concentration of 10 μM.

RESULTS

cPLA$_2\alpha$ Inhibition Reduces PAR4- and GPVI-Mediated Platelet Activation. To determine the contribution of cPLA$_2\alpha$ activity to platelet activation, we tested the effect of the cPLA$_2\alpha$ inhibitor giripladib on platelet activation induced by PAR, collagen receptor GPVI, and purinergic receptor stimulation. P-Selectin expression and GPIIbIIIa inside-out activation (detected with PAC1) were used as readouts of platelet activation. Preincubation with giripladib selectively inhibited PAR4-AP-mediated platelet P-selectin expression by 46% (Figure 1A) with minimal effects on other agonists. Although technically significant, the effect of giripladib on P-selectin expression by 10 nM thrombin (7% reduction) and convulxin (10% reduction) was meager. No significant effects were noted on PAR1-AP, and ADP-induced P-selectin expression was enhanced (Figure 1A). In contrast to P-selectin expression, GPIIbIIIa activation by all agonists was affected; however, the largest reductions in activity occurred with PAR4-AP (62% reduction), followed by convulxin (45% reduction), ADP (33% reduction), PAR1-AP (30% reduction), and thrombin (29% reduction) stimulated platelets (Figure 1B). No inhibitory effects of giripladib were observed when platelet activation was monitored by aggregation regardless of the agonist employed (data not shown). However, a small but significant reductions in the release of ATP from giripladib-treated platelets were noted for all agonists (Figure 1C). Free arachidonic acid is quickly utilized or reincorporated back into GPL sources and therefore transient and difficult to measure. To confirm efficacy, we measured the effect of preincubation with giripladib on TXB$_2$ production. TXB$_2$ is the stable product of TXA$_2$, which is rapidly hydrolyzed in aqueous solutions. TXA$_2$ production relies on the liberation of arachidonic acid; therefore, an effective cPLA$_2\alpha$ inhibitor should blunt the amount of TXB$_2$ detected in stimulated platelet supernatants. Preincubation with giripladib abolished TXB$_2$ production in response to platelet stimulation with thrombin or convulxin (Figure 1D). In parallel, we tested the effects of a TP antagonist, SQ29584, on platelet activation (Figure 1E,F). Despite complete inhibition of the thromboxane receptor agonist U46619-induced platelet activation (Figure 1E,F), SQ29584 had no effect on PAR-, GPVI-, or purinergic receptor-mediated platelet activation. Therefore, an eicosanoid other than TXA$_2$ must be responsible for modulating cPLA$_2\alpha$-dependent components of PAR4- and GPVI-mediated platelet activation.

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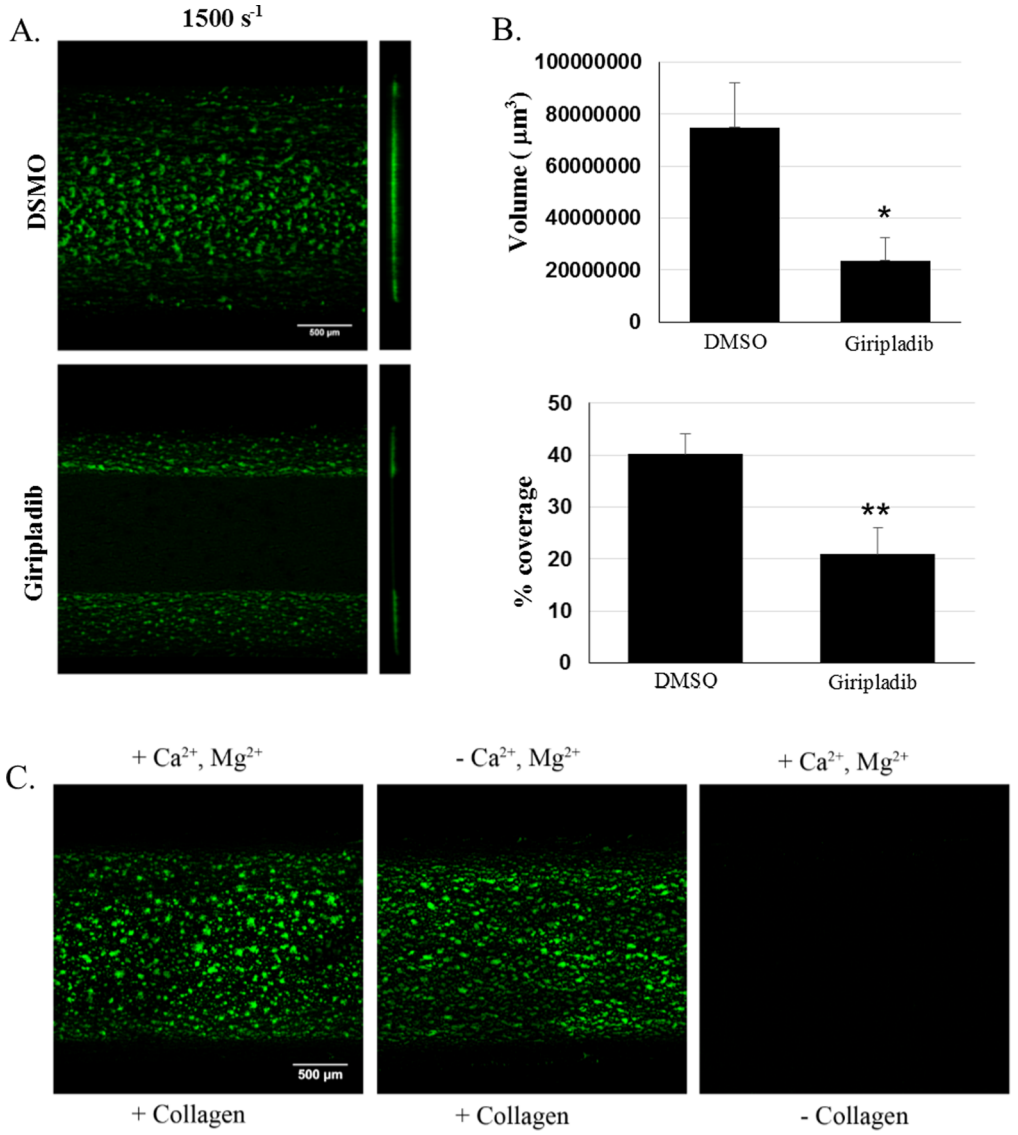

