0026-895X/07/7105-1399–1406
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 71:1399–1406, 2007

Vol. 71, No. 5
33365/3201686
Printed in U.S.A.

PAR1, but Not PAR4, Activates Human Platelets through a $G_{i/o}$/Phosphoinositide-3 Kinase Signaling Axis

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Received December 11, 2006; accepted February 15, 2007

ABSTRACT

Thrombin-mediated activation of platelets is critical for hemostasis, but the signaling pathways responsible for this process are not completely understood. In addition, signaling within this cascade can also lead to thrombosis. In this study, we have defined a new signaling pathway for the thrombin receptor protease activated receptor-1 (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 activation and 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 purinoceptor 12, as an antiplatelet/antithrombotic drug, our data suggest that specifically blocking only PAR1-mediated $G_{i/o}$ signaling could also be an effective therapeutic approach with the possibility of less unwanted bleeding.

Thrombin-mediated activation of platelets and the vascular endothelium is critical for the normal regulation of hemostasis (Brass, 2001, 2003; Ossovskaya and Bunnett, 2004; Coughlin, 2005). 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) whereas 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, because PAR1 and PAR4 are the "active" thrombin receptors on human platelets and might interact only 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 (Kawabata et al., 2000; Ma et al., 2005; Holinstat et al., 2006), which probably 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

This work was supported by National Institutes of Health grants HL060678, EY010291, and 1P50-HL081009-01 (to H.E.H.), American Heart Association Predoctoral Fellowship (to B.V.), and National Research Service Award (to M.H.), HL082068-01. R.Z. is funded by a MERIT Award from the Department of Veterans Affairs and by National Institutes of Health grant R01-DK069921.

This work was presented in part at the annual American Society for Biochemistry and Molecular Biology; April 1–5, 2006; San Francisco, CA (Voss B, McLaughlin JN, and Hamm HE, Calcium mobilization in human platelets is differentially modulated by PAR1 and PAR4 through $G_{i/o}$ and P13K).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.033365.

ABBREVIATIONS: PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PI3K, phosphoinositide-3 kinase; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; Bis-1, bisindolylmaleimide-1; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; Y-27632, 4-[(1*R*)-1-aminoethyl]-*N*-pyridin-4-yl-cyclohexane-1-carboxamide; AP, activating peptide; DMSO, dimethyl sulfoxide; GPIIbIIIa, integrin αIIbβ3; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; TRP, transient receptor potential.

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 α subunit deficiencies (Gabbeta et al., 1997) and studies that used cell permeable dominant-negative PAR peptides (Covic et al., 2002a,b) 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 et al., 1993; Odagaki and Koyama, 2002) 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 $G_{\alpha o}$ (M. Holinstat and B. Voss, 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, because the $G_{i/o}$ family of G-proteins is 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 but leave 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 that are different for PAR1- versus PAR4-mediated GPIIbIIIa activation in human platelets. We demonstrate that although 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, whereas 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, People's Republic of China). 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 Invitrogen (Carlsbad, CA). 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 min of collection, 5-μl aliquots of this fresh whole blood was added to 12 $\times$ 75-mm polystyrene tubes containing a saturating concentration (7 μg/ml) of the FITC-PAC1 antibody. The platelets in these samples were pretreated for 15 min with 5 μM Bis-1, 2 μM U-73122, 20 μM Y-27632, and 100 nM wortmannin), or vehicle (water or DMSO) followed by activation with PAR1-AP or PAR4-AP and incubated for 15 min 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 $MgCl_2$, and 0.3 mM Na_2HPO_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 8000 cells were analyzed for FITC fluorescence to quantify the amount of platelet-bound PAC-1. Each condition was performed a minimum of three times, using blood from a different donor.

