PAR1, but not PAR4, activates human platelets through a $G_{i/o}$/PI3K signaling axis

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Abbreviations:

1. Protease Activated Receptor-1 (PAR1)
2. PAR1-activating peptide (PAR1-AP)
3. Protease Activated Receptor-3 (PAR3)
4. Protease Activated Receptor-4 (PAR4)
5. PAR4-activating peptide (PAR4-AP)
6. Phospholipase C beta (PLCβ)
7. Phosphoinositide-3 Kinase (PI3K)
8. Protein Kinase C (PKC)
9. Integrin αIIbβ3 (GPIIbIIIa)
10. P2Y Purinocepter 12 (P2Y$_{12}$)
11. Bisindolylmaleimide-1 (Bis-1)
12. Fluorescence Activated Cell Sorting (FACS)
13. Fluorescein Isothiocyanate (FITC)
14. Phospholipase D (PLD)
15. Diacylglycerol (DAG)

Abstract

Thrombin-mediated activation of platelets is critical for hemostasis, but the signaling pathways responsible for this process are not completely understood. Additionally, signaling within this cascade can also lead to thrombosis. In this study we have defined a new signaling pathway for the thrombin receptor PAR1 in human platelets. We show that PAR1 couples to $G_{i/o}$ in human platelets and activates phosphoinositide-3 kinase (PI3K). PI3K activation regulates platelet integrin αIIbβ3 (GPIIbIIIa) activation, platelet aggregation, and potentiates the PAR1-mediated increase in intraplatelet calcium concentration. PI3K inhibitors eliminated these effects downstream of PAR1, but they had no effect on PAR4 signaling. This study has identified an important role for the direct activation of $G_{i/o}$ by PAR1 in human platelets. Given the efficacy of clopidogrel, which blocks the $G_{i/o}$ coupled $P2Y_{12}$ receptor, as an antiplatelet/antithrombotic drug, our data suggests that specifically blocking only PAR1-mediated $G_{i/o}$ signaling could also be an effective therapeutic approach with the possibility of less unwanted bleeding.

Introduction

Thrombin-mediated activation of platelets and the vascular endothelium is critical for the normal regulation of hemostasis (Brass, 2003; Brass, 2001; Coughlin, 2005; Ossovskaya and Bunnett, 2004). Thrombin signaling occurs largely through the activation of the four different isoforms of protease-activated receptors (PARs) characterized so far (Coughlin, 2000). PAR1 and PAR4 are expressed by human platelets (Coughlin, 2000) while mice express PAR3 and PAR4. PAR3 has been shown to act as a "cofactor" for PAR4-mediated thrombin signaling in mice (Nakanishi-Matsui et al., 2000), but this paradigm does not exist in human platelets, as PAR1 and PAR4 are the "active" thrombin receptors on human platelets and might only interact through oligomerization (Leger et al., 2006). These key differences in receptor expression and function underlie the importance of the study of platelet signaling using human platelets and not a mouse model.

The signaling pathways downstream of PAR1 and PAR4 in human platelets are not completely understood. These receptors were thought to couple to the same set of heterotrimeric G-proteins and signal in a redundant manner, however it has recently been shown that PAR1 and PAR4 signal differentially in human platelets and other tissues (Holinstat et al., 2006b; Kawabata et al., 2000; Ma et al., 2005) which likely results from differential G-protein coupling. Both PAR1 and PAR4 couple to G_q and $G_{12/13}$ family members in human platelets (Brass, 2003; Woulfe, 2005). The G_q pathway involves phospholipase C-β activation and concomitant intraplatelet calcium mobilization and PKC activation (Woulfe, 2005). The $G_{12/13}$ pathway involves Rho/Rho kinase activation and actin remodeling (Woulfe, 2005), causing platelet shape change. Both G_q and $G_{12/13}$-dependent signaling pathways play a critical role in GPIIbIIIa activation and clot formation (Dorsam et al., 2002; Woulfe, 2005). GPIIbIIIa is the most

important RGD-domain containing integrin in platelets, and in its active confirmation binds fibrinogen and other adhesion molecules to cause platelet aggregation and hemostasis (Lefkovits et al., 1995). Studies involving platelets from human subjects with G-protein alpha subunit deficiencies (Gabbeta et al., 1997) and studies that used cell permeable dominant negative PAR peptides (Covic et al., 2002a; Covic et al., 2002b) confirmed the importance of these pathways.

It is possible that the widely expressed $G_{i/o}$ family of heterotrimeric G-proteins couple to PAR1 or PAR4 in human platelets and play a role in platelet activation (Odagaki and Koyama, 2002; Odagaki et al., 1993) as PAR1 couples to G_o proteins in endothelial cells (Gilchrist et al., 2001; Vanhauwe et al., 2002), and human platelets express $G_{\alpha i}$ (Wettschureck and Offermanns, 2005) and $G_{\alpha o}$ (unpublished data). In contrast, some studies suggest that neither PAR1 nor PAR4 couple directly to $G_{i/o}$ in human platelets and that any $G_{i/o}$ signaling results from purinergic receptor signaling by secreted adenine nucleotides (Kim et al., 2002). However, these studies rely only on adenylyl cyclase inhibition by $G_{\alpha i/o}$ and do not consider the role of the $G_{\beta\gamma}$ subunits (Kim et al., 2002). Pertussis toxin is typically used to study $G_{i/o}$ mediated signaling, but human platelets lack the pertussis toxin receptor making the study of $G_{i/o}$ signaling in platelets difficult (Lapetina et al., 1986). However, the role of the $G_{\beta\gamma}$ subunits is critical as the $G_{i/o}$ family of G-proteins are thought to be the main significant source of $G_{\beta\gamma}$ mediated signaling (Wettschureck and Offermanns, 2005) which regulates PLC-β and phosphoinositide-3 kinase (PI3K) activity and the conductance of various ion channels (Wettschureck and Offermanns, 2005).