Figure 2. cPLA$_2\alpha$ inhibition reduces platelet adhesion and accumulation under shear stress. Citrated whole blood was preincubated with $DiOC_6$ for 20 min and giripladib or DMSO vehicle for 10 min prior to flowing through a capillary tube coated with 100 μg/mL collagen and blocked with 0.5% fatty acid free BSA. Blood was passed through the capillary tubes for 10 min at volumetric flow rates that yielded a shear rate of 1500 s^{-1}. Immediately prior to entry into the tube, blood was mixed with $CaCl_2$ and $MgCl_2$ to allow thrombin generation to occur. (A) Representative images from three independent experiments. Images were captured at 4×. The scale bar is 500 μm. (B) Quantification of three independent experiments: (top) volume and (bottom) percent coverage [SEM (n = 3)]. (C) Images of control samples without recalcification and collagen deposition.

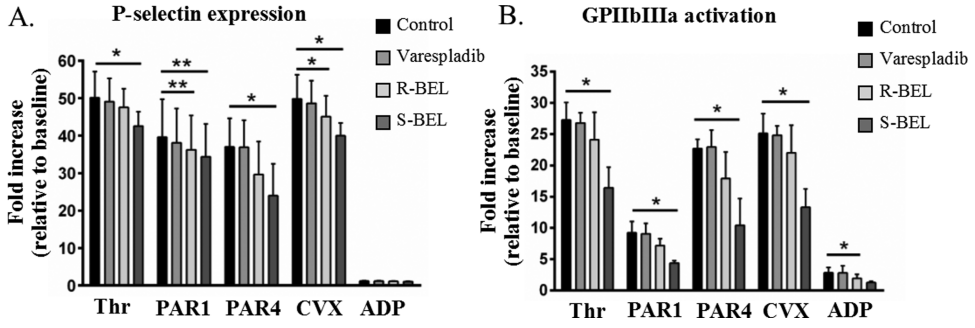

Figure 3. iPLA$_2\beta$ inhibitor reduces platelet activation. (A) Effect of 10 μM Varespladib, 5 μM R-BEL, and 5 μM S-BEL on P-selectin expression (A) and GPIIbIIIa activation (B) by 10 nM thrombin (Thr), 20 μM PAR1-AP, 200 μM PAR4-AP, 20 μM ADP, and 500 ng/mL convulxin (CVX) [SEM (n = 4)].

cPLA$_2\alpha$ Inhibition Reduces the Accumulation of Platelets on Collagen under Shear Stress. To further demonstrate the importance of arachidonic acid in hemostasis and thrombosis, we tested the effect of giripladib on platelet