Platelet Aggregation. Platelet aggregations were measured using washed platelets (Kahn et al., 1998). In brief, blood was centrifuged in a Forma 400-ml GP centrifuge at 170*g* for 15 min at room temperature. The platelet rich plasma 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*g* for 10 min at room temperature. The pelleted platelets were resuspended in Tyrode's buffer and adjusted to a concentration of 3×10^8 platelets/ml using a Coulter counter (Beckman Coulter, Fullerton, CA). Some platelets were treated with 100 nM wortmannin for 30 min or 100 μM LY294002 for 15 min in the dark. After 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 (Chronolog, Inc., Philadelphia, PA). Each result is representative of at least three independent experiments.

Measurement of Intraplatelet Calcium Concentration. Intraplatelet calcium concentration was measured in washed platelets (Santoro et al., 1994), at a concentration of 3×10^8 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 min in a 37°C 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 min before stimulation, except for conditions in which extracellular calcium was intentionally left out. Fura-2-loaded platelets receiving PI3K inhibition were preincubated with wortmannin or LY294009 for 15 min and then stimulated accordingly. A Fluorometer (Cary Instruments/Varian, Palo Alto, CA) was used to monitor the absorbance changes at 340 and 380 nm before and after stimulation with the agonists listed under *Materials*. The ratio of the absorbance at 340 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 (Figs. 1–3) or one-way ANOVA (Fig. 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 shown recently that phospho-

lipase D is important for GPIIbIIIa activation of PAR1, but not of PAR4 (Holinstat et al., 2007); therefore, we focused on identifying other important G-protein pathways that might be downstream of these receptors in human platelets. Because 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 after maximal PAR1 or PAR4 activation, in the presence or absence of the PKC inhibitors Bis-1 and chelerythrine using FACS. The FITC-PAC1 antibody only recognizes the activated conformation of GPIIbIIIa. Representative experiments for PAR1-AP (Fig. 1A, left) and PAR4-AP (Fig. 1A, right) 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) or PAR4-AP (Fig. 1B, right) stimulated platelets compared with 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.