Most therapeutic agents that target thrombin are direct inhibitors like hirudin and bivalirudin (Di Nisio et al., 2005), which have the unwanted side effect of hemorrhage. Ideal antithrombotic therapies should block pathological clotting, while leaving physiological clotting pathways intact. The identification of differences in the G-protein signaling pathways

downstream of PAR1 and PAR4 in human platelets could facilitate the development of a strategy to only block pathological clotting. Therefore, in this study we used a candidate approach to identify signaling pathways which are different for PAR1 vs. PAR4 mediated GPIIbIIIa activation in human platelets. We demonstrate that while G_q and $G_{12/13}$ signaling pathways did not vary between these receptors, PAR1 directly couples to $G_{i/o}$ family members to activate GPIIbIIIa and potentiate the increase intraplatelet calcium concentration in a PI3K-dependent manner while PAR4 does not. Thus, our data demonstrate a novel role for $G_{i/o}$ proteins in thrombin-mediated human platelet signaling.

Materials and Methods

Materials: PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai). Human α thrombin was purchased from Enzyme Research Labs (South Bend, IN). Wortmannin and epinephrine were purchased from Sigma (St. Louis, MO). Bisindolymaleimide-1 (Bis-1), U73122, and Y-27632 were purchased from Calbiochem (San Diego, CA). Fura-2-AM was purchased from Molecular Probes (Eugene, OR). FITC-PAC1 was purchased from BD-Pharmingen (San Jose, CA).

GPIIbIIIa activation: Human platelet GPIIbIIIa activation was assessed using fluorescence activated cell sorting (FACS) (Shattil et al., 1987). Venous blood was collected into 0.5 ml of 3.8% sodium citrate, and within 5 minutes of collection 5 μl aliquots of this fresh whole blood was added to 12 x 75-mm polystyrene tubes containing a saturating concentration (7 μg/ml) of the FITC-PAC1 antibody. The platelets in these samples were pre-treated for 15 minutes with Bis-1 (5 μM), U-73122 (2 μM), Y-27632 (20 μM), Wortmannin (100 nM), or vehicle (water or DMSO) followed by activation with PAR1-AP or PAR4-AP and incubated for 15 minutes at room temperature without stirring. The samples were then diluted with 500 μl of calcium-free

Tyrode's buffer (137 mM NaCl, 12 mM NaHCO$_3$, 5.5 mM glucose, 2 mM KCl, 1 mM MgCl$_2$, 0.3 mM Na$_2$HPO$_4$, pH=7.4) and submitted for immediate FACS analysis. The platelets were analyzed using the single fluorochrome FACS method, and were distinguished from red and white blood cells on the basis of their side- and forward-light scatter profile. A gate was set around the platelets, and 8,000 cells were analyzed for FITC fluorescence to quantify the amount of platelet-bound PAC-1. Each condition was performed a minimum of 3 times, utilizing blood from a different donor.

Platelet Aggregation: Platelet aggregations were measured using washed platelets (Kahn et al., 1998). Briefly, blood was centrifuged in a Forma 400 mL GP centrifuge at 170 x G for 15 minutes at room temperature. The platelet rich plasma (PRP) was placed into 15 mL conical tubes containing a 10% acid citrate dextrose solution (39 mM citric acid, 75 mM sodium citrate and 135 mM glucose; pH 7.4) and centrifuged at 800 x G for 10 minutes at room temperature. The pelleted platelets were resuspended in Tyrode's buffer and adjusted to a concentration of 3 x 10^8 platelets/ml using a Coulter counter. Some platelets were treated with 100 nM Wortmannin for 30 minutes or 100 μM LY294002 for 15 minutes in the dark. Following stimulation with either 10 nM thrombin, 20 μM PAR1-AP or 200 μM PAR4-AP, the change in light transmission was monitored with an aggregometer (Chrono-log, Inc.). Each result is representative of at least 3 independent experiments.

Measurement of Intraplatelet Calcium Concentration: Intraplatelet calcium concentration was measured in washed platelets (Santoro et al., 1994), at a concentration of 3x10^8 platelets per milliliter, using the cell permeable fluorescent calcium dye Fura-2-AM. Washed platelets were incubated with Fura-2-AM (5 μg/ml) for 30 minutes in a 37ºC cell culture incubator. Any Fura-2-AM remaining outside of the platelets was washed away as described previously (Santoro et

al., 1994). Calcium (1 mM final concentration) was then added to the Fura-2 treated platelets 15

minutes prior to stimulation, except for conditions where extracellular calcium was intentionally

left out. Fura-2 loaded platelets receiving PI3K inhibition were preincubated with Wortmannin

or LY294009 for 15 minutes and then stimulated accordingly. A Fluorometer (Cary

Instruments/Varian; Palo Alto, CA) was used to monitor the absorbance changes at 340 nm and

380 nm before and after stimulation with the agonists listed in the Materials section. The ratio of

the absorbance at 340 nm and 380 nm was then calculated for each time point and graphed

against time.