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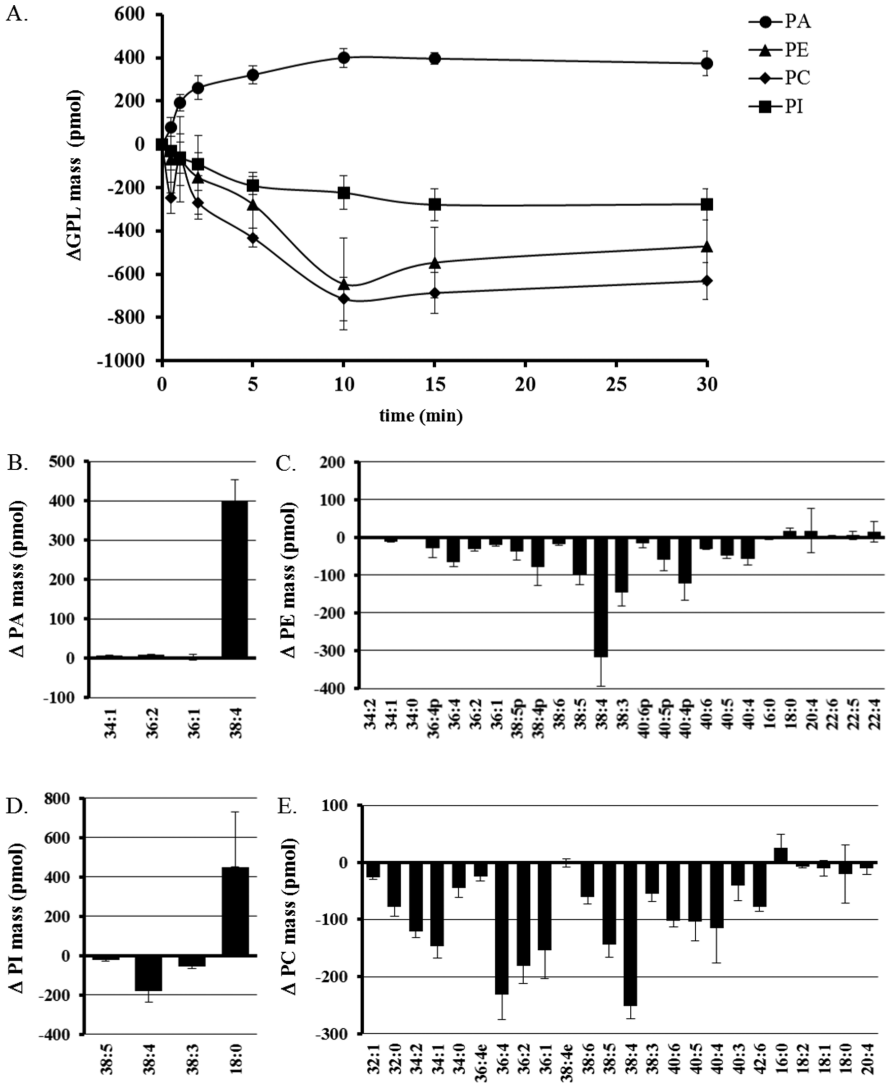

Figure 4. Thrombin induces changes in glycerophospholipid mass. Human platelets were stimulated with 2 nM thrombin for the indicated amounts of time. Glycerophospholipids (GPLs) were isolated and prepared for mass spectrometry as detailed in Experimental Procedures. GPLs were quantitated according to known amounts of injected internal standards. Only species with putative arachidonyl are represented. The data are presented as the change in GPL mass relative to the unstimulated control for each experiment (n = 3). (A) Changes in the mass of each of five classes of arachidonyl-glycerophospholipid species, including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). (B) Changes in mass of PA separated into individual species. (C) Changes in mass of PE separated into individual species. (D) Changes in mass of PI separated into individual species. (E) Changes in mass of PC separated into individual species. Ether-linked species are denoted as XX:Xe and plasmenyl-linked species as XX:Xp.

accumulation on collagen under flow (Figure 2). Whole blood treated with giripladib or vehicle control was pushed through capillary tubes at volumetric flow rates that yielded a shear rate of 1500 s^{-1}. Citrated blood was recalcified prior to entering the capillary tube to allow thrombin generation to occur, so that fibrin would be generated and PARs would also be engaged. Preincubation with giripladib significantly reduced platelet adhesion (as measured by percent coverage) and accumulation (as measured by volume) (Figure 3A,B). These data demonstrate the significant role that cPLA$_2\alpha$ activity plays in the hemostatic response of blood.

An iPLA$_2\beta$ Inhibitor Reduces Platelet Activation. We also tested the effect of sPLA$_2$ and iPLA$_2$ inhibitors on platelet activation (Figure 3A,B). The sPLA$_2$ inhibitor Varespladib had no effect on platelet activation. However, the iPLA2 inhibitors R-BEL and S-BEL had significant effects on PAR-, collagen receptor-, and purinergic receptor-mediated platelet activation. R-BEL, which is specific to iPLA$_2\gamma$, significantly reduced P-selectin expression in response to PAR1-AP; however, the magnitude of the reduction was negligible. S-BEL, which is 10-fold more specific to iPLA$_2\beta$, significantly reduced platelet activation in response to each agonist.

Changes in Glycerophospholipid Mass in Response to Stimulation with Thrombin. Given the dramatic effect of giripladib on PAR- and collagen-mediated platelet activation, we sought a biochemical understanding of cPLA$_2\alpha$ activity on different GPL pools. We did not utilize arachidonic acid labeling as this technique relies on arachidonic acid reacylation and reincorporation into the GPL pool through the Land's pathway, which will occur at different rates for each GPL class resulting in the preferential labeling of GPL pools with high rates of turnover under resting conditions. Free arachidonic acid is rapidly oxidized by cyclooxygenase and lipooxygenase enzymes to make a diverse array of eicosanoids. Because of its