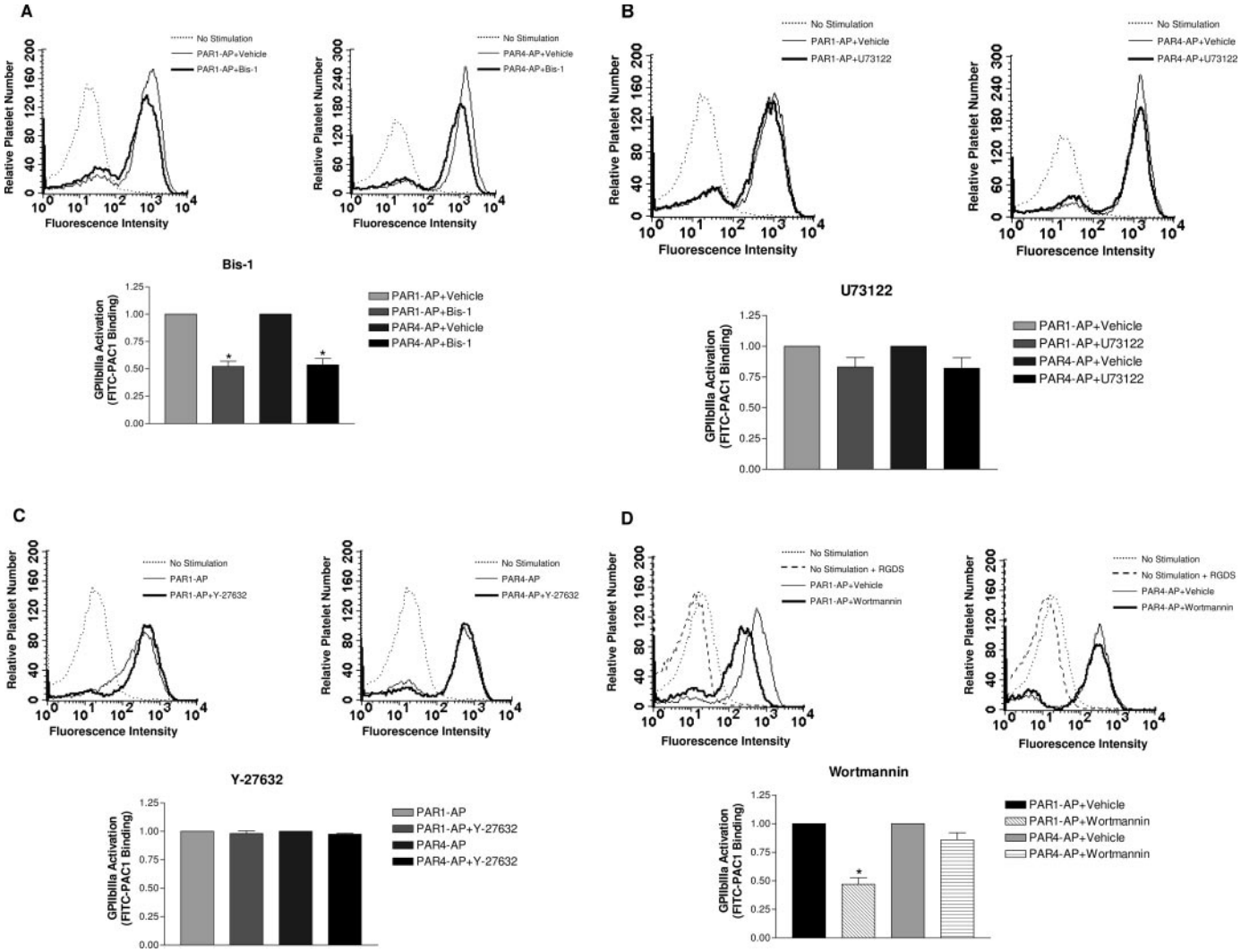

Fig. 1. PAR1 activates GPIIbIIIa through PI3K. Whole blood samples were treated with Bis-1 (A), U73122 (B), Y-27632 (C), wortmannin (D), or vehicle (water, DMSO, or ethanol) as described under *Materials and Methods*. A saturating concentration of FITC-PAC1 (5 μg/ml) was added before stimulation with maximal doses of PAR1-AP (left, 20 μM) or PAR4-AP (right, 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 of multiple experiments ($n \geq 3$) and are quantified in a bar graph for each respective data set (5 μM Bis-1, 2 μM U73122, 20 μM Y-27632, and 100 nM wortmannin) ($n = 3$). *, $P < 0.05$.

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 (Kovacsovics et al., 1995; Jackson et al., 2004); 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) before assessing PAR1- versus PAR4-mediated PAC-1 binding. Wortmannin inhibited GPIIbIIIa activation mediated by PAR1-AP (Fig. 1D, left) by ~50% but had no significant effect on that mediated by PAR4-AP (Fig. 1D, right). These data were quantified in the corresponding bar graph (Fig. 1D), and similar results were obtained with another PI3K inhibitor, LY294002 (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). 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 (nonphysiological) extracellular calcium concentration (Fig. 3B) did not differ significantly, whereas the PAR1-AP-mediated increase in intraplatelet calcium concentration was significantly attenuated in the presence of a low extra-

