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PCTP contributes to human platelet activation by enhancing dense granule secretion

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ABSTRACT

PCTP (phosphatidylcholine transfer protein) was discovered recently to regulate aggregation of human platelets stimulated with PAR4 activating peptide (PAR4AP). However, the role of PCTP following thrombin stimulation, the mechanisms by which PCTP contributes to platelet activation, and the role of PCTP with other receptors remained unknown. As mouse platelets do not express PCTP, we treated human platelets with various agonists in the presence of the specific PCTP inhibitor A1. We observed that PCTP inhibition significantly reduced dense granule secretion in response to thrombin, PAR1AP, PAR4AP, convulxin (GPVI agonist) and FcγRIIA cross-linking. In contrast, among these agonists, PCTP inhibition reduced aggregation only to low dose thrombin and PAR4AP. Unlike its effects on dense granule secretion, PCTP inhibition did not reduce alpha granule secretion in response to thrombin or PAR4AP. PCTP inhibition reduced both the increase in cytoplasmic Ca^{2+} as well as PKC activity downstream of thrombin. These data are consistent with PCTP contributing to secretion of dense granules, and to being particularly important to human PAR4 early signaling events. Future studies will address further these molecular mechanisms and consequences for hemostasis and thrombosis.

1. Introduction

The role of PCTP in platelet activation first came to attention when a team including one of us (LE) discovered that the level of PCTP protein correlated with platelet aggregation to PAR4AP [1]. The higher the PCTP level, the greater the reactivity to PAR4AP, at doses of 50 μM and 75 μM. PCTP protein is absent in mouse platelets [1]. Therefore, in order to learn more about the role of PCTP, the investigators treated human platelets with a well characterized PCTP inhibitor, A1, prior to their being stimulated with either PAR1AP or PAR4AP. There was no effect of A1 on PAR1AP-mediated aggregation at A1 doses of 25, 50 and 100 μM. In contrast, A1 at 100 μM fully inhibited platelet aggregation to PAR4AP. Lower doses of A1 were either ineffective (25 μM) or partially effective (50 μM) in inhibiting PAR4AP stimulation. Thus, the A1 dose response and the role of PCTP in PAR4-mediated aggregation were established. However, thrombin is the physiologic agonist and works on both PAR1 and PAR4 on human platelets. Recognizing the different affinity of thrombin for PAR1 and PAR4 and the differences in signaling is critical to understanding the mechanisms of PARs in platelet activation and thrombosis.

The role of PCTP in thrombin-mediated activation events including granule secretion and aggregation, the mechanisms by which it contributes to platelet activation downstream of PAR4, and the role of PCTP with other agonists such as those stimulating ITAM-linked receptors, remains unknown.

In this work, we have studied the role of PCTP in thrombin stimulation of dense granule secretion, aggregation and alpha granule secretion. We compared the effects of PCTP inhibition on thrombin stimulation with that of PAR4AP, PAR1AP and agonists of ITAM-linked (immunoreceptor tyrosine-based activation motif) receptors GPVI and FcγRIIA. We report for the first time that PCTP inhibition in human platelets reduces dense granule secretion by blunting the increase in intracellular Ca^{2+} and decreasing PKC activity.

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2. Materials and methods

2.1. Reagents

PCTP inhibitor-A1 was purchased from Tocris Bioscience (Minneapolis, MN, USA) and dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO, USA). PAR1AP and PAR4AP from GL-Biochem (China), Human alpha thrombin from Enzyme Research laboratories (South Bend, IN, USA) and PGE_1 from Cayman Chemicals (Ann Arbor, MI, USA). Chronolume and ATP were from Chrono-log (Havertown, PA, USA). Anti-CD62P-PE was purchased from eBiosciences/Thermo Fisher Scientific (Waltham, MA, USA). Acid citrate dextrose (ACD) tubes were obtained from BD (Franklin Lakes, NJ, USA). BCA kit and 5× reducing gel loading buffer from Pierce/Thermo Fisher Scientific. Protease inhibitor cocktail tablets and Phosphatase inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA), sodium fluoride, sodium orthovanadate and PMSF from Sigma/Thermo Fisher Scientific. Phospho PKC substrate and p-PKD2 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and GAPDH antibody from Proteintech (Rosemont, IL, USA). Goat anti-rabbit and Goat anti-mouse antibodies were obtained from LICOR (LI-COR Biosciences, Lincoln, Nebraska). Calcium 6 QF assay kit, 96-well black, clear-bottom plate, and 96-well black pipette tips for Flex station were purchased from Molecular Devices, (San Jose, CA, USA). Convulxin was obtained from K J Clemetson (Theodor Kocher Institute, University of Berne, Switzerland), IV.3 antibody from Stemcell Technologies, (Vancouver, Canada), and goat anti-mouse Antibody (GAM) from Jackson Immunoresearch labs, (Westgrove, PA). Qiagen Blood Core Kit was purchased from Qiagen (Germantown, MD, USA). PAR4 primers were synthesized by Eurofins (Horsham, PA, USA), as follows: forward primer 5′ AAGTCTGTGCCAATGACAGT 3′, reverse primer 5′ GCTCGGGTCCGAGTAATG 3′.

All other chemicals were purchased from Sigma or Fisher Scientific.