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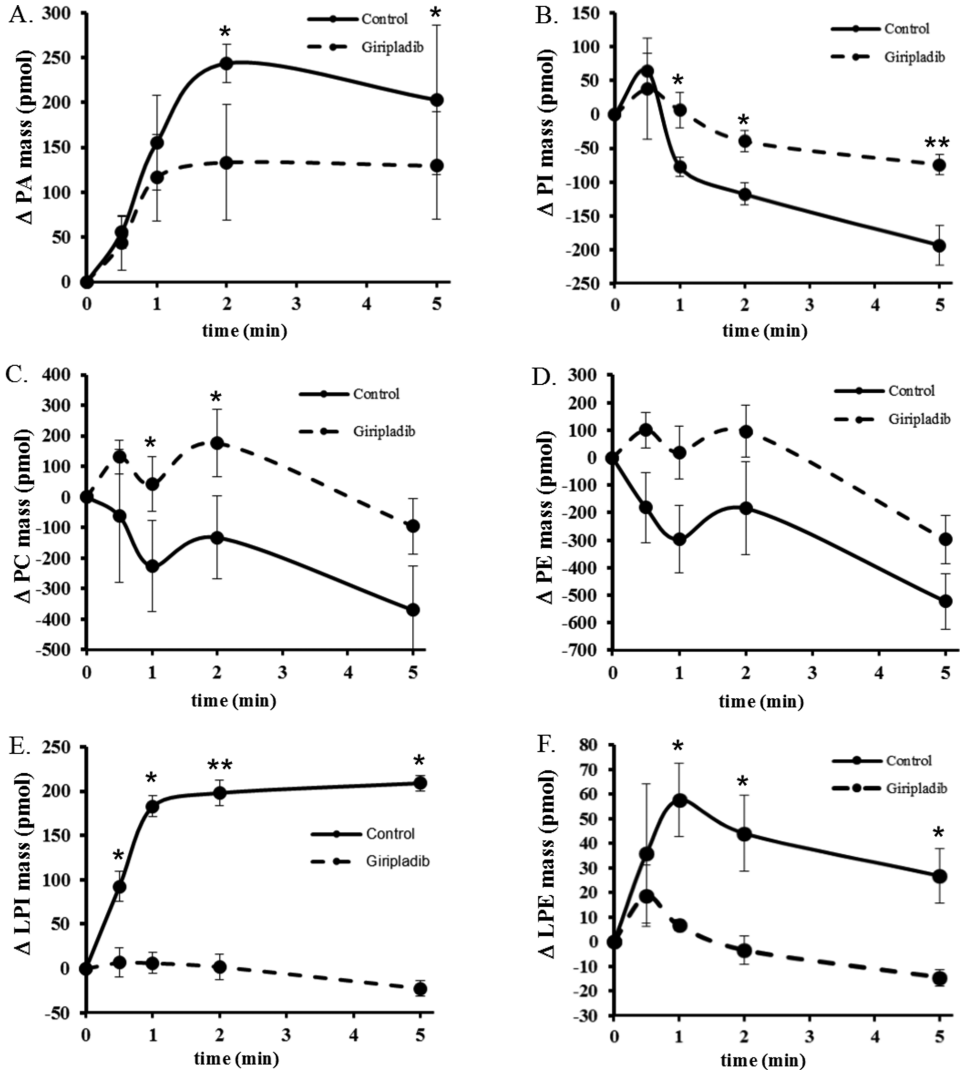

Figure 5. cPLA$_2$ inhibition blunts changes in putative arachidonyl-glycerophospholipid mass induced by thrombin. Platelets were preincubated with 10 mM giripladib or vehicle control for 10 min prior to stimulation with 2 nM thrombin. The data are presented as the change in glycerophospholipid (GPL) mass relative to the unstimulated control for each experiment (n = 3). (A) Changes in the 38:4 phosphatidic acid (PA) mass. (B) Changes in the 38:4 phosphatidylinositol (PI) mass. (C) Changes in the sum of putative arachidonylphosphatidylcholine (PC) mass. (D) Changes in the sum of putative arachidonylphosphatidylethanolamine (PE) mass. (E) Changes in the 18:0 lysophosphatidylethanolamine (LPE) mass. (F) Changes in the 18:0 lysophosphatidylinositol (LPI) mass.

transient nature, measurement of arachidonic acid is not a reliable assessment of cPLA$_2\alpha$ activity. We observed changes in GPL mass from the perspective of arachidonate. This analysis prevents the determination of the mass and degree of saturation for individual acyl chains. However, determination of the combined mass of both acyl chains allows the inference of a GPL containing one 20:4 acyl chain. Therefore, we present here only those lipids detected that putatively contain arachidonyl according to the appearance of their "−20:4" lysolipid counterpart during stimulation. Changes in all GPLs detected are presented in the Supporting Information and summarized in Figure 4C−E. Figure 4A presents a time course of changes in putative arachidonyl-GPL species mass in human platelets challenged with 2 nM thrombin for up to 15 min. Changes in putative arachidonyl-GPL mass reach maximal levels by 5 min. The greatest changes in mass occur in the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) pools, which experience a loss of roughly 1000 pmol. There were also significant losses in mass in the phosphatidylinositol (PI) pool. In contrast to the PE, PC, and PI pools, the phosphatidic acid (PA) pool displayed a significant and persistent increase in mass of roughly 400 pmol in response to thrombin stimulation. Although we attempted to measure changes in phosphatidylglycerol (PG), lipid levels were barely above the detection limit and were therefore excluded from this work. Changes in phosphatidylserine (PS) mass were inconsistent between donors; these results are presented in the Supporting Information.

