Supplemental Methods

Human platelet isolation

Human blood (30 – 50 mL) was obtained from consented healthy volunteers via venipuncture, anti-coagulated with citrate (0.38% final) and spun at 150 x *g* to isolate platelet-rich plasma (PRP). PRP was warmed 5 min and then incubated with 50 ng/mL prostaglandin I_2 (PGI_2) for an additional 5 min at 37°C before centrifugation at 500 x *g* to pellet platelets. For gel filtration, the pellet was gently resuspended in HEPES-Tyrode's buffer (5 mM HEPES pH 7.4, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 1 mM $MgCl_2$ and 0.1% fatty acid- and endotoxin-free BSA) containing PGI_2 and 1 U/mL apyrase (Grade VII). Platelets were then loaded on a Sepharose 2B gel filtration column, collected in the void volume and counted electronically with a Z1 Particle Counter (Beckman Coulter). For washed platelets, PRP was treated as above, but platelets were resuspended in 30 mL of Tyrode's buffer pH 6.5 containing 1 U/mL apyrase and treated with PGI_2 as above. Platelets were centrifuged at 500 x *g* and resuspended in 15 mL pH 6.5 Tyrode's buffer with 0.5 U/mL apyrase and treated with PGI_2 as above. Platelets were centrifuged as above and resuspended in 2 mL HEPES-Tyrode's buffer pH 7.4 containing 1 mM $CaCl_2$, and adjusted to a final concentration of 3×10^8 platelets/mL.

Mouse and rat platelet isolation

Mouse platelets were isolated from wild-type, *RasGRP2*$^{-/-}$ or *P2Y12*$^{-/-}$ mice (3 – 5 months old) as described.[1] Platelets were adjusted to 2×10^8/mL and pretreated with 10 μM HAG or 0.5% DMSO for 10 min at 37°C in aggregation cuvettes before stimulation with 0.5 μg/mL convulxin (gift of Dr. Ken Clemetson) as indicated in figure legends. Aggregation was performed as described in the main text.

For rat platelet aggregation, blood was obtained via thoracotomy followed by cardiac puncture of 4.5 – 6 months old male Wistar rats. Blood was drawn into 0.48 mg/mL heparin (209 USP units/mg) in PBS with an average blood volume of 6 – 10 mL per rat. Platelets were isolated using a centrifugation/washing protocol similar to the mouse protocol above. Briefly, blood was

centrifuged at 200 x *g* for 15 min to obtain platelet rich plasma (PRP). PRP was treated with 50 ng/mL PGI_2 for 5 min at 37°C prior to centrifugation at 500 x *g* to obtain a platelet pellet. Platelets were washed in 5 mL HEPES-Tyrode's buffer pH 6.5 (under the same conditions as human platelets above) containing 0.2 units/mL apyrase (Sigma Grade VII). Platelets were treated with 50 ng/mL PGI_2 for 5 min at 37°C and centrifuged again at 500 x *g* to obtain a platelet pellet. Platelets were resuspended in 1.5 mL of the same buffer without apyrase. Platelets rested for 30 min before treatment with JW480 and aggregation was performed as described in the main text.

AADACL1 overexpression

AADACL1 was overexpressed according to Chiang et al.[2] Briefly, HEK293T cells were cultured in DMEM supplemented with 9% fetal bovine serum and nonessential amino acids at 37°C at 5% CO_2. Subconfluent cells ($1x10^6$/well) were transfected with 2 μg of pcDNA3.1+ AADACL1 plasmid (gift of Dr. Ben Cravatt) using a 3:1 ratio of X-tremeGene HP (Roche) to DNA (mL:mg), whereas control cells were transfected with X-tremeGene HP alone. After 24 h, the media was replaced with fresh media. After 2-3 days, cells were harvested by pipetting into ice-cold PBS, sonicated with a Misonix probe tip sonicator at 70% power using two, five-second pulses and centrifuged at 13,000 x *g* for 5 min at 4°C. To collect membranes, the supernatant was centrifuged at 100,000 x *g* for 30 min at 4°C in a TLA100.3 rotor in a Beckman Coulter ultracentrifuge. The resultant membrane pellet was resuspended in 50 mM Tris pH 7.4, sonicated again and membrane protein concentration was determined using BCA protein quantification.