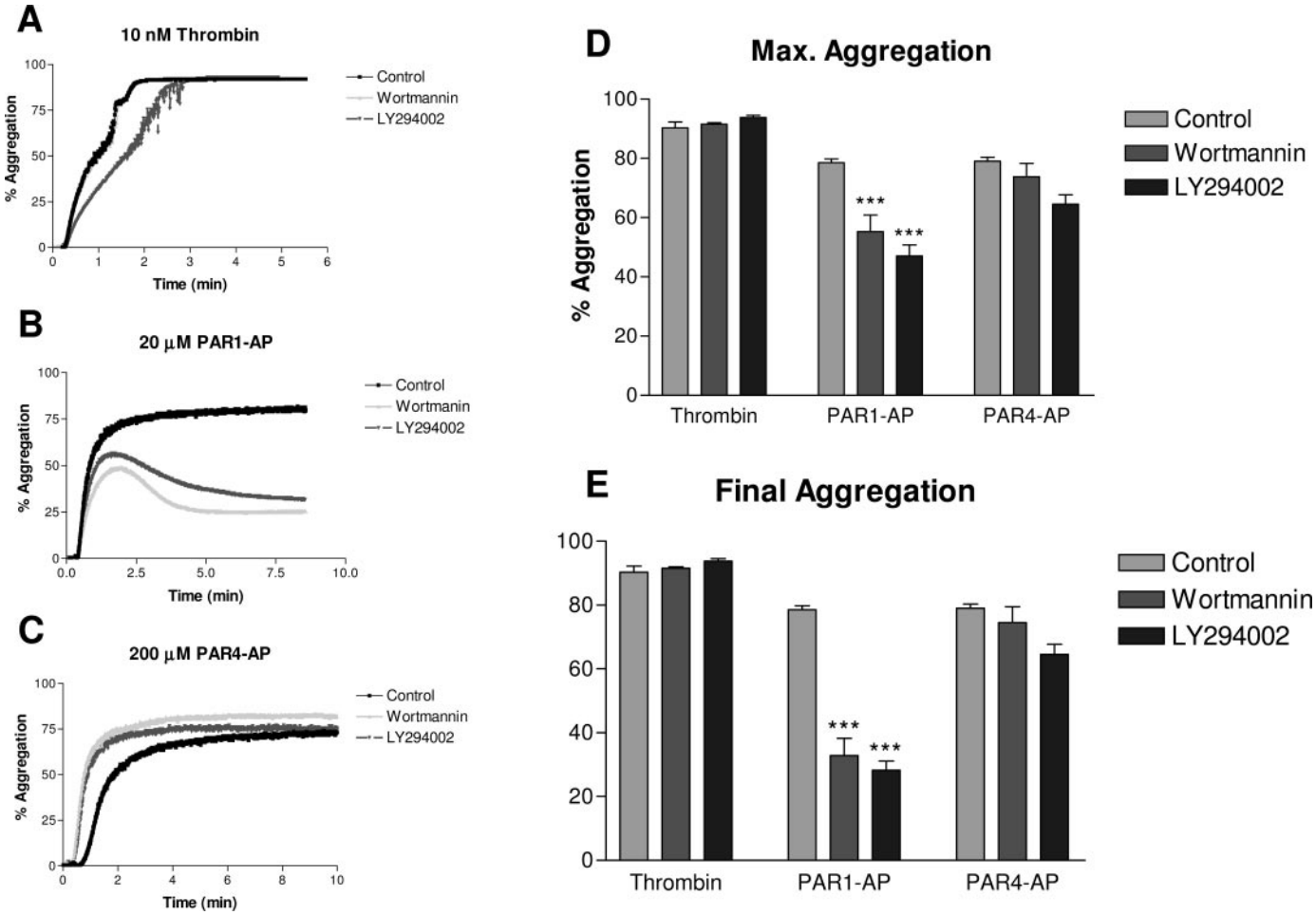

Fig. 2. PI3K differentially regulates PAR1- and PAR4-mediated platelet aggregation. Washed platelets were treated with or without 100 nM Wortmannin for 30 min or 100 μM LY294002 for 10 min followed by stimulation with 10 nM thrombin (A), 20 μM PAR1-AP (B), or 200 μM PAR4-AP (C). D, maximal aggregation was measured after agonist stimulation for each condition. E, final aggregation was measured at 6 min after agonist stimulation ($n = 3$). ***, $P < 0.001$.