Figure 4B−F shows the same results as Figure 4A segregated into GPL species at the 5 min time point. Across all classes of GPLs, the largest changes in mass occurred in the 38:4 species. Multiple plasmenyl- and ether-linked arachidonyl-GPL species were detected in the PC and PE pools (Figure 4C,E); however, there did not appear to be a preference for the breakdown of these particular species. Intriguingly, the PA mass created in response to thrombin stimulation was composed exclusively of a 38:4 PA species (Figure 4B).

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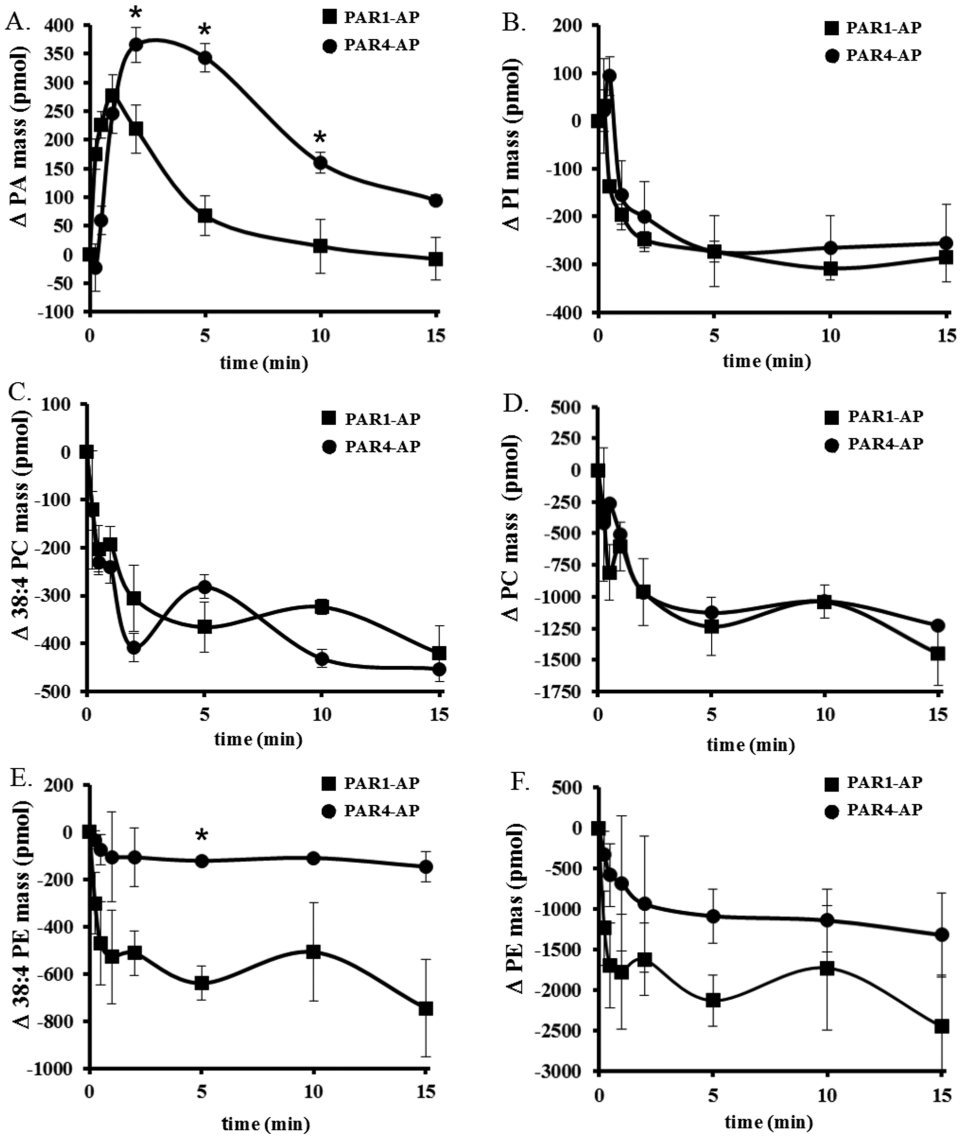

Figure 6. Differences in PAR1- and PAR4-induced changes in glycerophospholipid mass. Platelets were stimulated with 20 μM PAR1-activating peptide (AP) or 200 μM PAR4-AP for the indicated amounts of time, and samples were prepared for mass spectrometry as detailed in Experimental Procedures. (A) Changes in 38:4 phosphatidic acid (PA) mass. (B) Changes in 38:4 phosphatidylinositol (PI) mass. (C) Changes in 38:4 phosphatidylcholine (PC) mass. (D) Changes in putative arachidonyl-PC mass. (E) Changes in 38:4 phosphatidylethanolamine (PE) mass. (F) Changes in putative arachidonyl-PE mass.

We also tracked lysolipid species of each of the five major GPL classes. Most notably, we observed a persistent increase in lysophosphatidylinositol (LPI). When the samples were separated into specific molecular species, all of the LPI mass could be ascribed to an 18:0 LPI (Figure 4D), a strong indication that it is a product of the breakdown of 38:4 PI for the release of arachidonic acid (20:4). Various species of lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) were also detected (Figure 4C,E).