Preparation of lipid extracts

At designated time points, platelets were removed from the aggregometer and lysed with cold 2X lysis buffer (20 mM HEPES pH 7.4, 10 mM CHAPs, 150 mM NaCl, 5 mM $MgCl_2$, 5 % glycerol, 1 mM Na_3VO_4, 1 mM NaF, 1 mM β-glycerophosphate, 0.5 mM Na pyrophosphate and protease inhibitor cocktail) on ice. Platelet aggregates were further disrupted with a probe-tip sonicator as

above and total platelet lipids were extracted via the Bligh and Dyer method[3]. Purified lipids were dried under N_2 and stored at -20°C until further MS analysis.

NPLC-ESI/MS (for HAGP and PI analysis in the negative ion mode)

Normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μm, 25 cm x 2.1 mm (Sigma-Aldrich, St. Louis, MO). Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v); mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Applied Biosystem, Foster City, CA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: IS= -4500 V; CUR= 20 psi; GSI= 20 psi; DP= -55 V; and FP= -150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Applied Biosystem, Foster City, CA). Integrated peak areas of extracted ion chromatograms were used for relative quantitation.

RPLC-ESI/MS (for HAG and cholesterol analysis in the positive ion mode)

Reverse phase LC-ESI/MS was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to a TripleTOF5600 mass spectrometer (as above). LC was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of

methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 μm, 2.1 x 50 mm) was obtained from Agilent (Palo Alto, CA). Instrumental settings for positive ion ESI and MS/MS analysis of lipid species were as follows: IS= +5000 V; CUR= 20 psi; GSI= 20 psi; DP= +55 V; and FP= +150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst as above. Integrated peak areas of extracted ion chromatograms were used for relative quantitation.

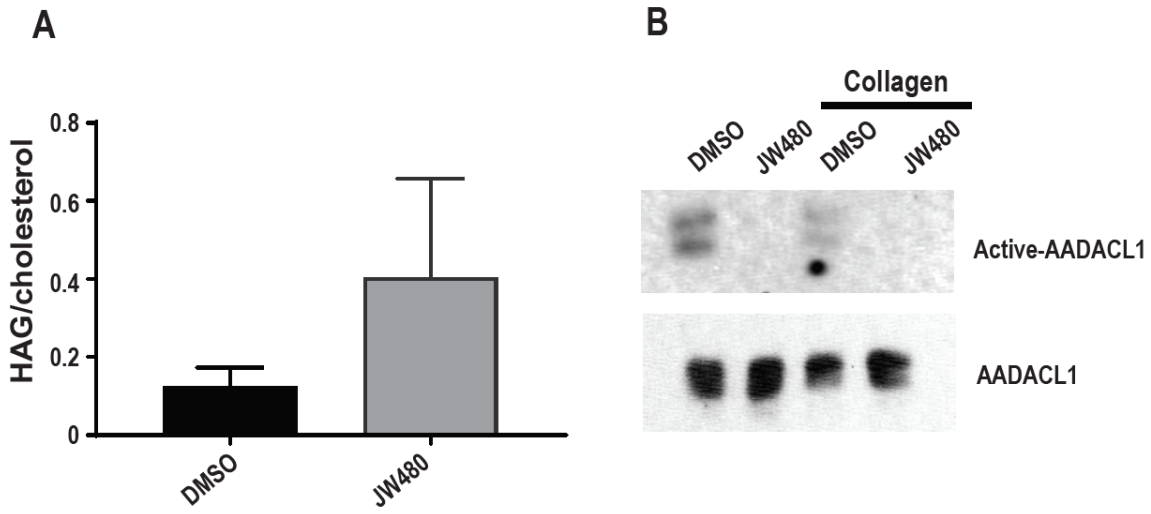
SUV composition and tryptophan fluorescence