2.2. Preparation of washed human platelets

Healthy volunteers were recruited according to the protocols approved by the Institutional Review Board at Thomas Jefferson University, Philadelphia, PA. Informed consent forms were signed prior to blood draw. Venous blood from each volunteer was drawn into vacutainer tubes containing ACD and washed platelet suspensions were prepared as follows. Briefly, whole blood was centrifuged at 800 rpm for 20 min with no brakes and the platelet rich plasma (PRP) separated. PRP was diluted with HEN buffer (10 mM HEPES, 150 mM sodium chloride and 1 mM EDTA, pH 6.5) at a ratio 4:1 and 1 μM PGE1 was added to the PRP. Platelet pellet was obtained following centrifugation at 1300 rpm for 10 min. Platelets were resuspended in modified Tyrode's buffer (137 mM NaCl, 25 mM HEPES, 1 mM $MgCl_2$, 2.7 mM KCl, 0.1% Glucose, 0.485 mM NaH_2PO4, 12 mM $NaHCO_3$, 1 mM $CaCl_2$, pH 7.4) to a final concentration of 3×10^8/ml or as indicated below. After final resuspension, the platelets were rested at 37 °C for 30 min before use in experiments.

2.3. Genotyping of PAR4 single nucleotide variant (SNV) rs773902 (G/A; Ala/Thr120)

We used whole blood obtained in ACD tubes, then centrifuged at 250*g* for 15 min with no brake. The buffy coat layer was isolated and genomic DNA (gDNA) was extracted via the Blood core kit. The gDNA was amplified with PAR-4 specific primers surrounding the SNV, and the sequence of the PCR product was obtained. All of the 5 donors in this study, 4 Caucasians and 1 Asian, were homozygous for rs773902 GG (Ala120/Ala120). Recent studies have indicated that the PAR4 SNV contributes to differential response of human platelets at thrombin doses ≤0.8 nM and PAR4AP doses ≤75 μM [2–4]. In this report, we used thrombin at ≥1 nM and PAR4AP at ≥100 μM, so there was no expected contribution of the SNV genotype.

2.4. Platelet treatment with inhibitor A1

Washed human platelets were pre-incubated with PCTP inhibitor A1 at 100 μM for 1 h at 37 °C. Control platelets were in the same buffer (modified Tyrode's buffer and final concentration 0.5% DMSO) but received no inhibitor. Platelets were then stimulated with agonists thrombin, PAR1AP, PAR4AP, convulxin or IV.3 + GAM at concentrations indicated in each experiment. We used A1 at 100 μM because published dose response studies identified that dose as the minimum effective concentration in inhibiting human platelet aggregation [1].

2.5. Platelet dense granule secretion and aggregation

For this assay, 3×10^8 cells/ml were stimulated with agonists following pre-treatment of platelets in a Chronolog lumi-aggregometer (Chronolog-Log Corporation, Havertown, PA) at 37 °C under stirring conditions (1200 rpm). Agonists and concentrations employed were as follows: thrombin at 1 nM and 2 nM, PAR1AP at 1 μM and 10 μM, PAR4AP at 100 μM and 200 μM, convulxin at 50, 100 and 200 ng/ml, and IV.3 + GAM at (1 + 5) or (2 + 10) μg/ml. For FcγRIIA stimulation and its known inter individual variability [5], the concentration of the ligand used was 1 + 5 μg/ml (n = 3) or 2 + 10 μg/ml (n = 2) based on our criterion that we obtain at least 50% aggregation in the no inhibitor control.

Platelet dense granule secretion was measured as release of ATP on agonist stimulation. Briefly, 5 μl of Luciferin/Luciferase reagent-Chronolume was resuspended per manufacturer's instructions and added to 245 μl of pre-treated platelets for 2 min before stimulation with agonists. The ATP-Luciferin-Luciferase reaction was standardized with 2 nmole of ATP.

2.6. Flow cytometry for P-Selectin expression

Washed human platelets resuspended in Tyrode's buffer with 1 mM $CaCl_2$ were incubated with A1 or no inhibitor control as described earlier. For P-selectin expression, the cells (50 μl) were stimulated with agonists for 10 min at 37 °C and subsequently incubated with 2 μl of PE conjugated anti-CD62P (1:5 dilution) for another 10 min. The samples were fixed by the addition of 450 μl of Tyrode's buffer containing 1% PFA and 0.1% BSA. Samples were analyzed on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and the data processed using BD Accuri C6 software.

2.7. Intracellular Ca^{2+} flux assay

Washed human platelets (3×10^8 cells/ml) were pre-incubated with A1 inhibitor vs control at 37 °C for 1 h, assessed for changes in cytosolic Ca^{2+} concentrations upon agonist stimulation using a Ca^{2+} sensitive fluorescent dye. The manufacturer's instructions for preparing the dye loading buffer and flex station 3 plate reader were followed. Briefly, platelets were preincubated with 100 μM A1 vs. no inhibitor control for 30 min at 37 °C, then an equal volume of FLIPR Calcium 6 QF loading dye added, and incubation continued for 20 min at 37 °C in the dark. Platelets in buffer with Ca^{2+} at 2 mM final concentration were seeded onto a 96-well, black clear bottom plate (180ul/well), and incubation continued for another 10 min at 37 °C in the Flex station 3 plate reader (Molecular Devices). The total incubation time following dye addition was 30 min. At 10× concentration, the agonist was added to a 96-well plate and loaded to the Flex station 3. Fluorescence readings were recorded with excitation at 485 nm, emission at 525 nm, and automatic emission cut off at 515 nm. The platelet samples were analyzed in Flex Station 3, baseline fluorescence measured (60s) prior to the addition of the agonist (20 μl/well) robotically, and fluorescence signal recorded for additional 4 min following agonist stimulation. Relative Ca^{2+}