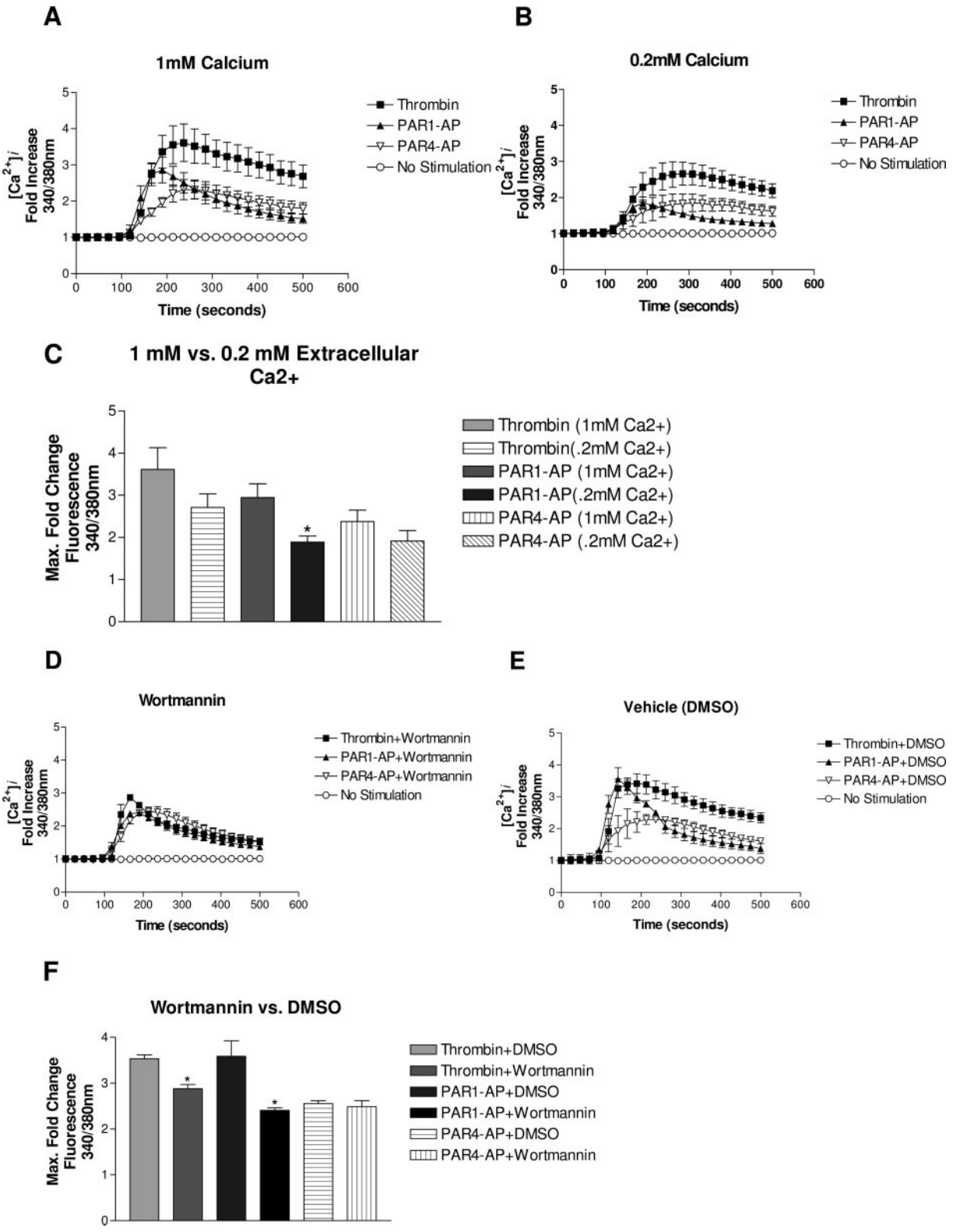

Fig. 3. PAR1 causes extracellular calcium entry via a $G_{i/o}$/PI3K signaling pathway. A, platelets loaded with Fura-2-AM were stimulated with either Tyrode's buffer (negative control), 10 nM thrombin, 20 μM PAR1-AP, or 200 μM PAR4-AP in the presence of 1 mM extracellular calcium, and the absorbance ratio at 340 and 380 nm 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 340 and 380 nm 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 (DMSO; E) for 15 min 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 340 and 380 nm 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 ($n = 3$). $*$, $P < 0.05$.