Small unilamellar vesicles (SUV) were made for binding experiments with PKC C1 peptides. Briefly, purified lipids 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS, Avanti Polar Lipids, Inc.) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids Inc.) in chloroform were mixed such that all SUV contained 5 mol% DOPS and 20 mol% dioleoyl-rac-glycerol (DAG, Cayman Chemical) or 20 mol% HAG or 20 mol% HAGP (custom synthesized by Avanti Polar Lipids, Inc.). The remaining lipid consisted of DOPC. Negative control SUV were 95 mol% DOPC and 5 mol% DOPS. Lipid mixtures were dried under nitrogen, resuspended in cyclohexane plus methanol, frozen for 30 min and lyophilized overnight. Lipids were resuspended in binding buffer and sonicated as previously described.[4] SUV were centrifuged, diluted in binding buffer (20 mM HEPES pH 7.4, 100 mM NaCl) and incubated with PKC C1 peptides that naturally contain a single tryptophan in the middle of the sequence. Tryptophan emission increased proportionally with increasing peptide concentration in the absence of lipids (control, data not shown). Increasing concentrations of SUV were incubated with 200 – 500 nM peptide in binding buffer (20 mM HEPES pH 7.4, 100 mM NaCl) under stirring conditions for 10 replicates. Fluorescence maxima were normalized to fluorescence in the absence of lipid (F_0) and fitted to hyperbolic decay curves to calculate a dissociation constant (K_d) using SigmaPlot software.

Western blotting

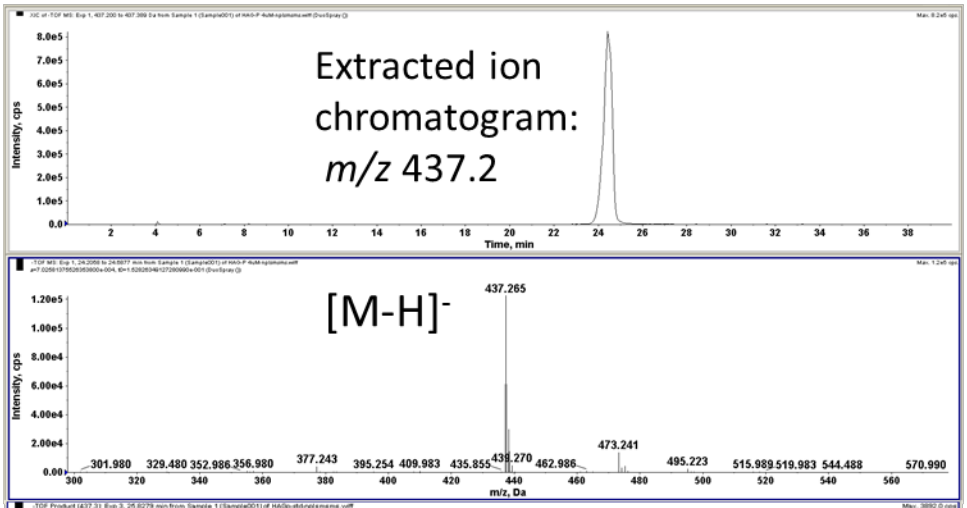
For detection of phosphorylated PKCδ or PKCθ, human platelets (7.5×10^7) were stimulated with the indicated concentrations of agonist with stirring. Aggregation traces were collected for 2.5 min post-agonist (Supplemental Figure 3 below) before lysis in cold 2X buffer (same used for "Preparation of lipid extracts" above) and incubated on ice for 15 min. Lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 30 min in 5% BSA in TBS/0.1% Tween-20 (TBST) before addition of PKC isoform-specific polyclonal antibodies per manufacturer's instructions and anti-rabbit secondary antibodies diluted 1:10,000 in Tris-buffered saline/0.1% Tween-20 (all antibodies from Cell Signaling Technology). Bands were visualized with ECL-Plus (GE) and autoradiography. Quantitation of phospho-PKC was performed with ImageJ software. AADACL1 was detected with monoclonal antibody 16C8 as described in Holly et al.[5]

Supplemental Figures



Supplemental Figure 1. HAG does not accumulate in platelets treated with JW480. A) Endogenous HAG was quantified from the same human platelet samples in which HAGP was detected in Fig. 3A. Platelets were pretreated with 10 μM JW480 or 0.5% DMSO for 10 min at 37°C prior to stimulation with 10 μg/mL collagen. Platelets were immediately lysed and total lipids were extracted. HAG (359.3 *m/z*) levels were normalized to cholesterol (369.3 *m/z*) for relative quantitation ($p = 0.34$ for DMSO vs. JW480, $n = 3$). B) JW480 inhibits fluorophosphonate-desthiobiotin (FP)-biotin labeling of endogenous platelet AADACL1. Human platelets (2×10^8/mL) were treated with either DMSO (0.1%) or JW480 (10 μM) for 10 min at 37°C. Samples were then stirred for 5 min in the presence or absence of 10 μg/mL collagen, lysed in ice-cold lysis buffer, sonicated, precleared with streptavidin-agarose beads for 1h at 4°C and then frozen in N_2 (l). Endogenous AADACL1 was reacted with FP-biotin and precipitated with streptavidin agarose per the manufacturer's instructions (Thermo Pierce). Bound AADACL1 was eluted with Laemmli buffer, subjected to SDS-PAGE and detected via Western blotting with a monoclonal antibody (clone 16C8, gift of Dr. Ben Cravatt).