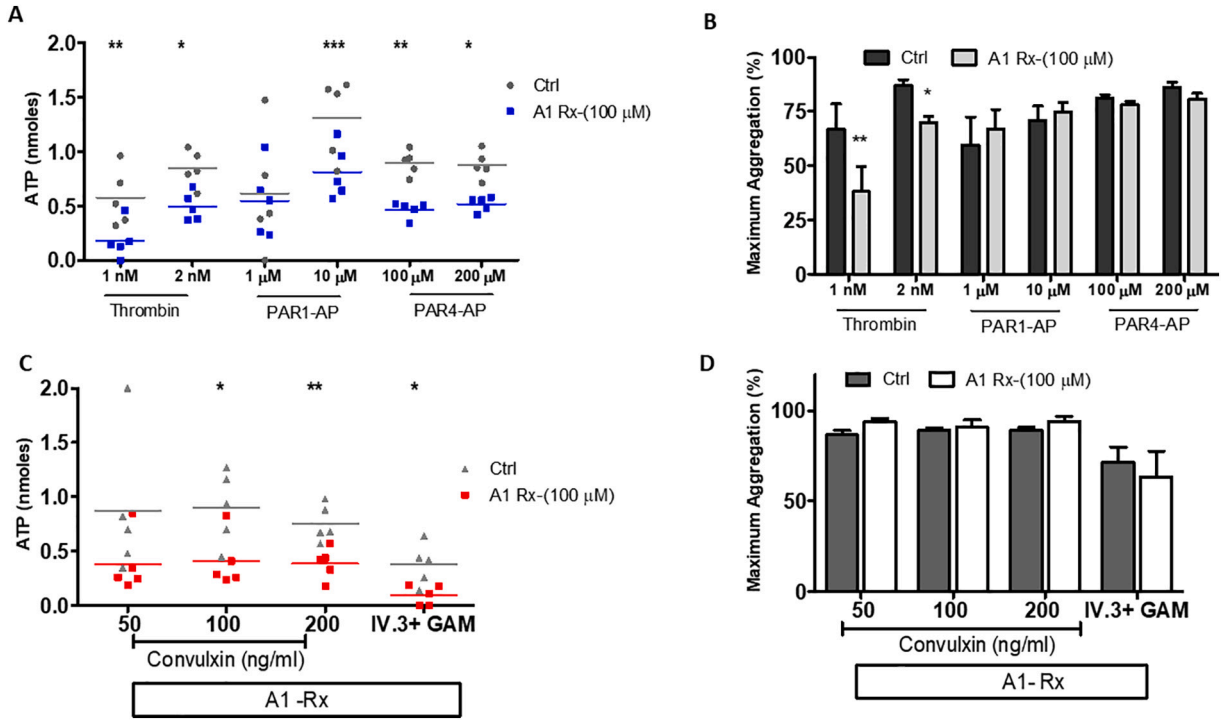

Fig. 1. PCTP regulates dense granule secretion to multiple agonists and aggregation to thrombin. ATP release was assessed in washed human platelets (3×10^8 cells/ml) treated with PCTP inhibitor A1 (100 μM) vs no inhibitor control before stimulation with thrombin, PAR1-AP, or PAR4-AP (A) and convulxin or FcγRIIA receptor ligand (IV.3 + GAM; see Materials and methods for dose) agonists at the concentrations indicated (C). A1 reduced ATP secretion for all agonists tested (mean of each condition shown by horizontal line, $p < 0.05$). Maximum aggregation to thrombin, PAR1AP or PAR4AP (B) and convulxin or IV.3 + GAM (D) showed significant reduction by A1 with thrombin stimulation. Two-way ANOVA was used to assess statistical significance with Bonferroni post-hoc tests for (B) and (D); One sample *t*-test was used for (A) and (C), and p-values <0.05 were considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fluorescence values were quantified as fold change, the ratio of the peak magnitude signal to the basal signal in each well.

2.8. Immunoblotting

Total protein lysates from human (3×10^8/ml) platelets were obtained by addition of 1× RIPA buffer (150 mM NaCl, 1% NP 40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EGTA) with protease inhibitors (PMSF, NaF, Na vanadate, protease inhibitor cocktail, phosphatase inhibitor cocktail) following stimulation of A1 treated platelets with 1 nM thrombin for time points −1, 3 and 5 min. The lysates were kept on ice for 20 min and stored at −80 °C until use. Protein concentration of the cell lysates were measured by BCA Kit. Equal amount of protein (30 μg) was loaded in each lane with gel loading buffer (5×) containing 100 mM DTT. Prior to loading, the lysates were heated at 100 °C for 10 min.

