Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets

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Abbreviations: PAR, Protease activated receptor; FV, Factor V; PS, Phosphatidyl-serine; AP, activating peptide; ELISA, enzyme linked immunosorbent assay; PLC, phospho lipase C; PIP₂, phosphatidyl inositol 4,5-biphosphate; IP₃, inositol 1,4,5-triphosphate; DAG, Diacylglycerol; MFI, mean fluorescence intensity; FII, prothrombin; F, factor
Abstract

With the recent interest of protease-activated receptors (PAR) 1 and PAR4 as possible targets for the treatment of thrombotic disorders, we compared the efficacy of protease-activated receptor (PAR)1 and PAR4 in the generation of procoagulant phenotypes on platelet membranes. PAR4-activating peptide (AP) stimulated platelets promoted thrombin generation in plasma up to 5 min earlier than PAR1-AP stimulated platelets. PAR4-AP mediated factor V (FV) association with the platelet surface was 1.6-fold greater than for PAR1-AP. Moreover, PAR4 stimulation resulted in a 3-fold greater release of microparticles compared to PAR1 stimulation. More robust FV secretion and microparticle generation with PAR4-AP was due to stronger and more sustained phosphorylation of myosin light chain at serine 19 and threonine 18. Inhibition of Rho-kinase reduced PAR4-AP mediated FV secretion and microparticle generation down to PAR1-AP mediated levels. Thrombin generation assays measuring prothrombinase complex activity demonstrated 1.5-fold higher peak thrombin levels on PAR4-AP stimulated platelets, compared to PAR1-AP stimulated platelets. Rho-kinase inhibition reduced PAR4-AP mediated peak thrombin generation by 25% but had no significant effect on PAR1-AP mediated thrombin generation. In conclusion, stimulation of PAR4 on platelets leads to faster and more robust thrombin generation than PAR1 stimulation. The greater procoagulant potential is related to more efficient FV-release from intracellular stores and microparticle production driven by stronger and more sustained myosin light chain phosphorylation. These data have implications about the role of PAR4 during hemostasis, and are clinically relevant in light of recent efforts to develop PAR antagonists to treat thrombotic disorders.
Introduction

Thrombin activates platelets through proteolytic cleavage of protease-activated receptors (PARs) resulting in the generation of a tethered ligand. Human platelets express two PARs (PAR1 and PAR4). PAR1 contains a hirudin-like sequence within its exodomain that interacts with thrombin’s anion binding exosite-1 (Vu et al., 1991, Liu et al., 1991). Due to this high affinity interaction PAR1 is engaged at lower concentrations of thrombin than PAR4, which lacks the hirudin-like domain (Hammes and Coughlin, 1999, Faruqi et al., 2000, Xu et al., 1998). PAR1 and PAR4 differ not only in temporal engagement but also in downstream signaling pathways (Holinstat et al., 2009, Voss et al., 2007, Holinstat et al., 2007, Holinstat et al., 2006, Ma et al., 2005, Bilodeau and Hamm, 2007, Coughlin, 2000, Covic et al., 2000).

