FIGURES S1 – S10

Figure S1. KODA-PC induces the phosphorylation of numerous proteins in human platelets. Human platelets isolated by gel filtration were incubated with 50 μM PLPC or 50 μM KODA-PC for 5 and 20 minutes. Equal amount of protein were separated by gel electrophoresis and probed with antibodies raised against (A) phospho-tyrosine (Cell Signaling, catalog # 9411) and (B) phospho-Ser PKC substrate (Cell Signaling, catalog # 2261) as indicators of changes in tyrosine, serine, and threonine phosphorylation.

Figure S2. Abundance of proteins identified by phosphoproteomics in platelets based on Burkhart et al (Blood, 120: e73-82, 2012). Phosphorylated proteins identified in this study were matched with protein abundance in platelets as reported by Burkhart et al (Blood, 120: e73-82, 2012). Unique proteins from the original reference or proteins identified in our study were grouped based on their abundance (estimated copies/platelet) and the percentage of each group calculated.

Figure S3. Motif-X analysis of sites that were (de)phosphorylated after platelet activation by KODA-PC and thrombin. The tryptic peptides identified by phosphoproteomics were grouped based on phosphorylation and dephosphorylation changes and used for motif extraction with Motif-X as described in Materials and Methods S1. Sequence logos generated by Motif-X software and the predicted substrate/binding motifs are shown.

Figure S4. Selected GO-terms enriched from the list of genes identified by phosphoproteomic in platelets activated by KODA-PC and thrombin. The list of proteins identified by phosphoproteomics (all) and proteins that changed in phosphorylation for the indicated agonist (changed) were analyzed for GO-term enrichment in (A) cellular component, (B) biological process, and (C) molecular function. Only selected terms are shown in the graphs. The complete list of terms and enrichment values is shown in Table S4 in File S2.

Figure S5. PLC inhibitor blocks platelet P-selectin expression induced by different ligands. Human platelets isolated by gel filtration were incubated with 10 μM PLC inhibitor (U73122), or control (DMSO) for 30 min. Then, 50 μM KODA-PC, 10 μM ADP, or 0.05 U/mL thrombin were added for 30 min. Platelet P-selectin expression was determined using PE-conjugated antibody to P-selectin.

Figure S6. ERK kinase is not required for P-selectin expression induced by KODA-PC. Human platelets isolated by gel filtration were incubated with 10 μM SFK inhibitor (PP2), 10 μM ERK kinase inhibitor (PD98059), or control (DMSO) for 30 min. Then, 30 μM KODA-PC was added for 30 min. Platelet P-selectin expression was determined using PE-conjugated antibody to P-selectin. (A) Flow
cytometry histograms from representative experiments are shown. (B) Quantitation of flow cytometry data presented as mean ± SD of at least 3 independent experiments. * P < 0.05.

**Figure S7. KODA-PC induces Src-family kinases (SFK) phosphorylation.** Human platelets isolated by gel filtration were incubated with DMSO or 10 μM SFK inhibitor (PP2); then, 50 μM PLPC or 50 μM KODA-PC were added to the platelets for 7 minutes. Equal amount of protein were separated by gel electrophoresis and probed with antibodies raised against phosphorylated SFK (pTyr416).

**Figure S8. SYK and PLCγ2 phosphorylation induced by KODA-PC at different time points in the presence of CD36 blocking antibody and chemical inhibitors to SFK and SYK.** (A) Human platelets isolated by gel filtration were incubated with CD36 blocking antibody clone FA6-152 (CD36), or a negative control antibody (IgG); then, 50 μM PLPC or 50 μM KODA-PC were added to the platelets for 5 and 30 minutes. Equal amount of protein were separated by gel electrophoresis and probed with antibodies raised against phosphorylated SYK (pTyr525/526) and PLCγ2 (pTyr1217). The blots were reprobed with SYK and PLCγ2 antibodies for normalization. (B) Human platelets isolated by gel filtration were incubated with DMSO, 10 μM SFK inhibitor (PP2), or 0.2 μM SYK inhibitor (BAY61-3606) for 30 min; then, 50 μM PLPC or 50 μM KODA-PC were added to the platelets for 5 and 30 minutes. Equal amount of protein were separated by gel electrophoresis and probed with antibodies raised against phosphorylated SYK (pTyr525/526) and PLCγ2 (pTyr1217). The blots were reprobed with SYK and PLCγ2 antibodies for normalization.