For immunoblotting, cell lysates were fractionated onto a 10% SDS-PAGE, then transferred to a nitrocellulose membrane, blocked with TBS containing 3% BSA for 30 min to 1 h at room temperature (RT). The blots were probed with p-PKC substrate antibody (1:500) or p-PKD2 antibody (1:250) incubated overnight at 4 °C. The proteins were detected with a goat anti-rabbit secondary antibody (1:15,000; LICOR) which was incubated at RT for 1 h. The immunoblots were imaged by LICOR Odyssey system and analyzed using LICOR image Lite Software ver. 5.2 (LI-COR Biosciences, Lincoln, NE). Membranes were then stripped with stripping buffer (25 mM glycine-pH 2.5, 1% SDS) and analyzed for equal loading of protein with GAPDH antibody, as primary antibody, followed by goat anti-mouse (1: 15,000), as secondary antibody.

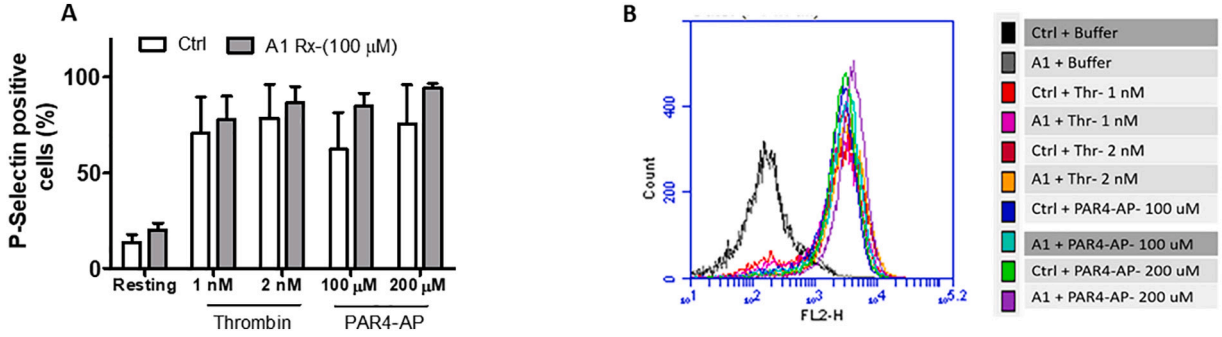

Fig. 2. PCTP inhibitor A1 does not inhibit alpha granule release to thrombin or PAR4AP. Alpha granule secretion was determined in washed human platelets (6 × 10^7 cells/ml) treated with A1 (100 μM) or vehicle followed by stimulation with thrombin or PAR4AP at concentrations indicated. P-Selectin expression was assessed with CD62-PE antibody by flow cytometry on Accuri C6. (A) The bar graph represents P-selectin positive cells (n = 4). (B) A representative flow cytometry data. Two-way ANOVA with Bonferroni post-hoc test detected no statistical significance, and p-value <0.05 considered as significant.

2.9. Statistical analysis

Data analysis was performed using PRISM v5 (GraphPad, CA). All data are expressed as mean ± SEM as indicated in each figure legend. The statistical significance was calculated using two-way ANOVA, Student's *t*-test or one sample *t*-test as indicated in each figure legend; $p < 0.05$ was considered statistically significant.

3. Results

3.1. PCTP regulates dense granule secretion by multiple agonists

Dense granule secretion was inhibited by PCTP inhibitor A1 in platelets stimulated by thrombin at doses of 1 and 2 nM (Fig. 1A, $p < 0.05$). A1 also inhibited dense granule secretion by PAR1AP, PAR4AP, convulxin (GPVI agonist) and IV.3 + GAM (Fig. 1A and C, $p < 0.05$). The trend with PAR1AP at 1 μM (mean reduction to 28% of no inhibitor control) did not reach statistical significance, but the difference was significant at 10 μM. These observations reveal a role for PCTP in dense