Monroe et al (Monroe et al., 2002) described a model of hemostasis implicating platelets in the “amplification/priming” and “propagation” of thrombin generation. Platelet activation results in the expression of a procoagulant surface and assembly of the prothrombinase and intrinsic Xase complexes, leading to cleavage of fibrinogen to fibrin and formation of a hemostatic clot. In addition to the provision of phosphatidyl-serine (PS) rich membranes for the assembly of coagulation complexes, platelets possess a unique, APC-resistant, pre-activated form of factor (F) V (Duckers et al., 2010, Alberio et al., 2000), which is concentrated in α-granules from plasma sources. Platelet-FV represents 20% of the total amount of FV circulating in whole blood, and FV concentrations in the platelet exceed that of the plasma by 100 fold (Weiss et al., 2001). The importance of platelet-factor V is illustrated in clinical cases of bleeding associated with defects in FV storage or release (Diamandis et al., 2008, Grigg et al., 1989, Nesheim et al., 1986, Tracy et al., 1984, Weiss and Lages, 1997, Weiss et al., 2001). More recently, patients with severe congenital FV deficiency were shown to be protected against a loss of thrombin generation and severe bleeding by residual FV concentrated in their platelet granules (Duckers et al., 2010).
Activated platelets shed microparticles which mediate a number of processes involved in coagulation, platelet adhesion, angiogenesis and vascular smooth muscle cell proliferation (Flaumenhaft et al., 2010, Italiano et al., 2010). The concentrations of circulating platelet-derived microparticles correlate with the severity of a number of cardiovascular diseases including: acute coronary syndrome (Mallat et al., 2000), atherosclerosis (Tan and Lip, 2005) hypertension (Preston et al., 2003), peripheral artery disease (van der Zee et al., 2006, Zeiger et al., 2000, Tan and Lip, 2005), aortic valve stenosis (Diehl et al., 2008), metabolic syndrome (Diamant et al., 2002), and Type II Diabetes (Nomura et al., 1995, Tan et al., 2005, Koga et al., 2006, Cohen et al., 2002). Platelet-derived microparticles possess binding sites for coagulation factors FVa (Alberio et al., 2000), FVIIIa (Gilbert et al., 1991), FIXa (Hoffman et al., 1992a). Platelet-derived microparticles are generated in response to a number of strong platelet agonists, including thrombin (Flaumenhaft et al., 2010), however the individual capacity of PAR1 and PAR4 to induce microparticle formation have not been explored.

Platelet degranulation involves activation of phospholipase C (PLC) and subsequent cleavage of phosphatidyl inositol 4, 5-biphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3). DAG directly activates PKC and IP3 mobilizes intracellular calcium that synergistically contribute to platelet secretion (Walker and Watson, 1993). Accordingly, impaired platelet secretion is observed in G$_{\alpha q}$ or PLC$\beta$ deficient mice and humans (Offermanns et al., 1997, Gabbeta et al., 1997). New data are shedding light on a role for the G$_{\alpha 12/13}$ pathway and RhoA activation in platelet secretion. In addition to defects in hemostasis and protection against thrombosis, G$_{\alpha 13}$ deficient mice display defective agonist induced secretion (Moers et al., 2003). In mice a megakaryocyte-specific RhoA deficiency causes impaired platelet granule secretion (Pleines et al., 2012). Kunapuli et al demonstrated that supplementation of G$_{\alpha q}$ signaling with G$_{\alpha 12/13}$ activation is necessary for dense granule secretion which can be blocked with a RhoA inhibitor (Jin et al., 2009). Active RhoA, through its
effector Rho-kinase, maintains phosphorylation of myosin light chain by inactivating myosin light chain phosphatase. Inhibition of Rho-kinase results in impaired thrombin mediated secretion (Getz et al., 2010).

The signaling pathways involved in platelet microparticle production have not been clearly defined. Several mechanisms are implicated in microparticle production including destabilization of membrane-cytoskeletal attachments in a Ca<sup>2+</sup>/calpain-dependent manner and changes in membrane composition, particularly acute decreases in PIP<sub>2</sub> membrane composition (Flaumenhaft et al., 2009, O'Connell et al., 2005, Flaumenhaft et al., 2010). Although the possibility of a Rho/Rho-kinase/myosin signaling axis contributing to microparticle production has been proposed (Flaumenhaft et al., 2010), involvement of this pathway has not been demonstrated.

Activated platelets support thrombin generation and in turn are stimulated by thrombin through PARs. Therefore platelet activation and thrombin generation are interdependent biologic processes. We hypothesize that PAR1 and PAR4 play distinct roles in thrombosis and hemostasis and therefore present unique procoagulant phenotypes when stimulated individually. A careful assessment of the efficacy of the PARs to modulate biochemical events involved in thrombin generation has not been conducted. We conducted a series of experiments with PAR-activating peptides (AP) that have been extensively used to describe the signaling of PARs (Faruqi et al., 2000, Holinstat et al., 2006, Holinstat et al., 2007, Voss et al., 2007). We demonstrate that PAR4 stimulation leads to more FV secretion and microparticle generation than PAR1 stimulation in human platelets. The stronger procoagulant phenotypes exhibited by PAR4 were due to more sustained and robust MLC phosphorylation driven by Rho-kinase activity, a previously undocumented observation. We also demonstrate a novel role for Rho-kinase in platelet microparticle production.
Materials and Methods