A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Time, min
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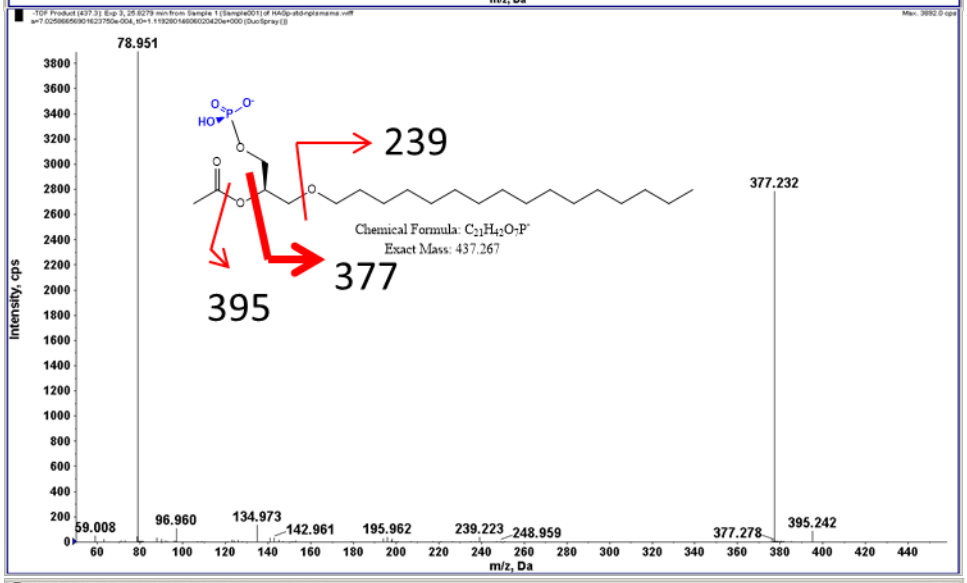
m/z, Da