cellular calcium concentration (Fig. 3, A and 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, whereas 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 suggest that thrombin causes extracellular calcium entry in human platelets in a PI3K-dependent manner (Lian et al., 2005); however, it has not been established whether this occurs downstream of PAR1 or PAR4. 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). It is noteworthy that, compared with vehicle, the thrombin- and PAR1-AP-mediated calcium transients were significantly attenuated in the presence of wortmannin, whereas PAR4-AP was not significantly affected. This result was confirmed with LY294002. In addition, 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.

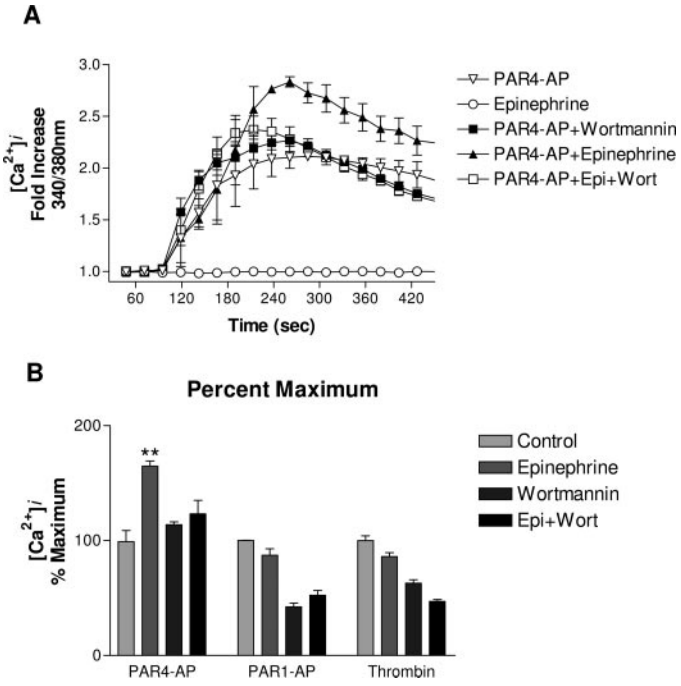

Fig. 4. PAR1 couples to $G_{i/o}$ in human platelets to modulate calcium flux. A, platelets loaded with Fura-2-AM were treated with 200 μM PAR4-AP, 200 nM epinephrine, or both in the presence of 1 mM extracellular calcium, and the absorbance ratio at 340 and 380 nm was monitored fluorimetrically. Additional samples of Fura-2-loaded platelets were pretreated with 100 nM wortmannin for 15 min 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 percentage change in maximum intraplatelet calcium concentration is shown ($n = 3$). **, $P < 0.01$.

PAR1, but Not PAR4, Couples to $G_{i/o}$ in Human Platelets to Modulate Calcium Flux. Our data suggest 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. Because PAR4 seems 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 Fig. 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 have already activated this $G_{i/o}$-PI3K pathway via PAR1 coupling to $G_{i/o}$, whereas 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. Because 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, uses 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. (2002), who used cAMP signaling as the readout for $G_{i/o}$ coupling, the data are 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, whereas 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). In addition, we have shown recently that phospholipase D is the source of diacylglycerol, which causes PKC activation downstream of PAR1 in human platelets (Holinstat et al., 2007). During PLCβ inhibition, this path-

