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GPIbα negatively regulates thrombin-mediated platelet function through PKCδ signaling

Younes Zaid[1,2,3,*], *Mounia Oudghiri*[2], *Abdallah Naya*[2], *Rachida Hassikou*[1]

[1] *Botany Laboratory, Biology Department, Faculty of Sciences, Mohammed V University, Rabat, Morocco.*

[2] *Immunology and Biodiversity Laboratory, Department of Biology, Faculty of Sciences Ain Chock, Hassan II University, Casablanca, Morocco.*

[3] *Research Center of Abulcasis University of Health Sciences, Rabat, Morocco.*

Abstract

Protein kinase Cδ (PKCδ) negatively and positively regulates platelet function in response to GPVI and protease-activated receptors (PAR) stimulation, respectively. However, its importance in response to thrombin stimulation via the high-affinity GPIbα binding site remains unknown. We showed that in human platelets, pre-treatment with the specific PKCδ inhibitor δ(V1-1)TAT significantly potentiates platelet aggregation in response to a low concentration of α-thrombin, but not to δ-thrombin or stimulation of PAR1 and PAR4, in a GPIbα-dependent, since blockade of GPIbα thrombin-binding site prevents this effect. This study reveals for the first time that GPIbα negatively regulates thrombin-mediated platelet responses through PKCδ signaling. This adds novel insights into the previously documented role of GPIbα as a positive regulator of platelet function in response to thrombin.

Keywords: Platelets, Protein kinase Cδ, Glycoprotein Ibα, Throbin, Protease-activated receptors.

Full length article **Corresponding Author*, e-mail: *y.zaid@um5r.ac.ma*

1. Introduction

Thrombin signaling in platelets is mediated by a specialized family of G-protein-coupled receptors known as protease-activated receptors (PARs) and by GPIbα [1]. Indeed, GPIbα, PAR-1 and PAR-4 constitute the high-, medium- and low-affinity thrombin receptors in human platelets, respectively [2]. Platelets are anucleated blood cells that play a central role in hemostasis and inflammation [3-4]. These processes are mediated by multiple cell surface adhesive receptors, which enable platelets to interact with the damaged vascular wall and with other platelets, thereby leading to platelet activation and aggregation. Platelet activation is mediated through different signaling pathways among which the protein kinase C (PKC) family of serine/threonine kinases represents a central and redundant element of platelet signaling [5-8]. Members of this family are classified into three subfamilies according to the structure of their regulatory domains.[9] Conventional PKC (cPKC) isoforms (α, βI/II and γ) contain both C1 and C2 domains and thus require both DAG and Ca^{2+} for activation. Novel PKC (nPKC) isoforms (δ, θ, η and ε) also contain C1 domains, but lack the ability to bind Ca^{2+} at the C2-like domain. Atypical PKC (aPKC) isoforms (ζ and ι/λ) lack the C2 domain and have an atypical C1 domain. Multiple PKC isoforms are expressed in platelets and have been shown to regulated distinct elements of platelet function such as platelet aggregation, secretion and shape change [10-12]. For instance, it has been documented that PKCδ negatively and positively influences platelet function in response to collagen and protease-activated receptor (PAR) stimulation, respectively [13-14].

The platelet glycoprotein (GP) Ib/IX/V complex is required for platelet adhesion under shear stress, as demonstrated by the increased bleeding time seen in patients suffering from the Bernard-Soulier syndrome, who are deficient for the GPIb subunit [15]. Although the von Willebrand factor (vWf) represents the main GPIbα ligand, additional ligands of significant importance have been described, namely thrombin. Indeed, this receptor contains a high affinity binding site for thrombin, which has been localized to a sequence spanning the amino acid residues 271 to 284 within the extracellular domain of GPIbα [16]. Peptidomimetics of this site inhibit α-thrombin binding to GPIbα, as well as platelet activation and aggregation induced by subnanomolar thrombin concentration [16]. Although several authors argue that PAR-1 and PAR-4 are sufficient to explain all aspects of platelet activation by thrombin [17-18], others have highlighted the contribution of GPIbα as a

positive regulator of thrombin signaling in platelets without elucidating its exact contribution as well as the underlying cellular mechanism in this response [19-20].