Materials  The thrombin substrate z-GGR-AMC was purchased from Bachem (Basel, Switzerland). Sheep anti-human FV antibody, FII, FX, FXa, and Antithrombin (AT) were from Haematologic Technologies (Essex Junction, VT). Anti-PKC (S)-substrate, phospho myosin light chain (pMLC) T18 and pMLC S19, and p44/p42 ERK antibodies were from Cell Signaling Technologies Inc. (Danvers, MA). Anti-Rabbit and Anti-Mouse secondary antibodies and ECL reagent were from Perkin Elmer (Waltham, MA). Alexa Fluor 488 donkey anti-sheep were from Invitrogen (Carlsbad, CA). Activating peptides for PAR1 (PAR1-AP; SFLLRN) and PAR4 (PAR4-AP, AYPGKF) were purchased from GL Biochem (Shanghai, China). Human plasma was purchased from George King Bio-medical Inc. (Overland Park, KS). PAR-1 span IgY and PAR-4 span IgY were kind gifts from Dr. Fred Ofosu.

Blood collection and platelet isolation  Human platelets were obtained from healthy volunteers. The studies were approved by the Vanderbilt University Internal Review Board. Informed consent was obtained from all individuals prior to the blood draw. Blood was collected into sodium citrate anticoagulant (final concentration 0.32%) through a 19 gauge needle. PGE_1 (5 μg/ml, final concentration) was added to citrated blood, and gel filtered platelets were prepared as described (Hoffman et al., 1992b). Blood samples were overlaid onto Accuprep Lymphocyte separation medium (Accurate Chemical and Scientific Corp., Westbury, NY) and centrifuged for 30 min at 500 x g. The middle band containing platelets and mononuclear cells was isolated and mixed with an equal volume of CGS buffer (13 mM Citrate, pH7.4, 123 mM NaCl, 33 mM dextrose) containing 5 μg/ml PGE_1 (final concentration). Nucleated cells were removed by centrifugation at 120 x g for 10 min. The platelet-rich supernatant was isolated and layered onto a Sepharose 4B column (Sigma-Aldrich, St. Louis, Missouri), equilibrated with Tyrode’s Buffer (15 mM HEPES, 0.33 mM NaH_2PO_4, pH7.4, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2, 5.5 mM
dextrose) with 0.1% BSA. Platelets were collected, counted on a Coulter Counter, and diluted in Tyrode’s with 0.1%BSA to the indicated concentrations.

**Immunocytochemistry (ICC)** Gel filtered platelets at a density of 1.5 x10^7 cells/ml were incubated with agonist or vehicle control for 15 min before fixation with 1% paraformaldehyde (PFA). Samples were diluted in PBS (137mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.46mM KH2PO4) with 0.1% BSA and added to Lab-Tek II chamber slides (NUNC, Rochester, NY) pre-coated with poly-lysine. Chamber slides were incubated overnight at 4°C to allow platelets to adhere. After seeding, chambers were washed once with an equal volume of PBS. Samples were then blocked for 30 min at room temperature with 1% BSA in PBS. After blocking, samples were incubated with antibodies diluted in PBS with 1% BSA for 1 hr followed by three wash cycles with PBS before incubation with the appropriate fluorescent secondary antibody in PBS with 1% BSA for 30 min. Samples were washed three more times before mounting in aqua polymount (Polysciences Inc, Arrington, PA). Images were taken with a 63x / 1.40 Plan-APOCHROMAT oil objective on a Zeiss LSM 510 Inverted confocal microscope. Microscopy was performed through the use of the VUMC Cell Imaging Shared Resource, (supported by NIH grants CA68485, DK20593, DK50404, Hd15052, Dk59637, and Ey008126).