Statistical Analysis: The experiments were performed at least three times and comparisons were

made between experimental groups with the paired t-test (Figures 1-3) and one way ANOVA

(Figure 4) using Prism software. All statistically significant differences between groups had

$p<0.05$. Given the heterogeneity and well established experimental inconsistency of human

platelets the p-values in the data are similar to those previously published for other studies using

human platelets.

Results

PAR1, but not PAR4, causes platelet GPIIbIIIa activation and clot formation in a PI3K-

dependent manner: A candidate approach was taken to determine whether there are differences

in PAR1 and PAR4-mediated GPIIbIIIa activation. We have recently shown that phospholipase

D (PLD) is important for PAR1, but not PAR4, -mediated GPIIbIIIa activation (Holinstat et al.,

2006a), therefore we focused on identifying other important G-protein pathways that might be

downstream of these receptors in human platelets. As PKC has previously been shown to be

critical for thrombin mediated platelet activation (Woulfe, 2005), we assessed the role of the G_q-

PKC pathway downstream of PAR1 and PAR4 in the activation of human platelet GPIIbIIIa. To

do this, we determined FITC-PAC1 antibody binding to platelets following maximal PAR1 or

PAR4 activation, in the presence or absence of the PKC inhibitors Bis-1 and chelerythrine

utilizing FACS. The FITC-PAC1 antibody only recognizes the activated conformation of

GPIIbIIIa. Representative experiments for PAR1-AP (Fig. 1A-left panel) and PAR4-AP (Fig.

1A-right panel) show that Bis-1 mediated PKC inhibition results in an approximately 40%

decrease in GPIIbIIIa activation downstream of both PAR1 and PAR4, indicated by the leftward

shift in the mean fluorescence intensity in the presence of Bis-1. These results are quantified in

the corresponding bar graph (Fig. 1A) and were confirmed using chelerythrine, another well

characterized PKC inhibitor (data not shown). Thus, PKC plays a similar, important role in

PAR1- and PAR4-mediated GPIIbIIIa activation.

PLC-β activity has also been shown to be important for thrombin mediated platelet

activation (Lian et al., 2005; Woulfe, 2005). When this pathway was inhibited with the specific

inhibitor PLC-β inhibitor, U73122, there was no significant decrease in PAC1 binding in either

PAR1-AP (Fig. 1B-left panel) or PAR4-AP (Fig. 1B-right panel) stimulated platelets when

compared to vehicle (ethanol). These data were also quantified in the corresponding bar graph

(Fig. 1B) and they suggest that PLC-β is not required downstream of either PAR1 or PAR4 for

GPIIbIIIa activation.

Rho kinase, which is downstream of $G_{12/13}$, also plays a role in thrombin mediated

platelet activation (Moers et al., 2003; Woulfe, 2005). The role of this pathway was determined

using the Rho-kinase inhibitor, Y-27632, and this compound did not significantly effect

GPIIbIIIa activation downstream of either PAR1 or PAR4 (Fig. 1C). These data were also

quantified in the corresponding bar graph (Fig. 1C). Thus, Rho kinase is not required

downstream of either PAR1 or PAR4 for GPIIbIIIa activation.

PI3K, which is activated by $G_{\beta\gamma}$ liberated from $G_{i/o}$ heterotrimeric G protein, plays a

significant role in thrombin-mediated GPIIbIIIa activation (Jackson et al., 2004; Kovacsovics et

al., 1995), however the distinct roles of PAR1 and PAR4 in this response have not been

characterized. To investigate this further, platelets were treated with the PI3K inhibitor

wortmannin or vehicle (DMSO) prior to assessing PAR1- vs. PAR4-mediated PAC-1 binding.

Wortmannin inhibited PAR1-AP (Fig. 1D-left panel) mediated GPIIbIIIa activation by ~50% but

did not have a significant effect on PAR4-AP (Fig. 1D-right panel) mediated GPIIbIIIa

activation. These data were quantified in the corresponding bar graph (Fig. 1D) and similar

results were obtained with another PI3K inhibitor, LY-294002 (data not shown). PAC1 binding

to activated GPIIbIIIa on activated platelets was specific, as the synthetic RGDS peptide blocked

its binding to activated platelets (Fig. 1D-left and right panel). These data suggest that PI3K is

required downstream of PAR1, but not PAR4, for human platelet GPIIbIIIa activation.

To confirm that PI3K is critical for PAR1, but not PAR4, mediated platelet activation we

assessed the effect of PI3K inhibitors on human platelet aggregation. Figure 2 shows

representative aggregations for thrombin (Fig. 2A), PAR1-AP (Fig. 2B) and PAR4-AP (Fig. 2C)

with and without pretreatment with the PI3K inhibitors, wortmannin and LY294002. These

inhibitors did not significantly effect thrombin or PAR4-AP mediated aggregation, but both

maximal (Fig. 2D) and final (Fig. 2E) PAR1-AP mediated aggregation was significantly

inhibited by these inhibitors. These data suggest that PAR1, but not PAR4, requires PI3K

activation for human platelet aggregation.