B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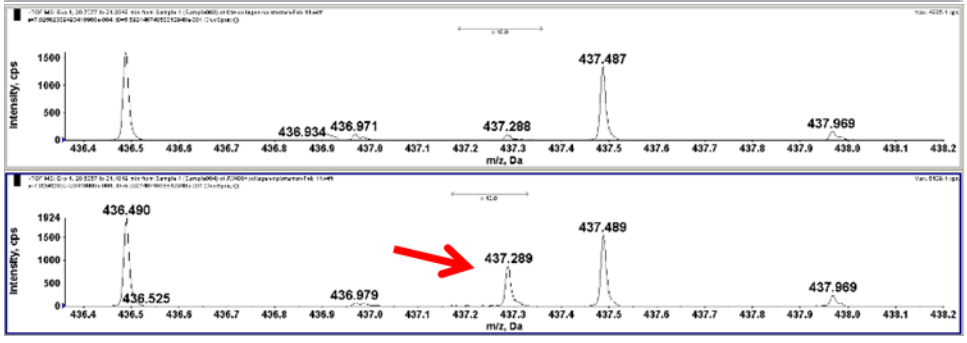
C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437.288
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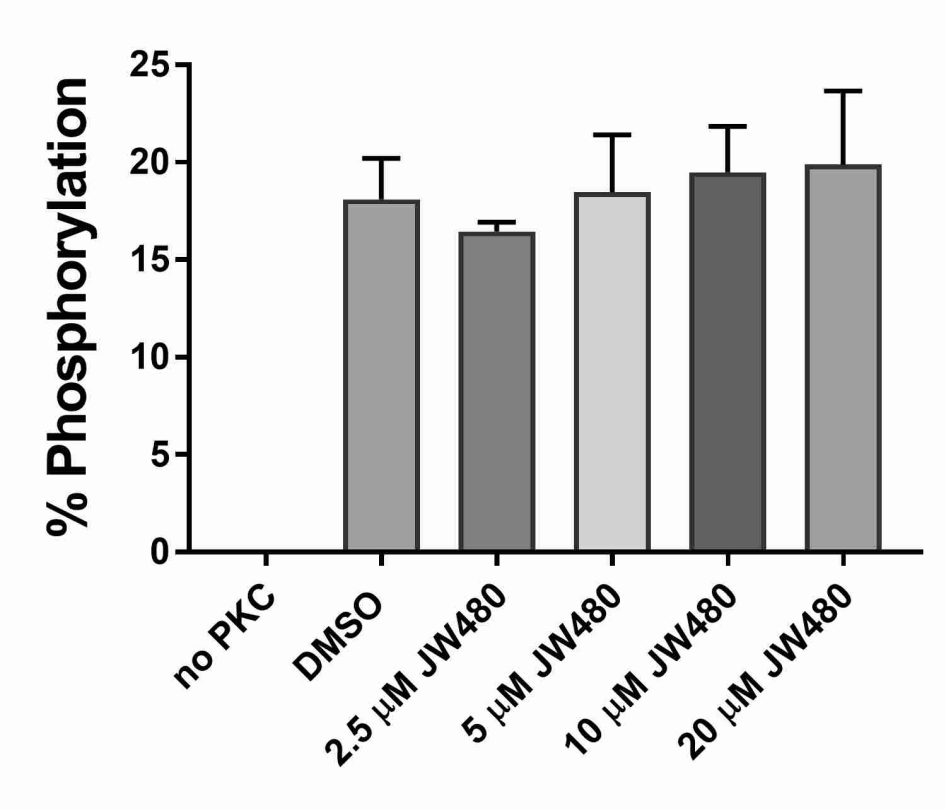
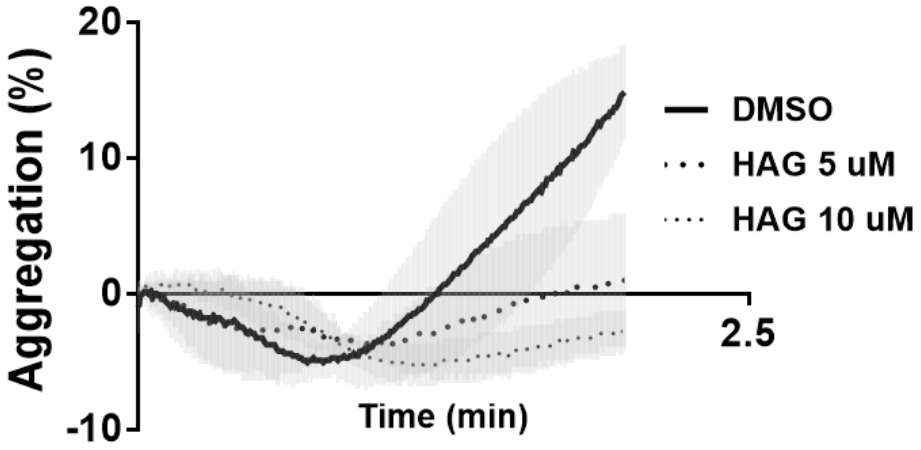
436.525
436.979
Intensity, cps
m/z, Da