**Flow cytometry** For detection of FV, gel filtered platelets at a density of 1.5x10^7 cells/ml were incubated with vehicle control or agonist for 15 min before fixation with 1% PFA for 20 min. After fixation and washing, samples were incubated with the appropriate concentration of primary antibody for 1 hr at room temperature. After washing with PBS, samples were suspended in 2 μg/ml of labeled secondary antibody and incubated for 30 min at room temperature. Samples were washed once more with PBS then suspended in 500 μl PBS. Samples were analyzed on a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ). For detection of P-selectin, platelets at 1.5x10^7 cells/ml were pre-incubated with APC conjugated CD62P before stimulation with the appropriate agonist for 15 min. Samples were fixed with 1%
paraformaldehyde in PBS for 20 min before dilution of the samples with Tyrode’s buffer. Data were analyzed using FACS DiVa acquisition software (Becton Dickinson) and Winlist software (Verity Software House) for analysis. Mean fluorescence intensity was determined by collecting the fluorescence recordings within the platelet gate for 100,000 events. % positive cells were determined by gating to unstimulated platelets incubated with an isotype matched IgG control for each primary antibody.

**Microparticle detection** Gel filtered platelets at a density of 2x10^8 cells/ml were supplemented with 2.5 mM CaCl₂ before incubation with the various concentrations of PAR agonists for 30 min at 37°C. Microparticle-enriched samples were prepared by centrifugation of platelet preparations for 10 min at 700xg. The supernatant was collected and incubated for 20 min with either FITC conjugated sheep IgG non-immune isotype control (Affinity Biologicals, Ancaster, Ontario), PE conjugated CD41 (Becton Dickson), and APC conjugated CD62P (Beckton Dickson) or FITC conjugated sheep FV (Affinity Biologicals), PE conjugated CD41, and APC conjugated CD62P. Samples were diluted in Tyrode’s buffer and analyzed on a FACS Canto II. Events were collected for a total of 1 min for each sample. Microparticles included CD41 and CD62P dual positive particles detected below 1 μm in size as determined by forward and side scatter analysis of 1 μm beads.

**Intracellular calcium mobilization** Washed human platelets are prepared as previously detailed (Holinstat et al., 2009) and diluted in Tyrode’s buffer + 0.1%BSA. 1 hour prior to stimulation, platelets are dye loaded with 1.25 μg/ml (final concentration) Fluo4-AM in calcium assay buffer (HBSS, 20mM HEPES, 2.5mM probenecid). 30 min prior stimulation platelets are mixed with a combination of PAR-1 span IgY and PAR-4 span IgY antibodies or chicken IgY isotype control. 60 μL of dye loaded platelets are added to each well of a standard surface BD falcon 384 well plate. The final platelet concentration was 0.8x10^8 cells/ml. Fluorescence measurements are
taken on a Functional Drug Screening System (Hamamatsu, Japan) at excitation/emission 480/540 for 240 sec at 25°C.

**Enzyme Linked Immunoabsorbance Assay (ELISA)** Gel filtered platelets at a density of 2.0x10^8 cells/ml were stimulated with the indicated concentration of PAR agonist for 15 min. Platelets were then pelleted at 13,000 x g for 10 min at room temperature. Supernatants were collected and stored for analysis at a later date. Immulon 2HB 96 well microtitre EIA plates were coated with a FV capture antibody (FV-EIA-C, Affinity Biologicals) in Carbonate buffer (50 mM Carbonate, pH 9.6) overnight at 4°C. Plates were blocked with 1% BSA before application of samples diluted 1/10 in HEPES buffered saline with 1% BSA and 0.1% Tween-20. Standards were prepared from purified FV (Haematologic Technologies) ranging from 70 ng/ml to 0.5 ng/ml. After washing with PBS-T, bound FV was detected with an anti-human FV antibody (FV-EIA-D, Affinity Biologicals), followed by another wash and incubation with 416 μg/ml OPD substrate (Sigma). Plates were read at 490 nm in a Spectramax 190 plate reader.