way 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 platelet aggregation mediated by PAR1-AP, but not that mediated by thrombin or PAR4-AP, 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 regarding why PAR1-AP (Kovacsovics et al., 1995), but not PAR4-AP, requires PI3K for human platelet aggregation. Although PI3K activation has been described previously to play a role in platelet aggregation, this was in the context of pathways activated after 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 use its unique $G_{i/o}$/PI3K pathway to bring additional calcium into the platelet, whereas 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 increase mediated by PAR1-AP, but not that by PAR4, in intraplatelet calcium concentration. The mechanism by which PI3K causes platelet calcium influx is believed to be through TRP channel activation/trafficking (Viard et al., 2004; Zhuang et al., 2004; Ramsey et al., 2006) 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 α-2A adrenergic receptor, a $G_{i/o/z}$ family-coupled receptor (Woulfe, 2005), to activate PI3K. In addition, epinephrine only minimally potentiated the thrombin- and PAR1AP-mediated increases in intraplatelet calcium concentration. It is noteworthy that 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 $G_{i/o}$, whereas 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 as a result of 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. In addition, 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.

References

Banfic H, Tang X, Batty IH, Downes CP, Chen C, and Rittenhouse SE (1998) A novel integrin-activated pathway forms PKB/Akt-stimulatory phosphatidylinositol 3,4-bisphosphate via phosphatidylinositol 3-phosphate in platelets. *J Biol Chem* **273:** 13–16.

Brass LF (2003) Thrombin and platelet activation. *Chest* **124:**18S–25S.

Brass S (2001) Cardiovascular biology. Platelets and proteases. *Nature (Lond)* **413:** 26–27.

Coughlin SR (2000) Thrombin signalling and protease-activated receptors. *Nature (Lond)* **407:**258–264.

Coughlin SR (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* **3:**1800–1814.

Covic L, Gresser AL, Talavera J, Swift S, and Kuliopulos A (2002a) Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci USA* **99:**643–648.

Covic L, Misra M, Badar J, Singh C, and Kuliopulos A (2002b) Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med* **8:**1161–1165.

Di Nisio M, Middeldorp S, and Buller HR (2005) Direct thrombin inhibitors. *N Engl J Med* **353:**1028–1040.

Dorsam RT, Kim S, Jin J, and Kunapuli SP (2002) Coordinated signaling through both $G_{12/13}$ and G_i pathways is sufficient to activate GPIIb/IIIa in human platelets. *J Biol Chem* **277:**47588–47595.

Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, and Rao AK (1997) Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. *Proc Natl Acad Sci USA* **94:**8750–8755.

Gilchrist A, Vanhauwe JF, Li A, Thomas TO, Voyno-Yasenetskaya T, and Hamm HE (2001) Gα minigenes expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. *J Biol Chem* **276:**25672–25679.

Holinstat M, Voss B, Bilodeau ML, and Hamm HE (2007) Protease activated receptors differentially regulate human platelet activation through a phosphatidic acid-dependent pathway. *Mol Pharmacol* **71:**686–694.

Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, and Hamm HE (2006) PAR4, but not PAR1, signals human platelet aggregation via Ca^{2+} mobilization and synergistic P2Y12 receptor activation. *J Biol Chem* **281:**26665–26674.

Jackson SP, Yap CL, and Anderson KE (2004) Phosphoinositide 3-kinases and the regulation of platelet function. *Biochem Soc Trans* **32:**387–392.

Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C, and Coughlin SR (1998) A dual thrombin receptor system for platelet activation. *Nature (Lond)* **394:**690–694.

Kawabata A, Kuroda R, Kuroki N, Nishikawa H, and Kawai K (2000) Dual modulation by thrombin of the motility of rat oesophageal muscularis mucosae via two distinct protease-activated receptors (PARs): a novel role for PAR-4 as opposed to PAR-1. *Br J Pharmacol* **131:**578–584.

Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, Cattaneo M, and Kunapuli SP (2002) 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:**3629–3636.

Kovacsovics TJ, Bachelot C, Toker A, Vlahos CJ, Duckworth B, Cantley LC, and Hartwig JH (1995) Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J Biol Chem* **270:**11358–11366.