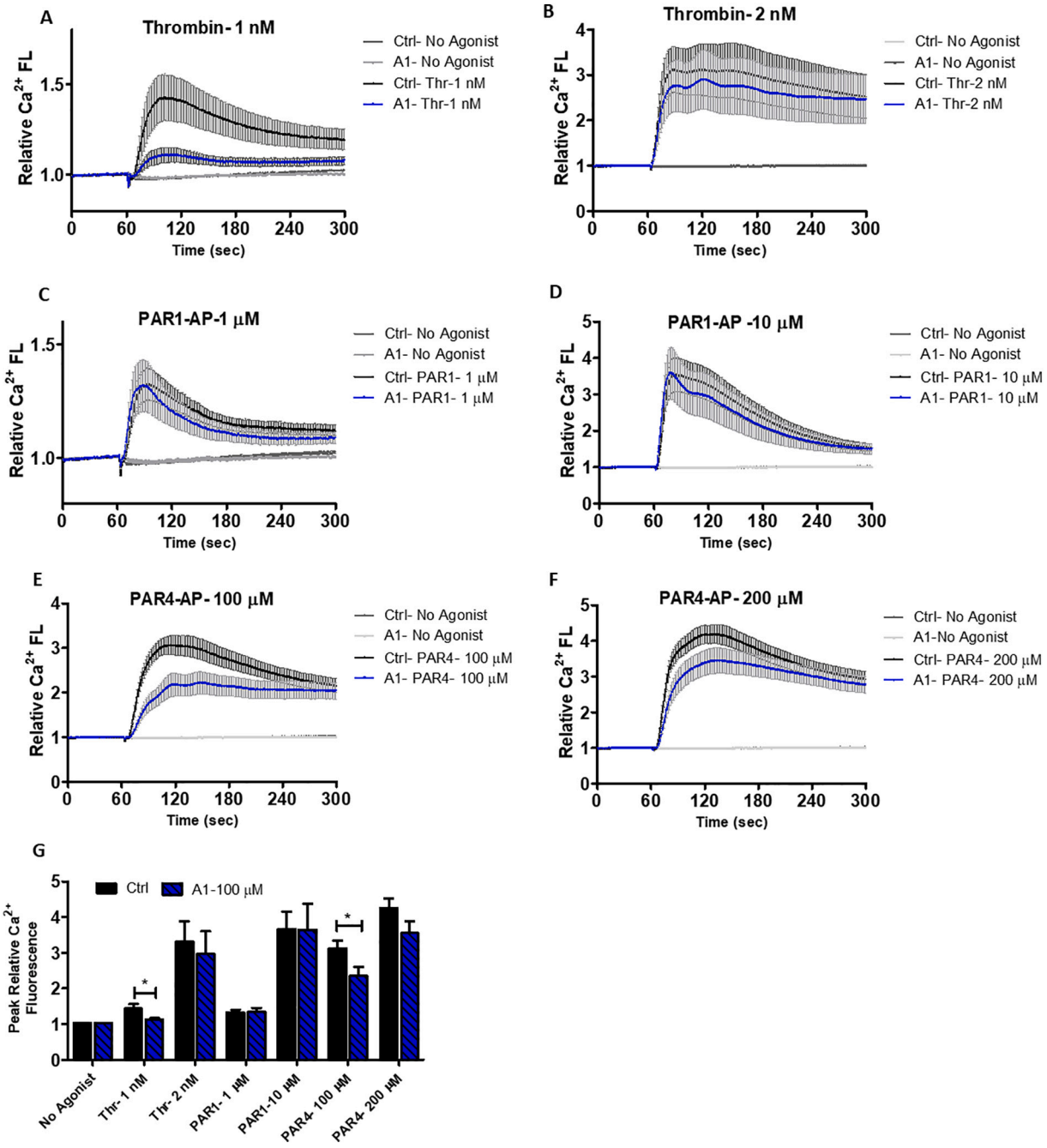

Fig. 3. PCTP regulates the Ca^{2+} response stimulated by thrombin and PAR4. Ca^{2+} mobilization in washed platelets (3×10^8 cells/ml) treated with A1 (100 μM) vs. no inhibitor control was measured in response to the agonists indicated using a calcium 6 QF assay. Fluorescence data are presented as relative calcium fluorescence (FL) detecting fold change in cytosolic calcium (A–F); the graphs represent mean ± SEM from n = 5 independent experiments performed in technical duplicates. The bar graph (G) represents peak relative calcium fluorescence with mean ± SEM for the agonists examined. Unpaired Student's *t*-test indicates statistical significance ($p < 0.05$) between control and A1-treated platelets.

granule secretion not only downstream of GPCRs (G-protein coupled receptors) PAR4 and PAR1 but also ITAM-linked receptors GPVI and FcγRIIA.

3.2. PCTP regulates aggregation by thrombin

A1 significantly inhibited aggregation upon stimulation with 1 nM or 2 nM thrombin (Fig. 1B; $p < 0.05$). The degree of inhibition was less at 2 nM thrombin than 1 nM thrombin. However, neither aggregation by PAR1-AP (1 μM and 10 μM) nor by higher doses of PAR4-AP (100 μM and 200 μM) were inhibited (Fig. 1B). Note that inhibition of aggregation by A1 (100 μM) was previously reported by Edelstein et al. when PAR4AP doses were lower, namely 50 and 75 μM [1].

A1 did not inhibit aggregation by convulxin or IV.3 + GAM, either (Fig. 1D). These findings indicate that PCTP regulates aggregation particularly at low to intermediate doses of thrombin and lower doses of PAR4AP.

3.3. PCTP does not regulate alpha granule secretion

We next investigated PCTP in alpha granule secretion using P-Selectin expression as a marker for release of alpha granule content. As shown in Fig. 2, the agonist-stimulated increase in P-selectin expression on the surface of platelets was the same in the presence or absence of PCTP inhibitor following stimulation with thrombin at 1 nM or 2 nM, as well as with PAR4-AP at 100 μM or 200 μM (n = 4; p not significant).

Together, these observations are consistent with PCTP having a special role in dense granule secretion for these agonists at these doses.

3.4. PCTP regulates the increase in intracellular Ca^{2+} in response to thrombin and PAR4AP

Previous studies reported a decrease in Ca^{2+} mobilization in megakaryocyte-like Meg-01 cells treated with siRNA against PCTP prior to PAR4AP stimulation [1]. In that system, siRNA to PCTP did not affect the increase in intracellular Ca^{2+} following stimulation by PAR1AP. We evaluated the consequences for Ca^{2+} mobilization in A1- treated platelets. In comparison with controls, A1 reduced the increase in Ca^{2+} significantly following stimulation with thrombin at 1 nM and PAR4AP at 100 μM (Fig. 3). In contrast, A1 did not inhibit the increase in Ca^{2+} in response to higher doses of thrombin at 2 nM or PAR4AP at 200 μM (Fig. 3). In addition, consistent with the findings in Meg-01 cells, A1 inhibition did not affect the increase in intracellular Ca^{2+} in response to PAR1AP at 1 or 10 μM in human platelets .