Effect of cPLA$_2\alpha$ Inhibition on the Loss of Arachidonyl-GPL and Production of Lysolipids in Response to Thrombin Stimulation. cPLA$_2\alpha$ is considered the primary PLA$_2$ enzyme responsible for the liberation of arachidonic acid from GPL sources in human platelets. To probe the contribution of cPLA$_2\alpha$ activity to the observed changes in mass of arachidonyl-GPL, we preincubated platelets with the specific cPLA$_2\alpha$ inhibitor giripladib. Giripladib inhibited the loss of mass in the PC and PE pools, although this difference was not statistically significant in the PE pool. At early time points in the presence of giripladib, arachidonyl-PC and arachidonyl-PE displayed increases in mass suggesting a flux of arachidonyl-PC/PE being created as it is consumed (Figure 5C,D). At later time points, the arachidonyl-PC mass and arachidonyl-PE mass continue to decrease, leaving open the possibility of an additional lipase working on these pools. Loss of arachidonyl-PI was partially but significantly inhibited by giripladib (Figure 5B), suggesting that the activity of another enzyme, most likely phospholipase C (PLC), is responsible for the changes observed with thrombin.

Preincubation with giripladib completely abolished the production of 18:0 LPI and 18:0 LPE (Figure 5E,F). Complete inhibition of the production of 18:0 LPI and 18:0 LPE by giripladib confirms the efficacy of this cPLA$_2\alpha$ inhibitor and cPLA$_2\alpha$ activity on the PE and PI pools. These results also confirm cPLA$_2\alpha$ activity as the source of the large 18:0 LPI signal. Finally, the increase in mass observed for 38:4 PA was

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blunted by giripladib, suggesting that cPLA$_2\alpha$ activity is partially required for the production of this GPL (Figure 5A).

Comparing PAR1-AP- and PAR4-AP-Mediated Changes in GPL Mass. To potentially gain some insight into the difference in the effect of giripladib on PAR1- and PAR4-stimulated platelets, we conducted a similar experiment using PAR1 and PAR4 specific peptides (PAR1-AP and PAR4-AP, respectively) tracking changes in GPL mass in response to the agonist over the course of 15 min by monitoring GPL pools that were affected by preincubation with giripladib. Figure 6 compares changes in GPL mass between PAR1-AP- and PAR4-AP-stimulated platelets. Looking across GPL classes, we noted major differences in metabolism between PAR1 and PAR4 in the PE and PA pools. Significantly more 38:4 PA was produced downstream of PAR4. We also observed a preference for the consumption of arachidonyl-PE downstream of PAR1 activation, in direct contrast to the effect of giripladib on PAR1 and PAR4 responses. In conclusion, cPLA$_2\alpha$ activity as assessed by the loss of putative arachidonyl-GPLs could not account for the difference in giripladib's effects on PAR1 and PAR4.

DISCUSSION

The liberation of arachidonic acid from GPL sources for the production of TXA$_2$ has long been a subject of interest in the cardiovascular field because of the importance of TXA$_2$ to normal hemostasis and the effectiveness of aspirin in the prevention of recurrent thrombotic events. However, the lack of a specific cPLA$_2\alpha$ inhibitor has prevented an assessment of the effect of blocking arachidonic acid release on platelet function as well as a detailed understanding of cPLA$_2\alpha$ substrates and therefore GPL sources of arachidonic acid. Giripladib was developed by Wyeth as part of a program to target various PLA$_2$ isoforms for the potential treatment of inflammatory diseases with an eicosanoid component. In addition to the reported efficacy of giripladib in two different models of arthritis [Drug Data Report 30(7), 611 (2008)], the patent that first claimed giripladib reported an IC$_{50}$ of 20 nM in a rat whole blood TXB$_2$ release assay and an IC$_{50}$ of 30 nM in a GLU micelle assay containing only lipid, detergent, and the enzyme. Efficacy was also demonstrated in the platelet function analyzer (PFA-100) that utilizes flow-based shear and collagen to activate platelets, a $FeCl_3$-induced model of arterial thrombosis, and a mouse experimental autoimmune encephalomyelitis model of multiple sclerosis (WO/2006/128142, example 14). We used giripladib to probe cPLA$_2\alpha$ function in human platelets.

Aspirin, which inhibits the conversion of free arachidonic acid into TXA$_2$, has previously been demonstrated to affect platelet accumulation but not adhesion in *ex vivo* and *in vivo* models of thrombosis.[20−25] The effects of TP antagonism on thrombosis have also been explored, demonstrating good efficacy but phenotypes similar to those of aspirin-treated blood.[26−28] However, the effects of blocking cPLA$_2\alpha$ activity and therefore arachidonic acid release on platelet function under flow have yet to be determined. The effect of giripladib on adhesion of platelets to collagen under flow highlights the importance of this pathway in platelet function during hemostasis and thrombosis. The inhibition of cPLA$_2\alpha$ has a much stronger effect on platelet adhesion than administration of aspirin or the TP receptor antagonist SQ29584, each of which has been reported to affect only platelet accumulation in such models. These data indicate there is an additional TXA$_2$-independent component of platelet signaling.