PAR1, but not PAR4, requires extracellular calcium for full change in intraplatelet calcium

concentration: We next assessed the role of PI3K signaling downstream of PAR1, but not

PAR4, in other important platelet signaling pathways including changes in the intracellular

calcium concentration. We initially determined whether PAR1 and PAR4 differ in their

requirement of extracellular calcium for a full change in intraplatelet calcium concentration. The

thrombin and PAR4-AP mediated increase in intraplatelet calcium concentration in the presence

of normal (physiological) extracellular calcium concentration (Fig. 3A) and low (non-

physiological) extracellular calcium concentration (Fig. 3B) did not differ significantly, while the

PAR1-AP mediated increase in intraplatelet calcium concentration was significantly attenuated

in the presence of a low extracellular calcium concentration (Fig. 3A-B). Figure 3C shows a

comparison of the maximum absorbance ratios under high and low extracellular calcium

conditions and the PAR1-AP mediated calcium transient was attenuated significantly in the

presence of low extracellular calcium while thrombin and PAR4-AP were not significantly

affected. These data suggest that the PAR1-mediated PI3K pathway may play an important role

in the potentiation of the increase in intraplatelet calcium concentration through the conditional

entry of extracellular calcium, possibly through TRP channel activation (Tseng et al., 2004;

Zhuang et al., 2004).

Recently published data suggests that thrombin causes extracellular calcium entry in

human platelets in a PI3K-dependent manner (Lian et al., 2005), however whether this occurs

downstream of PAR1 or PAR4 has not been established. To investigate this we treated Fura-2

loaded platelets with wortmannin (Fig. 3D) or vehicle (DMSO, Fig. 3E) and the maximum

absorbance ratios under wortmannin and vehicle conditions were compared (Fig. 3F).

Interestingly, the thrombin and PAR1-AP mediated calcium transients were significantly

attenuated in the presence of wortmannin when compared to vehicle, while PAR4-AP was not

significantly affected. This result was confirmed with LY-294002. Additionally, wortmannin

had no effect on the thrombin, PAR1-AP, or PAR4-AP-mediated increases in intraplatelet

calcium concentration in the presence of a low extracellular calcium concentration (data not shown). These data suggest that PAR1, but not PAR4, directly activates PI3K to potentiate the increase in intraplatelet calcium concentration via calcium from the extracellular milieu.

PAR1, but not PAR4, couples to $G_{i/o}$ in human platelets to modulate calcium flux: Our data suggests that PAR1, but not PAR4, directly couples to a $G_{i/o}$/PI3K signaling pathway and we confirmed this using a different G_i family coupled receptor. Since PAR4 appears to lack the $G_{i/o}$/PI3K pathway, we supplemented this pathway through epinephrine mediated activation of the G_i family coupled GPCR, the α2A adrenergic receptor (Woulfe, 2005). As shown in Figure 4A epinephrine alone cannot cause an increase in intraplatelet calcium concentration, but when used simultaneously with PAR4-AP it can potentiate the increase in intraplatelet calcium concentration. Wortmannin blocks this potentiation of the increase in intraplatelet calcium concentration (Fig. 4A). Figure 4B shows a comparison of thrombin, PAR1-AP and PAR4-AP in combination with epinephrine and the effect of wortmannin. Only the PAR4-AP mediated increase in intraplatelet calcium concentration is significantly potentiated by costimulation with epinephrine. Taken together, these data suggest that thrombin and PAR1-AP already have activated this $G_{i/o}$-PI3K pathway via PAR1 coupling to $G_{i/o}$, while PAR4 does not.

Discussion

Thrombin mediated activation of PAR1 and PAR4 on human platelets is critical for the maintenance of the hemostatic response, and is therefore essential to maintain the integrity of the cardiovascular system. Since thrombin is the most potent stimulator of platelets these receptors have become highly attractive targets for the development of novel therapeutic agents for the treatment of various cardiovascular diseases, given the high clinical relevance of their signaling pathways. In this study, we demonstrate that PAR1, but not PAR4, couples to $G_{\alpha i/o}$ to regulate

human platelet integrin GPIIbIIIa activation, *ex vivo* platelet aggregation, and increases in

intraplatelet calcium concentration. We also demonstrate that PI3K is a critical signaling

molecule downstream of the human platelet PAR1/$G_{i/o}$ pathway that mediates these effects.

Taken together, this study defines $G_{\alpha i/o}$, and its concomitant activation of PI3K, as a novel

signaling pathway directly downstream of PAR1 in human platelets.