The aim of this study was to evaluate the implication of PKCδ in thrombin-stimulated platelets. Interestingly, we found that PKCδ negatively regulates platelet function downstream of GPIbα in response to a low dose of thrombin. This novel finding adds new insights into the previously documented role of GPIbα as a positive regulator of platelet function in response to thrombin. The GPIbα/PKCδ axis may represent a critical regulator of hemostasis and a dysfunction of this pathway could lead to thrombotic disorders.

2. Materials and methods

2.1. Reagents

Total PKCδ were purchased from Cell Signaling Technology (Beverly, MA). The antibody against P-selectin clone AK4 (PE conjugated) was obtained from BD Biosciences (Mississauga, ON). The blocking anti-human GPIbα monoclonal antibody (SZ2, sodium azide free) was from Beckman Coulter (Mississauga, ON). Bovine α-thrombin was purchased from Sigma-Aldrich (Oaskville, ON). γ-thrombin was acquired from Abcam (Toronto, ON). The TRAP-1 (SFLLRN) and TRAP-4 (AYPGKF) peptides were acquired from Peptides International (Louiseville, KY). Ristocetin was purchased from Chrono-log Corp. (Havertown, PA).

2.2. PKCδ inhibitor peptide

PKCδ membrane translocation was selectively inhibited by the δ(V1-1)TAT peptide as previously described [21]. Briefly, the PKCδ δ(V1-1) interacting sequence of the receptors for activated C kinases (SFNSYELGSL) was coupled to the TAT HIV membrane permeable sequence (YGRKKRRQRRR) through a cysteine-cysteine bridge. Peptide sequencing was performed at CanPeptide Inc. (Pointe-Claire, QC). A scrambled sequence of δ(V1-1) was also synthesized and used as negative control.

2.3. Preparation of human platelets

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment. The protocol was approved by the human ethical committee of the Montreal Heart in accordance with the declaration of Helsinki for experiments involving humans. Washed platelets were prepared as previously described [22-26]. Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of acid citrate dextrose (ratio of 1:5) anticoagulated blood at 200 *g* for 15 minutes. Platelets were then pelleted from PRP, to which 1 μg/mL of PGE_1 was added, washed with HBSS-Hank's sodium citrate buffer (138 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM Na_2HCO_3, 5.6 mM Glucose, 10 mM HEPES, 12.9 mM sodium citrate, pH 7.4), also containing PGE_1 (0.5 μg/mL), and finally resuspended in HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Platelets were adjusted to 250 x 10^6/mL and allowed to rest at 37ºC for 30 minutes before further manipulation.

2.4. Measurement of platelet aggregation

Platelets were pre-incubated with or without δ(V1-1)TAT or its vehicle for 5 minutes at 37ºC. After addition of a platelet agonist, aggregation was monitored on a four-channel optical aggregometer (Chrono-log Corp.) under shear conditions (1000 rpm) at 37ºC. Traces were recorded until stabilization of platelet aggregation was reached.

2.5. Granule secretion

CD62P (P-selectin) translocation to the platelet surface from the α granules was measured by flow cytometry as previously described [27]. Briefly, platelets were stimulated with thrombin in the absence or presence of δ(V1-1)TAT for 5 minutes at 37ºC, fixed with 1% paraformaldehyde, washed and stained with saturating concentrations of anti-CD62P PE-conjugated antibody or its isotype-matched control IgG for 30 minutes. Samples were analyzed (20,000 events) on an Altra flow cytometer (Beckman Coulter, Mississauga, ON) and platelets were gated by their characteristic forward and side scatter properties. ATP release was measured by a Lumi-Aggregometer according to the manufacturer's instructions (Chrono-log Corp., Havertwon, PA). Briefly, 12.5 μL luciferin-luciferase (Chrono-Lume) reagent was added to a 487.5 μL platelet suspension 30 seconds before addition of thrombin. Dense granule release was measured by mepacrine uptake into platelets [28]. Platelets in HBSS-Hank's sodium citrate buffer were incubated with 5 μM mepacrine (Quinacrine dihydrochloride, Sigma-Aldrich) for 30 minutes at 37°C. Platelets were then removed from excess mepacrine by centrifugation and resuspended in final HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Secretion of dense body constituents was evaluated by flow cytometry as the fluorescence remaining in platelets upon treatment with thrombin in the presence or absence of δ(V1-1)TAT and anti-GPIbα antibody; in comparison to resting platelets.

2.6. Statistical analysis

The results are presented as mean ± SEM of at least 3 independent experiments. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnetts-*t*-test for comparison against a single group. Data with $P \leq 0.05$ were considered statistically significant.