Consistent with observations in the flow chamber model, preincubation with giripladib revealed that a large component of the PAR4- and GPVI-mediated GPIIbIIIa activation was dependent upon arachidonic acid release. The partial reduction in response is likely due to the inhibition of the production of an eicosanoid otherwise produced downstream of arachidonic acid release that synergizes with PAR- and GPVI-mediated responses. The greater impact of giripladib on PAR4- versus PAR1-mediated platelet activation likely reflects the ability of this unidentified eicosanoid to synergize with PAR4 signaling better than PAR1. Perhaps PAR1 signaling does not rely on the unidentified eicosanoid production as much as PAR4 signaling. The residual PAR- or GPVI-mediated GPIIbIIIa activation, P-selectin expression, and ADP secretion can be attributed to canonical receptor signaling that is independent of this putative eicosanoid-mediated augmentation of responses. Residual GPIIbIIIa activation, P-selectin expression, and ADP secretion could also be sufficient to induce full aggregation in response to maximal doses of the agonist and therefore explain the lack of an effect of giripladib treatment on aggregation. Aggregation is conducted in glass cuvettes with a stir bar, essentially a closed system in which the same partially activated platelets are continually exposed to one another. Platelets are also stimulated with maximal doses of agonist to induce full aggregation. In the flow chamber, however, naïve platelets are perfused past exposed collagen and previously activated/adherent platelets. Subtle gradients of agonists are generated in a flow system leading to partial platelet activation; activated platelets have fewer chances to adhere to and aggregate with other platelets before being pushed out of the capillary tube. Therefore, a much stronger response from an inhibitor in the flow chamber model versus aggregation is not unexpected.

A likely candidate for the elusive eicosanoid that synergizes with PAR4- and GPVI-mediated responses was TXA$_2$. TXA$_2$ generation has previously been demonstrated to be significantly greater downstream of PAR4-AP than downstream of PAR1-AP,[29] suggesting that the disparity in the effect of giripladib could be the result of more TXA$_2$ generation, presumably as a result of more arachidonic acid liberation. Comparing changes in GPL mass in response to PAR1-AP and PAR4-AP, we did not observe the consumption of more putative arachidonyl-GPLs downstream of PAR4-AP, indicating that augmented cPLA$_2\alpha$ activity does not account for the large cPLA$_2\alpha$-dependent component of the PAR4-AP response. Instead, this disparity is more likely due to the production of a unique eicosanoid downstream of PAR4 and not PAR1 or the activation of an unidentified receptor downstream of a commonly produced eicosanoid that synergizes with PAR4 and GPVI but not PAR1. In support of this, Holinstat and co-workers[30] have recently published observations with a LOX inhibitor (ML355) that indicated a large component of the response downstream of PAR4 and GPVI, but not PAR1, is dependent on LOX activity. Our data are in accord with this observation as we have blocked the production of this LOX-dependent eicosanoid by preventing the liberation of arachidonic acid, one step above LOX in the pathway. Moreover, they observe no effect of aspirin on platelet activation in agreement with our observations with the TP antagonist SQ29548, which had no effect on PAR- or GPVI-mediated platelet activation. The implication is that another eicosanoid that is an even stronger feed-forward signal than

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TXA_2 is also produced by platelets. This eicosanoid appears to represent a previously unappreciated component of PAR4- and GPVI-stimulated platelet activity. The molecular identity of this species remains undetermined. Identification of this eicosanoid represents a massive undertaking that is beyond the scope of this study.

In addition to profiling the effect of the cPLA$_2\alpha$ inhibitor giripladib on platelet activation, we also examined the potential involvement of sPLA$_2$ and iPLA$_2$ in platelet activation. sPLA$_2$ requires millimolar concentrations of Ca^{2+}, is stored in granules, and is typically active against GPLs found in plasma. Therefore, we anticipated no response with the sPLA$_2$ inhibitor Varespladib in washed platelets. We did not anticipate any effect of the iPLA$_2$ inhibitors R-BEL and S-BEL. S-BEL, which significantly reduced platelet activation regardless of the agonist employed, is an inhibitor of iPLA$_2\beta$ when used at a concentration of 5 μM.[31] These data are in accord with other circumstantial evidence and BEL-based studies suggesting a role for iPLA$_2$ in arachidonic acid release and platelet activation. However, the BEL series is known to have multiple cellular off-targets[32] despite its sparing of cPLA$_2\alpha$ activity; therefore, any conclusion with this inhibitor should not be taken as proof of this enzyme's role in platelet activation. Indeed, this conclusion is difficult to resolve with the fact that giripladib abolishes TXB_2 production in platelets.