Considering that GPIIbIIIa activation is the most critical step in platelet aggregation and

thrombus formation (Offermanns, 2000) we used a candidate approach to show that PAR1, but

not PAR4, utilizes PI3K to activate human platelet GPIIbIIIa These data suggest that PAR1, but

not PAR4, directly couples to $G_{i/o}$ in human platelets. While these findings do not agree with the

observations made by Kim et al (Kim et al., 2002), who utilized cAMP signaling as the readout

for $G_{i/o}$ coupling, the data is in agreement with previously published data from our lab on PAR1

mediated signaling in endothelial cells (Vanhauwe et al., 2002). We also demonstrated that the

G_q-PKC pathway is required for full activation of GPIIbIIIa by both PAR1 and PAR4 using Bis-

1 and chelerythrine, while the G_q-PLC and $G_{12/13}$-Rho-Kinase pathways are not required for

GPIIbIIIa activation by either PAR1 or PAR4 using U73122 and Y-27632, respectively. These

findings are in agreement with a previous study (Woulfe, 2005). Additionally, we have recently

shown that phopholipase D (PLD) is the source of diacylglycerol (DAG) which causes PKC

activation downstream of PAR1 in human platelets (Holinstat et al., 2006a). During PLCβ

inhibition this pathway could be a compensatory mechanism for the maintenance of PAR1-

mediated PKC activation. The mechanism by which PAR4 can activate PKC during PLCβ

inhibition is poorly understood but is an active area of research in our lab.

Our study demonstrated that PAR1-AP but not thrombin or PAR4-AP mediated platelet

aggregation was significantly inhibited by the PI3K inhibitors wortmannin and LY294002. These

data provide strong additional evidence that PAR1, but not PAR4, couples to $G_{i/o}$ to activate

PI3K in human platelets and would explain both our data and previously published results for

why PAR1-AP (Kovacsovics et al., 1995), but not PAR4-AP require PI3K for human platelet

aggregation. Although PI3K activation has been previously described to play a role in platelet

aggregation, this was in the context of pathways activated following integrin ligation by ligand

(Banfic et al., 1998). This contrasts with our study, where we propose that PI3K is critical for

PAR1-mediated integrin activation.

We demonstrated that PAR1, but not PAR4, requires extracellular calcium for a full

increase in intraplatelet calcium concentration. This suggested that PAR1 might utilize its

unique $G_{i/o}$/PI3K pathway to bring additional calcium into the platelet, while PAR4 lacks this

pathway and relies solely on the platelet dense tubular system for mobilizing calcium. We

confirmed this by showing that PI3K inhibition attenuates the PAR1-AP, but not the PAR4-AP,

mediated increase in intraplatelet calcium concentration. The mechanism by which PI3K causes

platelet calcium influx is believed to be through TRP channel activation/trafficking (Ramsey et

al., 2005; Viard et al., 2004; Zhuang et al., 2004) and/or reorganization of the cytoskeleton

(Rosado et al., 2004).

To further confirm that PAR1, but not PAR4, directly couples to $G_{i/o}$ and activates PI3K

we showed that epinephrine alone cannot elicit an increase in intraplatelet calcium concentration,

but in the presence of the G_q/$G_{12/13}$ coupled receptor agonist PAR4-AP, epinephrine potentiates

the increase in the intraplatelet calcium concentration considerably. Epinephrine activates the

alpha-2A adrenergic receptor, a $G_{i/o/z}$ family coupled receptor (Woulfe, 2005), to activate PI3K.

Additionally, epinephrine only minimally potentiated the thrombin- and PAR1AP-mediated

increases in intraplatelet calcium concentration. Interestingly, the potentiation of the increase in

intraplatelet calcium concentration by epinephrine in the presence of PAR4-AP was completely

blocked by wortmannin. These data suggest that thrombin and PAR1-AP have already activated

the PI3K/Calcium potentiation pathway through direct coupling to $G_{i/o}$, while PAR4 has not.

These data provide further evidence that PAR1, but not PAR4, directly couples to the $G_{i/o}$ family

of heterotrimeric G-proteins in human platelets. This has been a controversial topic for many

years (Brass, 2003; Woulfe, 2005).

PAR antagonists have been, and continue to be developed, for the treatment of

thrombosis and hemostasis. Targeting these receptors would be preferable to direct thrombin

inhibition, which has the side effect of unwanted bleeding due to the blockade of fibrin

formation. Therefore, delineation of this new pathway should be considered during the planning

and development of future therapeutic strategies targeting these receptors and their signaling.

Although we have focused on PAR1 and PAR4 signaling individually, PAR1/PAR4

oligomerization may also be important in hemostasis and this is an active area of interest in our

lab. Additionally, it is conceivable that blocking one or more of the G-protein pathways

downstream of PAR1 and/or PAR4, instead of blocking all receptor signaling, could yield a

safer, more efficacious antithrombotic agent for the prevention and treatment of cardiovascular

disease. Thus, our demonstration of a novel role for $G_{i/o}$ proteins in the signal transduction of

PAR1, but not PAR4, in human platelets where it activates PI3K which causes GPIIbIIIa

activation, platelet aggregation and the potentiation of the increase in the intraplatelet calcium

concentration is potentially important in the design of novel therapeutic agents that target

thrombin-dependent platelet activation.

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Footnotes:

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Legends for Figures

Figure 1: PAR1 activates GPIIbIIIa through PI3K
Whole blood samples were treated with Bis-1 (A), U73122 (B), Y-27632 (C), wortmannin (D), or vehicle (water, DMSO, or ethanol) as described under "Materials and Methods". A saturating concentration of FITC-PAC1 (5 μg/ml) was added prior to stimulation with maximal doses of PAR1-AP (left panels-20 μM) or PAR4-AP (right panels-200 μM). PAC1 binding was measured using flow cytometry and is expressed as mean fluorescence intensity. The small peptide RGDS (2 mM) was used to verify that FITC-PAC1 antibody binding was specific upon receptor stimulated integrin activation (D). The flow cytometric analyses are representative experiments of a minimum of an N of 3, which is quantified in a bar graph for each respective data set. (5 μM Bis-1, 2 μM U73122, 20 μM Y-27632, 100 nM wortmannin) (N=3)   *, $P<0.05$

Figure 2: PI3K differentially regulates PAR1 and PAR4-mediated platelet aggregation.