3. Results and Discussions

3.1. Inhibition of PKCδ membrane translocation potentiates human platelet aggregation induced by low dose of α-thrombin through GPIbα

We first sought to investigate the role of PKCδ in platelet aggregation in response to thrombin. As thrombin signaling in human platelets is mediated by the high-, medium- and low-affinity receptors, GPIbα, PAR-1 and PAR-4, respectively, we first used α-thrombin at low (0.01 U/ml) and high (0.1 U/ml) doses to evaluate thrombin signaling in platelets in the presence of a PKCδ inhibitor. As shown in Figure 1A, the low but not the high dose of α-thrombin potentiates platelet aggregation in the presence of the specific PKCδ membrane translocation inhibitor δ(V1-

1)TAT [21-29]. However, this potentiation of platelet aggregation was absent when platelets were treated with the TRAP-1 and TRAP-4 as specific agonists of PAR-1 and PAR-4, respectively, as well as by both low and high doses of γ-thrombin, which lacks GPIbα interaction (Figure 1A). In order to verify the specific role of PKCδ in this potentiation of platelet aggregation, we used a scrambled form of δ(V1-1)TAT, which showed no effect on platelet aggregation in response to a low dose of α-thrombin (Figure 1B). To further investigate the role of the GPIbα/thrombin interaction in this potentiation of platelet aggregation, we used ristocetin as a specific agonist of GPIbα. Our results show that activation of GPIbα by ristocetin, which has a different binding site on GPIbα than thrombin [20], had no effect on platelet aggregation in the presence of δ(V1-1)TAT (Figure 1C). On the other hand, blockade of the thrombin binding site on GPIbα by the specific anti-human GPIbα blocking antibody (SZ2) abrogated the potentiation of platelet aggregation induced by a low dose of α-thrombin in the presence of δ(V1-1)TAT (Figure 1D). These results indicate that in human platelets, inhibition of PKCδ membrane translocation potentiates platelet aggregation in response to a low dose of α-thrombin in a GPIbα dependent manner.

3.2. Inhibition of PKCδ membrane translocation potentiates human platelet degranulation induced by low dose of α-thrombin through GPIbα

Platelets harbor in their granules a plethora of compounds know to recruit and activate circulating quiescent platelets [30]. In an attempt to understand the mechanisms by which the absence of PKCδ activity at the membrane in platelets potentiates aggregation in response to a low dose of thrombin via GPIbα, we evaluated platelet α and dense granule release [31]. Our results indicate that a low dose of α-thrombin potentiates α-granule release as measured by CD62P expression (Figure 2A), as well as dense granule secretion as assessed for ATP release and loss of mepacrine fluorescence (Figures 2B and 2C), of human platelets pre-treated with δ(V1-1)TAT. Interestingly, blockade of the thrombin binding site on GPIbα with the site specific anti-human GPIbα antibody abrogates this potentiation of platelet degranulation, suggesting that signaling through GPIbα in the absence of PKCδ activity at the membrane probably leads to enhanced platelet function through the secretion of granule contents.

In platelets, the PKC family is an important signaling mediator required for different platelet functional responses, such as aggregation, secretion and thromboxane generation [12-22-25-32-33]. Specifically, the PKCδ isoform has been shown to negatively and positively regulate platelet function in response to GPVI and PAR stimulation, respectively. Indeed, PKCδ negatively regulates platelet aggregation and filopodia formation in response to collagen by interacting with the actin regulatory protein, vasodilator-stimulated phosphoprotein [34-35]. In contrast, Chari et al. [13] showed a partial reduction in granule secretion in platelets derived from PKCδ$^{-/-}$ mice and platelets treated with δ(V1-1)-TAT in response to PAR-4 stimulation. This reduction in secretion was only seen at low doses of the PAR-4 agonist, and it was lost at higher doses. These studies suggest that PKCδ regulates platelet function in an agonist nature- and concentration-dependent manner. The present work was undertaken to investigate the regulatory mechanism of this enzyme in response to different concentrations of thrombin.