**Thrombin generation** Gel filtered platelets at a density of 6.25x10^7 cells/ml were applied to prewarmed (37°C) black-sided 96 well plates (Greiner Bio-one). Platelets were stimulated with agonist or vehicle control for 5 min at 37°C before addition of an equal volume of citrate-anticoagulated human plasma or a mixture of purified coagulation factors in citrated Tyrode’s buffer, all prewarmed to 37°C. Thrombin generation was initiated immediately with the addition of excess calcium (final concentration 16.4 mM) and fluorogenic thrombin substrate z-GGR-AMC (440 μM). Thrombin generation was monitored in a FlexStation II 384 at 390/460 nm for 4 hours. After correction for substrate depletion and the inner-filter effect, the first order derivative was taken from raw fluorescent units and converted into concentrations of thrombin (nM) by comparison to a standard curve generated with fixed concentrations of thrombin (Hemker et al., 2003, De Smedt et al., 2008). Concentrations of thrombin were then plotted against time to establish a time-course of thrombin production. PAR-1 span IgY and PAR-4 span IgY (Ofosu et
al., 2008) were preincubated with platelets for 30 min prior to stimulation with PAR1-AP or PAR4-AP and the initiation of the assay.

**Western blot analysis** Gel filtered platelets at 3.0x10^8 cells/ml in Tyrode’s + 0.1% BSA were stimulated for indicated time periods at 37°C. Reactions were stopped with the addition of 0.6N HClO₄ to immediately precipitate all protein. Samples were centrifuged at 13,000 x g for 10 min at 4°C to isolate protein pellets. Pellets were resuspended and centrifuged 2 times with ice cold water. Finally, pellets were resuspended in 1X SDS Lamelli buffer and allowed to dissolve overnight. Samples were analyzed by SDS PAGE followed by western blotting. An estimated 25 μg was added to each well. Blots were probed with corresponding primary antibodies and HRP linked secondary antibodies before exposure with ECL reagent. Primary antibodies were used at 1/1,000 dilution as suggested by Cell Signaling.

**Results**

**Thrombin generation supported by platelet stimulation with PAR agonists** We examined the ability of PAR1 and PAR4 stimulated platelets to induce thrombin generation in plasma. Purified platelets were stimulated with increasing concentrations of PAR1-AP or PAR4-AP, and subsequently suspended in plasma. Maximal doses of PAR4-AP induced thrombin generation up to 5 minutes before PAR1-AP (Fig.1A and 1B). This data suggests the induction of a more procoagulant platelet phenotype with PAR4 stimulation compared to PAR1.

**Expression of FV on platelets in response to PAR activation** ICC analysis of unpermeabilized platelets demonstrated a clear increase in FV expression on the platelet surface in response to PAR stimulation (Fig. 2A). Having confirmed the expression of FV on the platelet surface in response to stimulation, we next quantified the amount of FV on PAR1-AP and PAR4-AP stimulated platelets, to determine if this could account for the more rapid thrombin generation observed with PAR4-AP stimulated platelets.
Gel filtered platelets were treated with increasing concentrations of PAR1-AP and PAR4-AP for 15 min before fixation, staining, and analysis by flow cytometry. Dose response curves were constructed to demonstrate that maximal responses had been reached. All data points comparing PAR1 and PAR4 were collected as matched samples with an unstimulated control from the same patient allowing for the use of the more powerful paired t-test for statistical analysis. Maximal fold-increase in mean fluorescence intensity (MFI) for PAR4-AP stimulated platelets (5.6±0.6 fold) was significantly higher than PAR1-AP stimulated platelets (3.8±0.3 fold) (p-value=0.021, n=4, paired t-test)(Fig. 2B), indicating a 1.6 fold increase in platelet bound FV on PAR4 stimulated platelets compared to PAR1-AP stimulated platelets. Maximal PAR1 stimulation increased the percentage of platelets staining positive for FV from 11.7% to 84.2% while maximal PAR4 stimulation increased the percent positive to 88.9% (Fig. 2D). Although PAR4 stimulation leads to slightly more positive cells, it does not account for the higher MFI values, indicating that PAR4 stimulation leads to a higher density of FV on the surface of platelets.