Washed platelets were treated with or without 100 nM Wortmannin for 30 minutes or 100 μM

LY294002 for 10 minutes followed by stimulation with 10 nM thrombin (A), 20 μM PAR1-AP

(B) or 200 μM PAR4-AP (C). D) Maximal aggregation was measured following agonist

stimulation for each condition. E) Final aggregation was measured at 6 minutes following

agonist stimulation. (N=3) ***, P<0.001

Figure 3: PAR1 causes extracellular calcium entry via a $G_{i/o}$/PI3K signaling pathway

A) Platelets loaded with Fura-2-AM were stimulated with either Tyrode's Buffer (negative

control), 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP in the presence of 1 mM

extracellular calcium and the absorbance ratio at 340nm and 380nm was monitored

fluorimetrically. (N=3) B) Platelets loaded with Fura-2-AM were stimulated with either

Tyrode's Buffer (negative control) 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP in

the presence of 0.2 mM extracellular calcium and the absorbance ratio at 340nm and 380nm was

monitored fluorimetrically. (N=3) C) The maximum absorbance ratios under high and low

extracellular calcium conditions were compared and the PAR1-AP mediated calcium transient

was attenuated significantly in the presence of low extracellular calcium. (N=3) *, P<0.05

Platelets loaded with Fura-2-AM were treated with either 100 nM wortmannin (D) or vehicle (E-

DMSO) for 15 minutes and then stimulated with either Tyrode's Buffer (negative control) 10 nM

thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP in the presence of 1 mM extracellular calcium

and the absorbance ratio at 340nm and 380nm was monitored fluorimetrically. (N=3)

F) The maximum absorbance ratios under wortmannin and vehicle conditions were compared

and the PAR1-AP mediated calcium transient was attenuated significantly in the presence of

wortmannin. (N=3) *, P<0.05

Figure 4: PAR1 couples to $G_{i/o}$ in human platelets to modulate calcium flux
A) Platelets loaded with Fura-2-AM were treated with 200 μM PAR4-AP, 200 nM epinephrine,

or both in the presence of 1 mM extracellular calcium and the absorbance ratio at 340nm and

380nm was monitored fluorimetrically. Additional samples of Fura-2 loaded platelets were

pretreated with 100 nM wortmannin for 15 minutes and stimulated with 200 μM PAR4-AP by

itself or in combination with 200 nM epinephrine. B) The experiments in Fig. 3A were also