We demonstrate a selective role for PKCδ in GPIbα signaling in human and mouse platelets in response to a low concentration of thrombin. It is well established that GPIbα is the high-affinity binding site for α-thrombin on platelets [36], but only recently the full implication of this interaction has emerged. Thrombin can activate platelets by either proteolytic or nonproteolytic mechanisms, and intact GPIbα facilitates the platelet response to low, but not high, doses of thrombin. Thrombin's interaction with GPIbα has two potential roles. First, thrombin is involved in regulating coagulation by localizing thrombin and factor XI on GPIbα. Second, thrombin's interaction with GPIbα can activate platelets in two ways: either by acting as a cofactor for the thrombin-dependent activation of PAR-1 or by signaling directly via GPIbα in the absence of GPV [37]. Our results show a potentiation of human platelet aggregation in response to a low but not a high dose of α-thrombin in the presence of the PKCδ inhibitor δ(V1-1)TAT. On the other hand, specific stimulation of PAR-1 and PAR-4 or stimulation with γ thrombin, which enable to bind GPIbα, had no effect on platelet aggregation in the presence of δ(V1-1)TAT. In addition, the response to ristocetin was not affected by PKCδ inhibition. All together, these data point to a specific effect of thrombin via GPIbα, since this was reversed by a site-specific antibody against GPIbα indicating that GPIbα negatively regulates platelet aggregation in response to a low dose of α-thrombin via PKCδ.

As platelets harbor a plethora of chemicals in their granules that potentiate platelet activation [38], we assessed the implication of the GPIbα/PKCδ axis in granule release. Our results show that GPIbα negatively regulate platelet granule release, as inhibition of PKCδ activity increases α and dense granule release in response to a low dose of α-thrombin, which is reversed by GPIbα blockade. The implication of PKCδ in GPIbα signaling in an *in vivo* setting in response to low levels of locally generated α-thrombin remains unknown. Here we provide novel evidence demonstrating a direct correlation between PKCδ, GPIbα and low levels of α-thrombin.