To examine if PS exposure could account for the difference in platelet FV surface expression after PAR1 and PAR4 stimulation, we compared the level of PS exposure by assessing FITC conjugated Annexin V binding to platelet membranes. Our data demonstrates a higher FITC mean fluorescence intensity for PAR1 stimulated platelets and generally low PAR1 and PAR4 Annexin V-FITC binding (Fig. 2E). These data indicate that PS exposure does not account for the difference in FV association with the platelet surface.

**Release of platelet-FV from intracellular granules in response to PAR activation** We compared the levels of FV in supernatants from PAR1 and PAR4 stimulated platelets as a direct measure of the FV release reaction. Samples were subjected to high speed centrifugation before assaying to remove membrane debris and any trace of platelets. Dose response curves constructed from ELISAs (Fig. 2F) indicated significantly more FV released from PAR4
stimulated platelets (83±4 ng/ml), compared to PAR1 stimulated platelets (54±3
ng/ml)(unstimulated 10±3 ng/ml)(p-value=0.0062, n=3, paired t-test). These data indicate 1.5old more FV is released from PAR4 stimulated platelets and suggest that PAR4 is more
efficient at liberating FV from intracellular stores.

Platelet-FV is reportedly stored in α-granules (Alberio et al., 2000, Duckers et al., 2010).
To determine if the difference in FV levels on the surface and in the supernatant of PAR1-AP
and PAR4-AP stimulated platelets was due to a difference in each receptor’s ability to induce
mobilization and secretion of α-granules we compared PAR1-AP and PAR4-AP induced P-
selectin levels on the platelet surface measured by flow cytometry. Dose-response curves
constructed with increasing concentrations of PAR1-AP and PAR4-AP indicate a significantly
higher maximal response for PAR4-AP (12.7±0.8 fold) versus PAR1-AP stimulated platelets
(10.1±0.8 fold)(p-value=.0049, n=3, paired t-test)(Fig. 2G). These data suggest that the
enhanced FV expression in response to PAR4-AP may be due to more efficacious mobilization
of α-granules by PAR4. The magnitude of the differential between PAR1-AP and PAR4-AP
mediated responses for FV and P-selectin expression were not identical suggesting
heterogeneity in cargo of the α-granule population.

**Platelet microparticle production in response to PAR activation**  Circulating microparticles
represent procoagulant surfaces and have been demonstrated to present binding sites for FV
(Alberio et al., 2000, Monkovic and Tracy, 1990, Sims et al., 1988, Sims et al., 1989). We used
flow cytometry to quantify the generation of platelet-derived microparticles in response to PAR
stimulation. Appropriate gating by size for microparticles was determined using forward and
side scatter analysis with 1 μm beads. Fig. 3A (left panel) shows a forward and side-scatter dot
plot of 1μm calibration beads and the size gate based on the forward scatter values used to
identify microparticles in subsequent experiments. The right panel shows the dot plot from
PAR1 stimulated platelets. Particles above the box are whole platelets, while particles within the box were candidates for microparticles. There is an increase in the number of particles under 1μm in response to both PAR1 and PAR4 stimulation (Fig. 3B). To confirm that these particles were platelet derived, we incubated the microparticle-enriched samples with antibodies against the platelet integrin GPIIb (CD41) and P-selectin (CD62P). Maximal PAR4 stimulation lead to a 10.3±0.0-fold increase in GPIIb and P-selectin positive microparticles compared to unstimulated platelets, while maximal PAR1 stimulation lead to a 4.3±0.3-fold increase (p-value=0.0142, n=2, t-test)(Fig. 3C). Consistently, we observed FV expression on the surface of GPIIb and P-selectin positive microparticles (Supplementary figure 1). The percent of PAR1-AP and PAR4-AP induced particles positive for FV was not significantly different; however, the number of FV-positive microparticles in PAR4 stimulated samples exceeds that in PAR1 stimulated samples by over 2-fold.