Lapetina EG, Reep B, and Chang KJ (1986) Treatment of human platelets with trypsin, thrombin, or collagen inhibits the pertussis toxin-induced ADP-ribosylation of a 41-kDa protein. *Proc Natl Acad Sci USA* **83:**5880–5883.

Lefkovits J, Plow EF, and Topol EJ (1995) Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med* **332:**1553–1559.

Leger AJ, Jacques SL, Badar J, Kaneider NC, Derian CK, Andrade-Gordon P, Covic L, and Kuliopulos A (2006) Blocking the protease-activated receptor 1–4 heterodimer in platelet-mediated thrombosis. *Circulation* **113:**1244–1254.

Lian L, Wang Y, Draznin J, Eslin D, Bennett JS, Poncz M, Wu D, and Abrams CS (2005) The relative role of PLCbeta and PI3Kgamma in platelet activation. *Blood* **106:**110–117.

Ma L, Perini R, McKnight W, Dicay M, Klein A, Hollenberg MD, and Wallace JL (2005) Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. *Proc Natl Acad Sci USA* **102:**216–220.

Moers A, Nieswandt B, Massberg S, Wettschureck N, Gruner S, Konrad I, Schulte V,

Aktas B, Gratacap MP, Simon MI, et al. (2003) G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat Med* **9:**1418–1422.
Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, and Coughlin SR (2000) PAR3 is a cofactor for PAR4 activation by thrombin. *Nature (Lond)* **404:**609–613.
Odagaki Y and Koyama T (2002) Epinephrine- and thrombin-stimulated high-affinity GTPase activity in platelet membranes from patients with psychiatric disorders. *Psychiatry Res* **112:**111–119.
Odagaki Y, Koyama T, and Yamashita I (1993) Pharmacological characterization of epinephrine-stimulated GTPase activity in human platelet membranes. *Biochem Pharmacol* **46:**2021–2028.
Offermanns S (2000) The role of heterotrimeric G proteins in platelet activation. *Biol Chem* **381:**389–396.
Ossovskaya VS and Bunnett NW (2004) Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* **84:**579–621.
Ramsey IS, Delling M, and Clapham DE (2006) An introduction to TRP channels. *Annu Rev Physiol* **68:**619–647.
Rosado JA, Lopez JJ, Harper AG, Harper MT, Redondo PC, Pariente JA, Sage SO, and Salido GM (2004) Two pathways for store-mediated calcium entry differentially dependent on the actin cytoskeleton in human platelets. *J Biol Chem* **279:**29231–29235.
Santoro SA, Zutter MM, Wu JE, Staatz WD, Saelman EU, and Keely PJ (1994) Analysis of collagen receptors. *Methods Enzymol* **245:**147–183.
Shattil SJ, Cunningham M, and Hoxie JA (1987) Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* **70:**307–315.
Tseng PH, Lin HP, Hu H, Wang C, Zhu MX, and Chen CS (2004) The canonical transient receptor potential 6 channel as a putative phosphatidylinositol 3,4,5-trisphosphate-sensitive calcium entry system. *Biochemistry* **43:**11701–11708.
Vanhauwe JF, Thomas TO, Minshall RD, Tiruppathi C, Li A, Gilchrist A, Yoon EJ, Malik AB, and Hamm HE (2002) Thrombin receptors activate G_o proteins in endothelial cells to regulate intracellular calcium and cell shape changes. *J Biol Chem* **277:**34143–34149.
Viard P, Butcher AJ, Halet G, Davies A, Nurnberg B, Heblich F, and Dolphin AC (2004) PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. *Nat Neurosci* **7:**939–946.
Wettschureck N and Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* **85:**1159–1204**.**
Woulfe DS (2005) Platelet G protein-coupled receptors in hemostasis and thrombosis. *J Thromb Haemost* **3:**2193–2200**.**
Zhuang ZY, Xu H, Clapham DE, and Ji RR (2004) Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *J Neurosci* **24:**8300–8309.

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