**Figure S9. Surface level expression of CD41 and CD61 is not affected by KODA-PC, thrombin, chemical inhibitors PP2 and BAY61-3606, or blocking antibody to CD36.** Human platelets isolated by gel filtration were incubated with 10 μM SFK inhibitor (PP2), 0.2 μM SYK inhibitor (BAY 61-3606), blocking antibody to CD36 (CD36), or controls (DMSO or IgG) for 30 min. Then, 50 μM KODA-PC, 0.05 U/mL thrombin, or buffer alone (Tyrode’s buffer) were added for 30 min. (A) Platelet CD41 (integrin α2b) expression was determined by flow cytometry using PE-conjugated antibody to CD41 (Millipore, catalog # MAB1207). (B) Platelet CD61 (integrin β3) expression was determined by flow cytometry using PE-conjugated antibody to CD61 (BD Biosciences, catalog # 555754). (A, B) PE-conjugated mouse IgG1 (PE-IgG, BD Biosciences, catalog # 555749) was used for control.

**Figure S10. Phosphoproteome network regulating integrin in platelets activated by KODA-PC.** Proteins and their sites of phosphorylation identified by mass spectrometry were mapped onto an integrin protein-interaction network (reference 32) as described in Materials and Methods. Graphical representation was done with Cytoscape. Proteins are colored based on the changes in phosphorylation (yellow circle for at least one phosphorylation event induced by thrombin; white circle for no measurable
change by thrombin). Phosphorylation sites are colored based on the up-regulation (red diamond), down-regulation (green diamond), or absence of modification (white diamond) by KODA-PC.
Figure S1

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Phospho-tyrosine

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Phospho-PKC substrate
Figure S2

Platelet proteins (Burkhart et al. Blood 2012)

Phosphorylated proteins identified in this study

Number of proteins (% of total)

Number copies / platelet

Platelet proteins (Burkhart et al. Blood 2012)

Phosphorylated proteins identified in this study
Figure S3

KODA-induced phosphorylation

Substrate/binding motif

Unknown

Calmodulin-dependent protein kinase II, PKA, PKC substrate motif
14-3-3 domain binding motif

Thrombin-induced dephosphorylation

Substrate/binding motif

ERK1, ERK2 Kinase substrate motif
WW domain binding motif

Unknown
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Figure S4

A

![Bar chart showing the percentage of total for different parts of the cell: plasma membrane part, cytosol, cytoskeleton, actin cytoskeleton, and cell junction. The chart compares KODA (all), KODA (changed), Thrombin (all), and Thrombin (changed).]

B

![Bar chart showing the percentage of total for different intracellular signaling cascades: intracellular signaling cascade, actin filament organization, regulation of cytoskeleton organization, and regulation of signal transduction. The chart compares KODA (all), KODA (changed), Thrombin (all), and Thrombin (changed).]

C

![Bar chart showing the percentage of total for different activities: protein kinase activity, phosphatase activity, actin filament binding, and small GTPase binding. The chart compares KODA (all), KODA (changed), Thrombin (all), and Thrombin (changed).]
Figure S5
Figure S6

A

B

DMSO:KODA
PD98059:KODA
PP2:KODA
Resting

Fluorescence intensity (log scale)

P-selectin expression (% of gated platelets)

DMSO  PD98059  PP2
PLPC  KODA

* ns
Figure S7

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![Image of a gel blot showing pTyr416 SFK levels with PLPC and KODA treatments]
Figure S8

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CD41 (Integrin $\alpha_{2b}$)
CD61 (Integrin β₃)
Figure S10