Figure 1

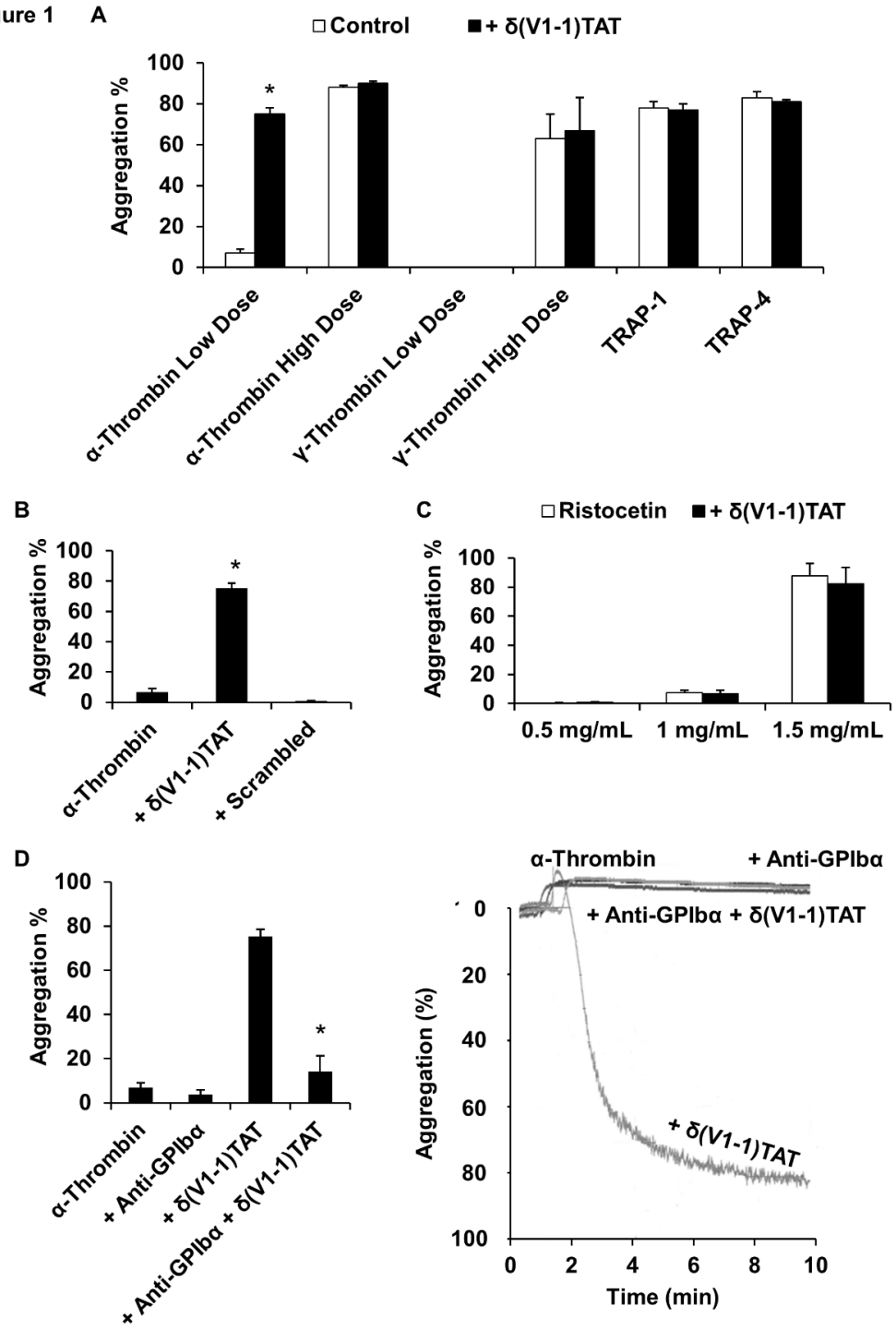

Figure 1. Inhibition of PKCδ membrane translocation potentiates human platelet aggregation induced by low dose of α-thrombin through GPIbα. A) δ(V1-1)TAT potentiates platelet aggregation in response to low but not high dose of α-thrombin, γ-thrombin, TRAP-1 and TRAP-4. Platelets were pre-incubated with δ(V1-1)TAT (1 µM) for 5 minutes at 37°C, then stimulated with a low (0.0125 U/mL) and high (0.1 U/mL) dose of α-thrombin, a low (1 µM) and high (10 µM) dose of γ-thrombin, TRAP-1 (5 µM) and TRAP-4 (100 µM). Histogram represents the mean of data ± SEM of aggregation traces (n > 4, * $P < 0.05$ vs. α-thrombin lose dose). B) The scrambled peptide of δ(V1-1)TAT has no effect on human platelet aggregation. Platelets were pre-incubated with δ(V1-1)TAT (1 µM) or its scrambled peptide (1 µM) for 5 minutes at 37°C. Aggregation was then initiated by a low dose of α-thrombin (0.0125 U/mL). Histogram represents the mean of data ± SEM of aggregation traces (n = 3, * $P < 0.05$ vs. all). C) The vWf binding site on GPIbα does not affect platelet aggregation in the presence of δ(V1-1)TAT. PRP was stimulated with the indicated concentration of ristocetin in the presence or absence of δ(V1-1)TAT (1 µM). Histogram represents the mean of data ± SEM of aggregation traces (n = 3). D) Pre-treatment with the human anti-GPIbα antibody prevents δ(V1-1)TAT-induced potentiation of platelet aggregation. Platelets were pre-treatment with δ(V1-1)TAT (1 µM) and/or the human anti-GPIbα antibody (20 µg/mL) for 5 minutes at 37°C. Aggregation was then initiated by a low dose of α-thrombin (0.0125 U/mL). Histogram represents the mean of data ± SEM of aggregation traces on the right (n > 4, * $P < 0.05$ vs. + δ(V1-1)TAT).