3.5. PCTP regulates phosphorylation of PKC substrates implicated in dense granule secretion

Thrombin and PAR4 stimulation of human platelets regulate dense granule release via PKC [6–8]. We assessed phosphorylation of PKC substrates pleckstrin and PKD2 in platelets stimulated with thrombin at 1 nM (Fig. 4A and C). Compared to controls, a statistically significant decrease in both pleckstrin and PKD2 phosphorylation was observed at 1 min and 3 min in A1-treated platelets (Fig. 4B and D, $p < 0.05$). Together with the Ca^{2+} results, these findings are consistent with a role for PCTP in proximal PAR4 signaling.

4. Discussion

PCTP was first identified as contributing to the regulation of human platelet aggregation via PAR4 stimulated by PAR4AP [1]. Specifically,

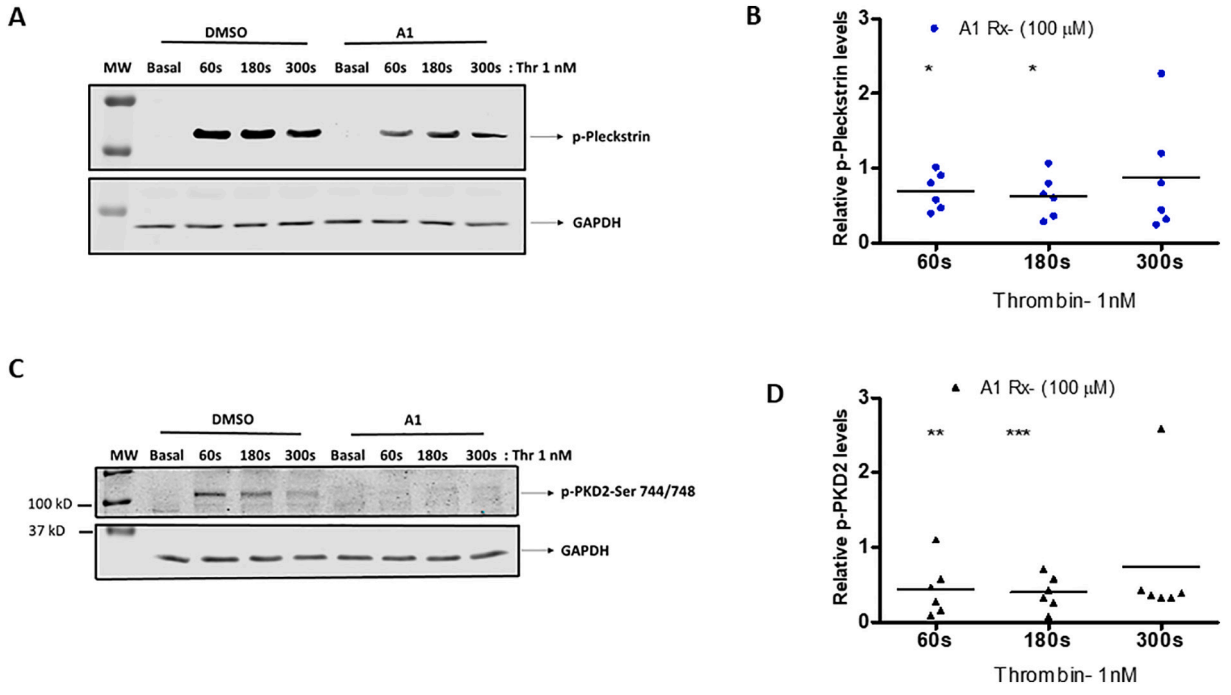

Fig. 4. PCTP regulates phosphorylation of PKC substrates Pleckstrin and Protein Kinase D2. Washed human platelets (3×10^8 cells/ml) were pre-incubated with PCTP inhibitor A1 (100 μM) vs. no inhibitor control for 1 h before stimulation with 1 nM thrombin. Cells lysed in RIPA buffer were fractionated on a 10% SDS-PAGE gel and immunoblotted with antibodies for PKC substrate (phospho-Pleckstrin) (A–B) and phospho-Protein Kinase D2 (pSer744/748) (C–D). The dot plots (B) and (D) depict the relative phosphoprotein level for the indicated time points following thrombin stimulation. Relative phosphoprotein levels were calculated as follows. First, each phosphoprotein band intensity was normalized for loading by the GAPDH band in its lane. Then, the ratio of the normalized phosphoprotein band intensity for A1-treated platelets to the normalized band intensity of control platelets at that same time point for that donor was computed. Those values are plotted, such that the respective untreated control for each time point has been set to 1. The mean is indicated by the horizontal line, with n = 6 independent experiments. Statistical significance analyzed by One sample *t*-test for each time point for p-values <0.05 (*), <0.01 (**), <0.001 (***).

the level of PCTP protein correlated positively with aggregation to PAR4AP at doses of 50 and 75 μM. In contrast to the prior report of A1 inhibition at low dose PAR4AP, we now show that aggregation is not inhibited at higher doses of PAR4AP, 100 and 200 μM. In order to identify the role of PCTP in response to the physiologic agonist thrombin, we treated human platelets with A1 and observed reduced dense granule secretion at thrombin doses of 1 and 2 nM. We then examined the effects of PCTP inhibition on dense granule secretion downstream of PAR1, GPVI and FcγRIIA. PCTP inhibition reduced dense granule secretion in response to agonists of each of these receptors, consistent with a more general role in this response.