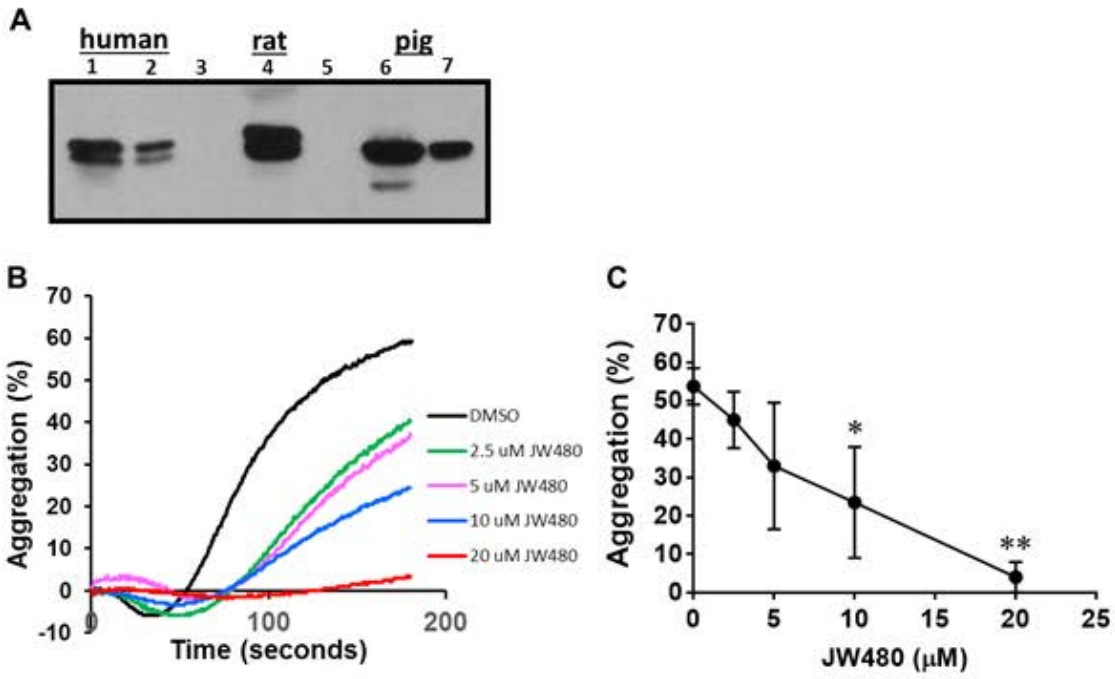
Supplemental Figure 2. HAG-phosphate (HAGP) is identified as a 437.2 *m/z* species by Tandem MS. A) Purified HAGP was subjected to LC-MS; an extracted ion chromatogram revealed a single negatively charged species at 437.2 *m/z*. B) MS/MS fragmentation of 437.2 *m/z* resulted in a major M2 ion at 377.2 *m/z*, which corresponds to the deacetylated lipid (arrows designate cleavage products). C) Platelet lipids were analyzed by NPLC-ESI/MS in a negative ion mode as described above and tandem MS was used to identify HAGP (arrow at 437.2 *m/z*) based on comparison with the pattern observed in B. Shown are lipid spectra from control (top panel) and JW480-treated platelets (bottom panel).



Supplemental Figure 3. JW480 does not significantly alter PKCα kinase activity. Recombinant, human PKCα (100 ng) was pretreated with the indicated concentrations of JW480 or DMSO (4%) for 10 min at 37°C before stimulation with 16 μM PMA for 50 min in the presence of ATP and a fluorescent peptide substrate. Phosphorylated and nonphosphorylated peptides were separated by agarose gel electrophoresis and quantitated to calculate percent phosphorylation (mean ± SEM, n = 3). Kinase activity for DMSO vs. any JW480 treatment were not statistically different ($p > 0.49$).



Supplemental Figure 4. HAG validation for PKC phosphorylation analysis. Washed human platelets were pretreated with 0.5% DMSO vehicle or the indicated concentrations of HAG for 30 min before stimulation with 0.375 μg/mL collagen. Platelet aggregation was observed for 2 min before lysis and analysis of PKC phosphorylation by Western blotting.



Supplemental Figure 5. JW480 dose-dependently inhibits rat platelet aggregation in response to collagen. A) Rat platelets were purified and adjusted to 2 x 10^9/mL in modified pH 6.5 HEPES-Tyrode's buffer before lysis by sonication. Lysates were analyzed by Western blotting as described in Methods. Human and pig lysates were included for reference. Lanes 1 and 4 contained 9 x 10^7 human or rat platelet equivalents respectively, whereas lane 2 contained 4 x 10^7 human platelet equivalents. Rat AADACL1 ran as a doublet similar to the human protein. B) Rat platelets were purified from the heparinized blood of 4.5 – 6 month old male Wistar rats before pretreatment with JW480 or DMSO control (0.5%) for 30 min at 37°C. Aggregation was initiated with collagen at a concentration that caused half-maximal aggregation (5 – 10 µg/mL). Shown are representative aggregation traces from platelets treated with the indicated concentrations of JW480 and stimulated with collagen. C) Data from four independent experiments are expressed as the mean maximum amplitude ± SEM as a function of JW480 concentration. JW480 potently blocked aggregation by 56% and 93% for 10 µM and 20 µM respectively compared to DMSO controls (∗ $p < 0.03$ for 10 µM and ∗∗ $p < 0.001$ for 20 µM).

References

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5. Holly SP, Chang JW, Li W, et al. Chemoproteomic discovery of AADACL1 as a regulator of human platelet activation. *Chemistry & biology.* 2013;20(9):1125-1134.