Figure 2

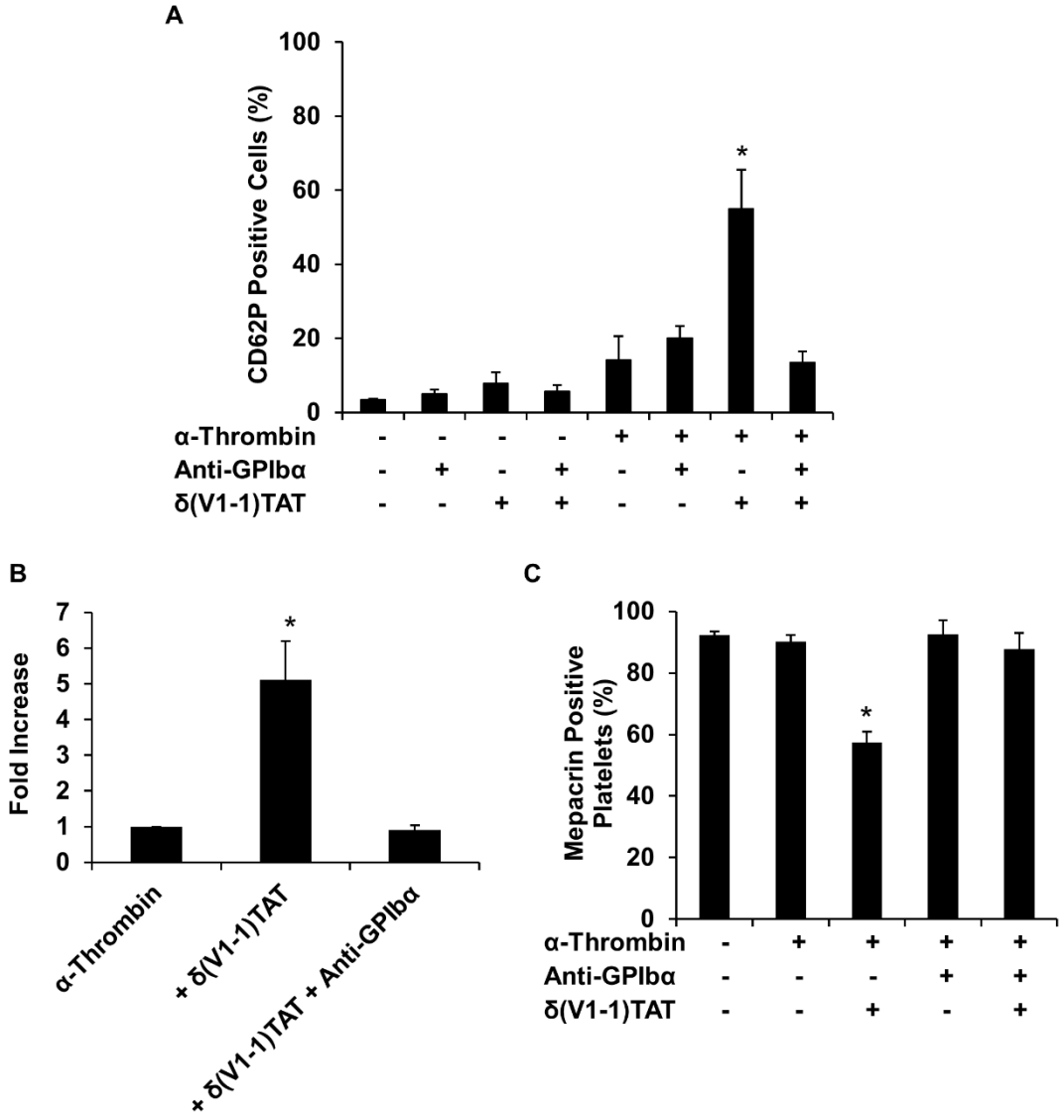

Figure 2. Inhibition of PKCδ membrane translocation potentiates human platelet degranulation induced by low dose of α-thrombin through GPIbα. A) δ(V1-1)TAT-induced increase in α-granule secretion in response to low dose of α-thrombin (0.0125 U/mL) is reversed by GPIbα blockade. Platelets were pre-treatment with δ(V1-1)TAT (1 µM) and/or the human anti-GPIbα antibody (20 µg/mL) for 5 minutes at 37°C. Degranulation was then initiated by a low dose of α-thrombin (0.0125 U/mL). Histogram represents the mean of data ± SEM of the percentage of CD62P positive platelets (n = 3, * $P < 0.05$ vs. all). B and C) δ(V1-1)TAT-induced increase in dense granule secretion in response to low dose of α-thrombin (0.0125 U/mL) is reversed by GPIbα blockade. Platelets pre-treated with δ(V1-1)TAT were incubated with the human anti-GPIbα antibody for 5 minutes at 37°C. Degranulation was then initiated by a low dose of α-thrombin (0.0125 U/mL). B) Histogram represents the mean of data ± SEM of ATP release represented as fold increase over α-thrombin alone (n = 3, * $P < 0.05$ vs. all). C) Histogram represents the mean of data ± SEM of remaining mepacrine labeling.

4. Conclusions

In conclusion, the present study provides further evidence for the role of PKCδ downstream of GPIbα. We show that this isoform plays a critical role in platelet function, where it negatively regulates platelet aggregation and activation in response to a low concentration of thrombin. Thus, PKCδ may eventually constitute a target in managing thrombotic events.

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Disclosure

The authors declare that there is no conflict of interest.

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