We observed that aggregation was inhibited by A1 both with low dose PAR4AP [Edelstein 2013] and thrombin. In contrast, PCTP inhibition did not result in decreased aggregation in response to PAR1, GPVI or FcγRIIA stimulation. Recent work by a team (including LE and SMcK) demonstrated that aggregation is at maximal response at these doses and that PAR4 contributes significantly [2]. This point is important, since thrombin acts on both PAR1 and PAR4, but the major role of PCTP tracks with its effects on PAR4. Our findings are in keeping with the requirement for ADP secretion downstream of PAR4, acting via P2Y12 to sustain aggregation to low dose thrombin [9–11]. Our model for PCTP action in stimulation of human platelets via PAR4 is depicted in Fig. 5.

One outstanding question is, what is PCTP doing in the platelet response to agonists? PCTP has a canonical function in transferring PC (phosphatidylcholine) from the endoplasmic reticulum to the plasma membrane and other intracellular phospholipid membranes, often at specific membrane contact points [12]. PCTP is also a cofactor for the acyl CoA thioesterase ACOT13 [13]. Acyl CoA thioesterases release longer chain fatty acids from CoA, where they can serve in intermediary metabolism or in dynamic phospholipid remodeling. A1 blocks docking of PC to PCTP, interfering with both its PC transfer function and with its cofactor activity for ACOT13 [13]. Future studies are needed to see if one or both of these functions of PCTP are responsible for its role in platelet activation (Fig. 5).

A second outstanding question is, how can PCTP function in both GPCR and ITAM pathways? PCTP actions in platelets are consistent with roles in the activity of both PLCβ, downstream of Gq-coupled GPCRs such as PAR4 and PAR1, and PLCγ2, downstream of ITAM receptors such as GPVI and FcγRIIA. In each case PCTP potentially facilitates generation of Inositol 3,4,5 phosphate (IP3) and diacyl glycerol (DAG) from phosphoinositol 4,5, bis phosphate (PI4,5,P2) to cause increased intracellular Ca^{2+} and PKC activity. PKC is a known regulator of dense granule secretion, consistent with our findings [9,11]. In this study, downstream of PAR4, PCTP inhibition reduced dense granule but not alpha granule secretion. Several groups have identified that dense granule secretion, but not alpha granule secretion, is regulated by PKC and PKD2 [7,8]. Phospho-PKC and -PKD2 likely function in dense granule secretion by phosphorylation of v-SNAREs, t-SNAREs and associated proteins such as SNAP23 and syntaxin-11 [14]. Future studies will be needed to explore how PCTP contributes to this difference.

In conclusion, future studies of this newly recognized role for PCTP in human platelets will enable greater insights into its role in hemostasis and thrombosis.