completed for 10 nM thrombin and 20 μM PAR1-AP and the percent change in maximum
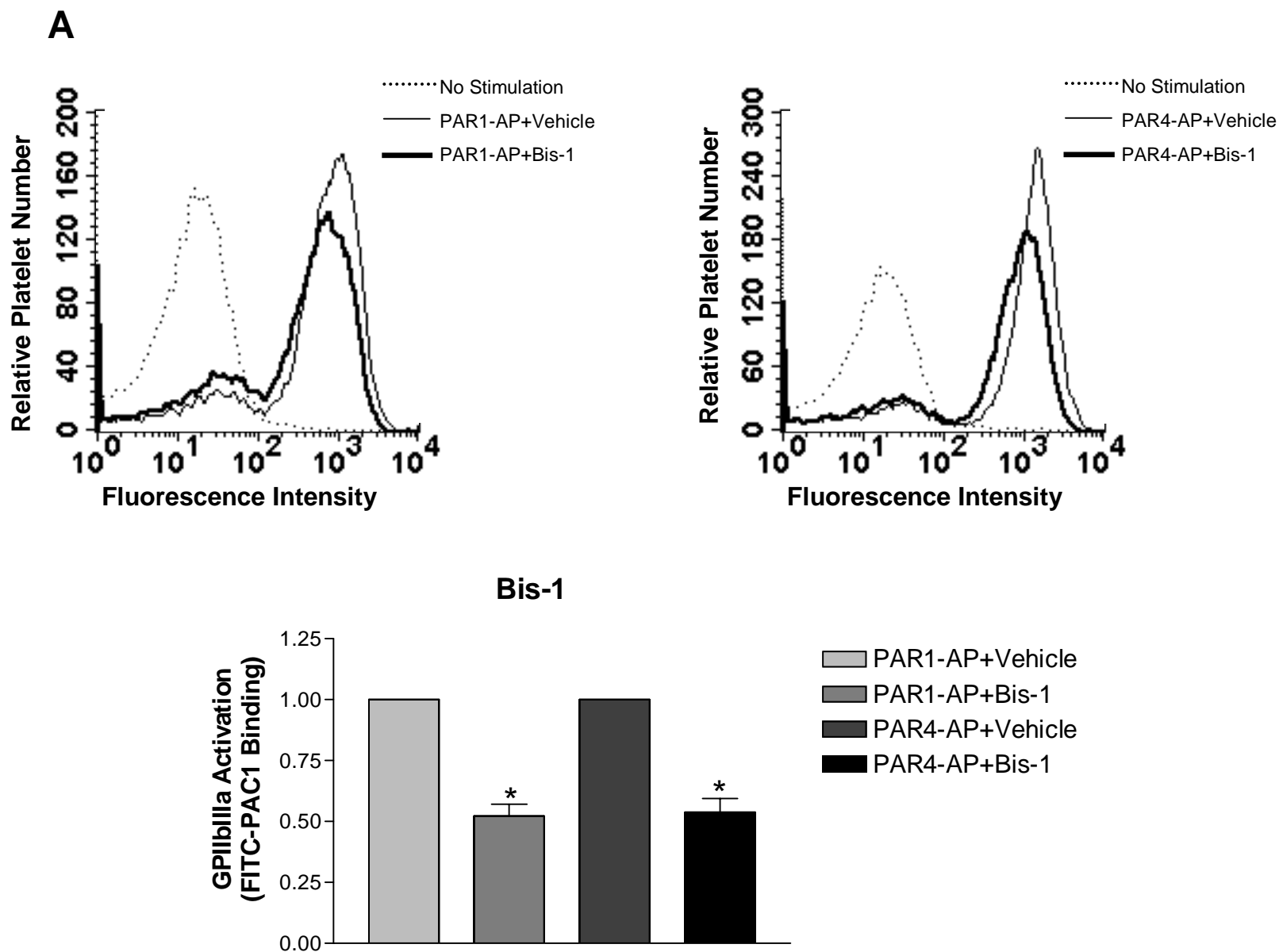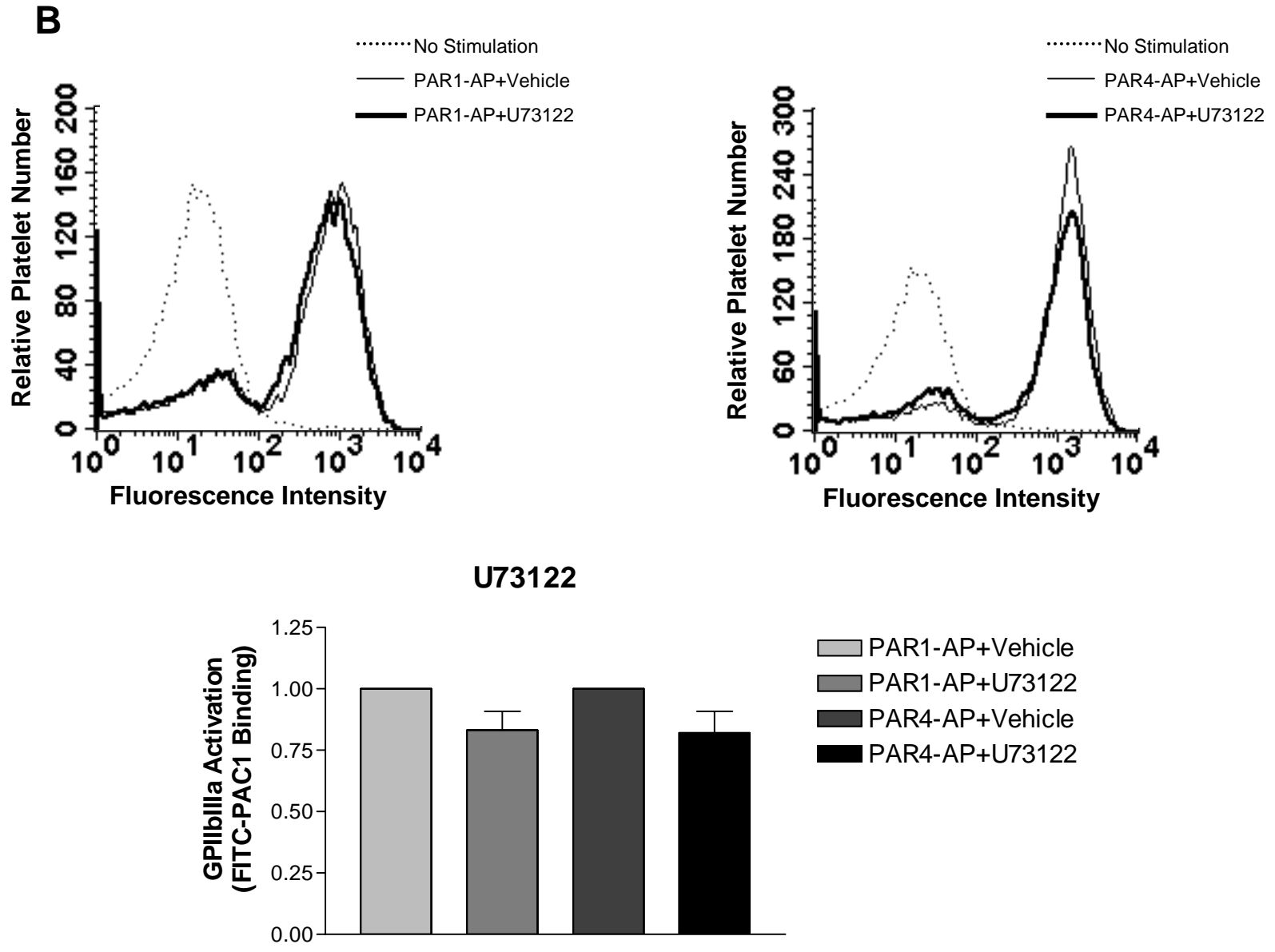
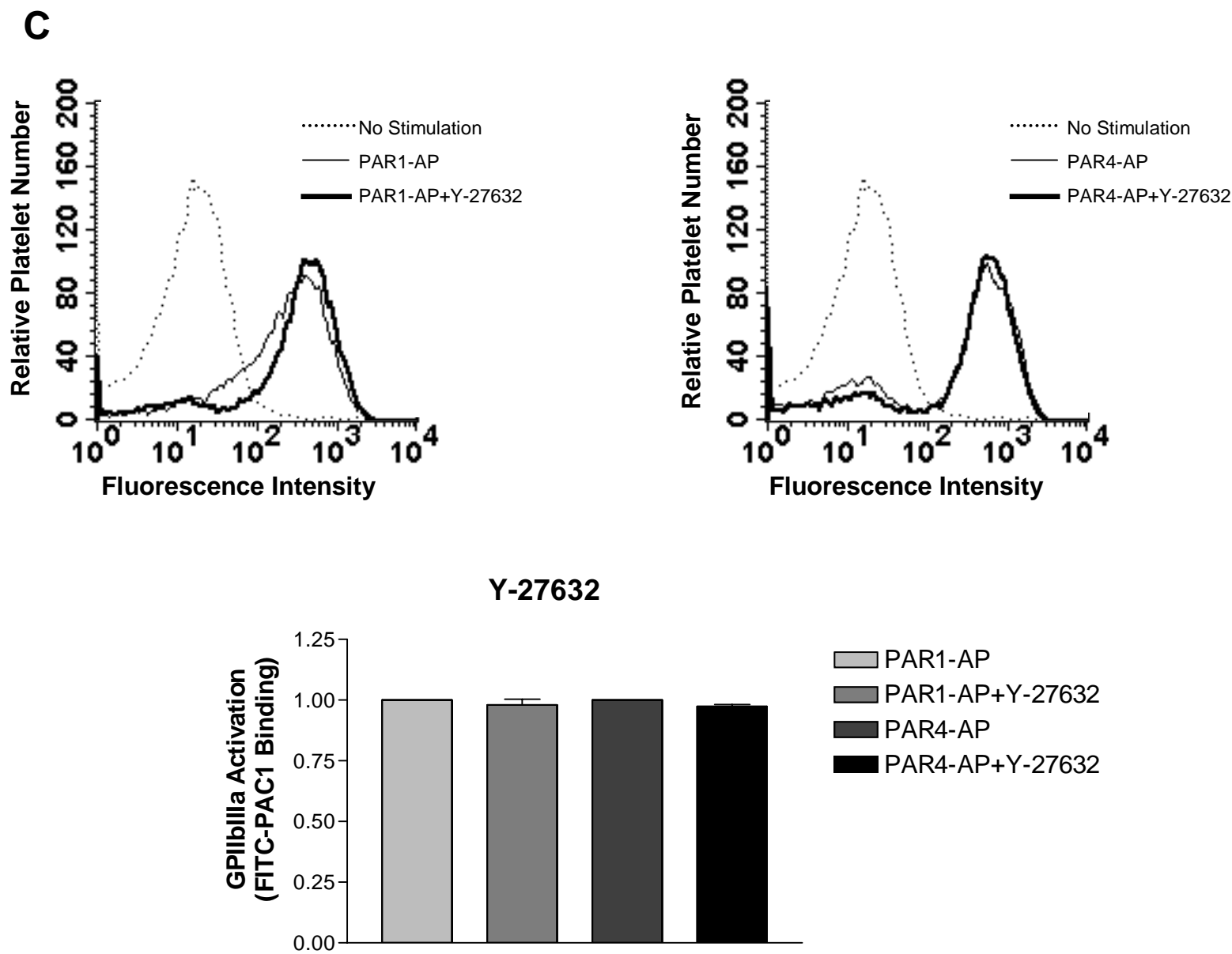intraplatelet calcium concentration is shown. (N=3) **, $P<0.01$

Figure 1

A

Figure 1

Figure 1

C

Y-27632

Figure 1

D

Figure 2

Figure 3

A

1mM Calcium

Legend:
- Thrombin
- PAR1-AP
- PAR4-AP
- No Stimulation

y-axis: $[Ca^{2+}]i$ Fold Increase 340/380nm

x-axis: Time (seconds)

B

0.2mM Calcium

Legend:
- Thrombin
- PAR1-AP
- PAR4-AP
- No Stimulation

y-axis: $[Ca^{2+}]i$ Fold Increase 340/380nm

x-axis: Time (seconds)

Figure 3

C

1 mM vs. 0.2 mM Extracellular Ca2+

Figure 3

D

Wortmannin

- ■ Thrombin+Wortmannin
- ▲ PAR1-AP+Wortmannin
- ▽ PAR4-AP+Wortmannin
- ○ No Stimulation

E

Vehicle (DMSO)

- ■ Thrombin+DMSO
- ▲ PAR1-AP+DMSO
- ▽ PAR4-AP+DMSO
- ○ No Stimulation

Figure 3

F

Wortmannin vs. DMSO

A Figure 4

B Percent Maximum