Seminal work in the field describes a hierarchical rank in the loss of mass from PE > PC > PI > PS during platelet stimulation with thrombin.[33] Our results do not disagree with this ranking. We observed the greatest loss of mass in the PE and PC pools with minor losses from the PI pool. The preference of cPLA$_2\alpha$ for the PC and PE pools is consistent with the current understanding of this enzyme. Both PE and PC losses in mass were inhibited but not abolished by giripladib. However, at later time points, reductions in mass relative to the baseline continued, which leaves room for the interpretation that another lipase could be acting on these GPL pools. Consistent with this hypothesis, arachidonic acid release is still observed in cPLA$_2\alpha^{-/-}$/sPLA$_2$-IIA$^{-/-}$ mice.[11] Moreover, a recent study demonstrated the effectiveness of the iPLA$_2$ inhibitor BEL in blocking PLA$_2$ hydrolysis of plasmenyl-GPLs in the absence of Ca^{2+}.[10] Yet another study recently reported that iPLA$_2\gamma$ deficient platelets demonstrated reduced ADP-dependent aggregation and ADP- or collagen-dependent TXA_2 production.[34] Indeed, we observed a reduction in platelet activation with the iPLA2β inhibitor S-BEL, suggesting that iPLA$_2$ activity may contribute to human platelet activation. The relative roles of these two PLA isoforms and the mechanism by which they contribute to platelet activation warrant further study.

Production of PC and PE species with cPLA$_2\alpha$ inhibition suggests a flux of arachidonyl GPLs through these pools and raises the possibility that arachidonyl-PC is produced during thrombin stimulation but is not observed because of its rapid consumption by cPLA$_2\alpha$. Although this process is difficult to demonstrate experimentally, it is possible that the arachidonate is shuffled from a non-PC/PE pool to the PC/PE pool prior to its liberation from GPL sources by cPLA$_2\alpha$. However, more specific inhibitors of transacylation enzymes are needed to definitively answer these hypotheses.

The formation of LPI has previously been observed in the field.[35] We have extended this observation and identified this species as 18:0 LPI. The cPLA$_2\alpha$ inhibitor significantly but only partially inhibited the loss of arachidonyl-PI, while abolishing the production of 18:0 LPI. Consistent with early observations in the field, our results indicate that two different enzymes are consuming 38:4 PI during thrombin stimulation. A part of the 38:4 PI mass lost during thrombin stimulation can be ascribed to cPLA$_2\alpha$ activity. This is indicated by the effectiveness of giripladib in reducing this loss in mass and the arithmetic relationship between the mass of 38:4 PI preserved during cPLA$_2\alpha$ inhibition and the mass of 18:0 LPI lost by cPLA$_2\alpha$ inhibition, roughly 200 pmol. The remaining 100 pmol of 38:4 PI lost during thrombin stimulation that was not affected by the cPLA$_2\alpha$ inhibitor is most likely caused by PLCβ activity, a known effector of PAR activation in the platelet, responsible for subsequent Ca^{2+} mobilization and PKC activation. However, because of the lack of a specific PLC inhibitor, it is not possible to test this. The effective PLC inhibitors currently available have an inconvenient off-target effect on PLA type lipases. Moreover, Ca^{2+} mobilization mediated by PLC activity likely contributes to cPLA$_2\alpha$ activity, rendering any experiment with a PLC inhibitor difficult to interpret when arachidonic acid release is being studied. However, these data definitively demonstrate that PI is a substrate of cPLA$_2\alpha$ in the human platelet and that the majority of PI lost during thrombin stimulation is a result of cPLA$_2\alpha$ activity.

Finally, it has recently been reported that thrombin stimulation of human platelets results in the selective hydrolysis of arachidonyl-plasmenylcholine and plasmenylethanolamine, with little activity toward diacyl phospholipids.[10] In accord with this study and others,[33,36] we detected multiple ether-linked PC species and plasmenyl-linked PE species that demonstrated a loss of mass in response to stimulation with thrombin. However, in direct contrast to this study, we did not observe a preference for these substrates over their diacyl counterparts.

This study demonstrates for the first time the effect of cPLA$_2\alpha$ inhibition on platelet activation, including defects in GPIIbIIIa activation, secretion, platelet adhesion, and aggregation on collagen under flow. Importantly, these data indicate that arachidonic acid release and subsequent eicosanoid production represent a much larger component of platelet activation than was ever suggested by aspirin, TP receptor antagonists, or previous cPLA$_2\alpha$ inhibitors. These effects appear to be unique to PAR4 and collagen receptor GPVI activation. Lipidomics suggest that there is no more loss of putative arachidonyl-GPL mass downstream of PAR4 versus PAR1, leading to the conclusion that perhaps a unique eicosanoid is produced downstream of PAR4 or GPVI activation or that an as yet unidentified eicosanoid receptor synergizes with PAR4 or GPVI, and not PAR1. These data suggest that there remains great potential in the pharmacological targeting of this signaling cascade to inhibit platelet activation and affect thrombosis. Moreover, they suggest caution be taken in a pharmacological strategy that would inhibit eicosanoid production and the platelet receptors with which they synergize.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00549.

A table of raw values of the mass of each glycerophospholipid species detected at each time point (XLSX)

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Notes

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ABBREVIATIONS

TXA_2, thromboxane A_2; PAR, protease-activated receptor; TP, thromboxane A_2 receptor; $cPLA_2$, cytosolic phospholipase A_2; GPVI, glycoprotein VI; COX-1, cyclooxygenase 1; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase 2; $iPLA_2$, inducible phospholipase A_2; GPL, glycerophospholipid; APC, allophycocyanin; PE, phycoerythrin; AP, activating peptide; ADP, adenosine diphosphate; CVX, convulxin; GPIIbIIIa, glycoprotein IIbIIIa; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; LPI, lysophosphatidylinositol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine.

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