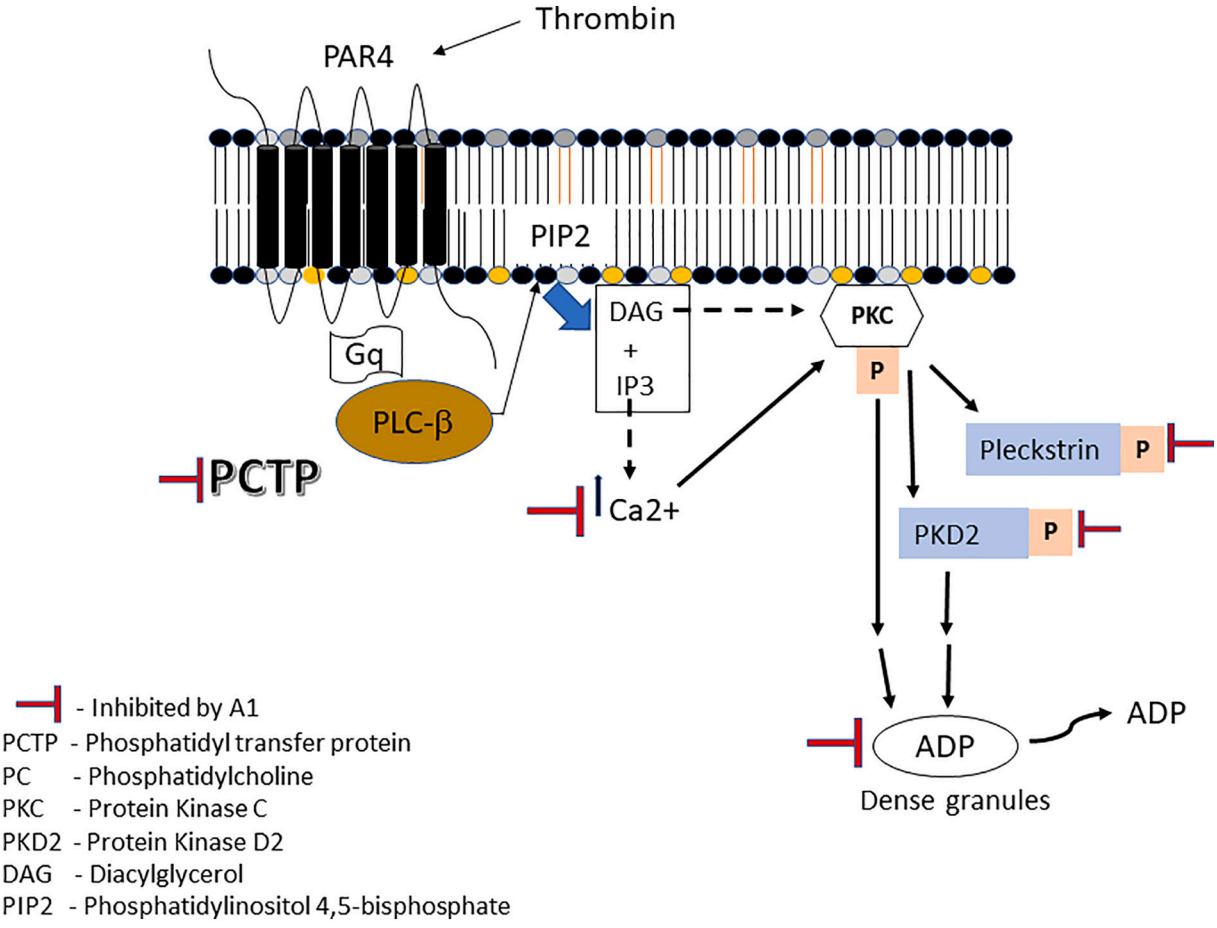

Fig. 5. Model of PCTP in human platelet PAR4 signaling.
Downstream of Gq-coupled PAR4, PCTP supports the increase in intracellular Ca^{2+} and phosphorylation of PKC substrates Pleckstrin and PKD2. Together they regulate dense granule release, providing ADP to signal to P2Y12 (not shown) for sustained aggregation.

CRediT authorship contribution statement

S Abraham performed experiments, analyzed data, and wrote and edited the manuscript. LM, XK and S Askari performed experiments and edited the manuscript. LE conceived the project, reviewed data and edited the manuscript. SMcK conceived the project, analyzed data, and wrote and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] L.C. Edelstein, L.M. Simon, R.T. Montoya, M. Holinstat, E.S. Chen, A. Bergeron, et al., Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c, Nat. Med. 19 (12) (2013) 1609–1616.

[2] M.J. Whitley, D.M. Henke, A. Ghazi, M. Nieman, M. Stoller, L.M. Simon, et al., The protease-activated receptor 4 Ala120Thr variant alters platelet responsiveness to low-dose thrombin and protease-activated receptor 4 desensitization, and is blocked by non-competitive P2Y12 inhibition, J. Thromb. Haemost. 16 (12) (2018) 2501–2514.

[3] S.L. French, C. Thalmann, P.F. Bray, L.E. Macdonald, A.J. Murphy, M.W. Sleeman, et al., A function-blocking PAR4 antibody is markedly antithrombotic in the face of a hyperreactive PAR4 variant, Blood advances. 2 (11) (2018) 1283–1293.

[4] Y. Morikawa, H. Kato, H. Kashiwagi, N. Nishiura, K. Akuta, S. Honda, et al., Protease-activated receptor-4 (PAR4) variant influences on platelet reactivity induced by PAR4-activating peptide through altered Ca(2+) mobilization and ERK phosphorylation in healthy Japanese subjects, Thromb. Res. 162 (2017) 44–52.

[5] Y. Zhou, S. Abraham, P. Andre, L.C. Edelstein, C.A. Shaw, C.A. Dangelmaier, et al., Anti-miR-148a regulates platelet FcgammaRIIA signaling and decreases thrombosis in vivo in mice, Blood. 126 (26) (2015) 2871–2881.

[6] O. Konopatskaya, K. Gilio, M.T. Harper, Y. Zhao, J.M. Cosemans, Z.A. Karim, et al., PKCalpha regulates platelet granule secretion and thrombus formation in mice, J. Clin. Invest. 119 (2) (2009) 399–407.

[7] O. Konopatskaya, S.A. Matthews, M.T. Harper, K. Gilio, J.M. Cosemans, C. M. Williams, et al., Protein kinase C mediates platelet secretion and thrombus formation through protein kinase D2, Blood. 118 (2) (2011) 416–424.

[8] D. Bhavanasi, S. Kim, L.E. Goldfinger, S.P. Kunapuli, Protein kinase Cdelta mediates the activation of protein kinase D2 in platelets, Biochem. Pharmacol. 82 (7) (2011) 720–727.

[9] S. Kim, C. Foster, A. Lecchi, T.M. Quinton, D.M. Prosser, J. Jin, et al., Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling, Blood. 99 (10) (2002) 3629–3636.

[10] L. Stefanini, D.S. Paul, R.F. Robledo, E.R. Chan, T.M. Getz, R.A. Campbell, et al., RASA3 is a critical inhibitor of RAP1-dependent platelet activation, J. Clin. Invest. 125 (4) (2015) 1419–1432.

[11] L. Stefanini, W. Bergmeier, Negative regulators of platelet activation and adhesion, J. Thromb. Haemost. 16 (2) (2018) 220–230.

[12] S. Cockcroft, P. Raghu, Phospholipid transport protein function at organelle contact sites, Curr. Opin. Cell Biol. 53 (2018) 52–60.

[13] N. Wagle, J. Xian, E.Y. Shishova, J. Wei, M.A. Glicksman, G.D. Cuny, et al., Small-molecule inhibitors of phosphatidylcholine transfer protein/StarD2 identified by high-throughput screening, Anal. Biochem. 383 (1) (2008) 85–92.

[14] S. Joshi, S.W. Whiteheart, The nuts and bolts of the platelet release reaction, Platelets. 28 (2) (2017) 129–137.