Regulation of myosin light chain phosphorylation by PAR stimulated platelets Platelet degranulation occurs downstream of Gαq mediated PKC activation and Ca²⁺ release and is enhanced by Gα12/13 mediated RhoA activation. The precise mechanism by which microparticle release occurs is largely unknown. It is known that the calcium response downstream of PAR4 is sustained compared to PAR1. As a possible mechanistic explanation of the difference between PAR1 and PAR4 in the procoagulant phenotypes described above, we examined PAR1 and PAR4 mediated PKC-substrate serine phosphorylation in addition to MLC phosphorylation at both serine 19 (pMLC S19) and threonine 18 (pMLC T18). PAR4 mediated phosphorylation of PKC substrates was more robust and more sustained than PAR1 (Fig. 4A). Both PAR1 and PAR4 induced phosphorylation of MLC at S19 and T18, however, the PAR4 response was markedly more robust and sustained over the course of 15 minutes (Fig. 4A). Figure 4B shows the quantification of three separate experiments and illustrates the rapid nature of MLC dephosphorylation downstream of PAR1 in comparison to PAR4.
The effect of PKC inhibition and RhoA pathway inhibition on platelet secretion and microparticle production

Due to the sustained nature of the PAR4 PKC activity and MLC phosphorylation we constructed a time course of FV release from the platelet. Interestingly, two distinct phases of secretion were noted, an initial response that occurs within the first 2 minutes and a more sustained response that persists for up to 15 minutes (Fig. 5B). The amount of FV released from PAR1 and PAR4-stimulated platelets at 2 minutes was essentially identical, however, at 10 and 15 minutes the amount of FV released was markedly different and reflected the differences between PAR1 and PAR4 stimulated platelets previously observed by flow cytometry and ELISA at single time points (Fig. 2). To determine if sustained phosphorylation of MLC downstream of PAR4 could account for the difference in FV secretion between the two agonists we determined the effect of the Rho kinase inhibitor Y-27632 on MLC phosphorylation and FV secretion. Pre-treatment with Y-27632 completely abolished PAR1 or PAR4 mediated MLC phosphorylation at T18 and reduced phosphorylation at S19 (Fig. 5A). Interestingly, at 10 and 15 minutes Y-27632 significantly reduced FV secretion downstream of PAR4 stimulation, close to that observed with PAR1 stimulated platelets, while the PAR1 response was not significantly inhibited by Y-27632 at any time point (Fig. 5B). The amount of FV released at 1 and 2 minutes by either agonist was not significantly reduced (Fig. 5B). In contrast to Y-27632, the pan PKC inhibitor BIM-1 completely abolished PAR induced secretion of FV (Fig. 5C). These data indicate biphasic dense granule secretion downstream of PAR stimulation in platelets with an essential role for PKC and an enhancing role for Rho kinase activity and MLC phosphorylation. In addition, these data suggests that PAR4 is capable of inducing more FV secretion from the platelet due to a stronger Rho signaling component initiated by the receptor.

We also examined the effects of the PKC inhibitor and the Rho kinase inhibitor on PAR induced microparticle production. The Rho kinase inhibitor had no significant effect on PAR1-AP induced microparticle production however, PAR4 mediated microparticle production was
reduced to PAR1-mediated levels in the presence of Y-27632 (Fig. 5D). These data suggest that at least a component of platelet microparticle production is driven by the Rho pathway, in particular Rho-kinase activity and that the difference in PAR1 and PAR4 mediated microparticle production is due to the sustained MLC phosphorylation downstream of PAR4, compared to PAR1. Inhibition of PKC with the pan PKC inhibitor BIM-1 also had opposing effects on PAR1 and PAR4 mediated microparticle production. Downstream of PAR1, BIM-1 caused a 15-fold enhancement of microparticle production, whereas downstream of PAR4, BIM-1 significantly reduced microparticle production (Fig. 5E).

**Prothrombinase complex activity on PAR stimulated platelets** In order to address the functional relevance of the presentation of the platelet procoagulant phenotype we evaluated assembly and activity of the prothrombinase complex on platelets using a modified thrombin generation assay. FXa and FVa associate on the surface of activated platelets to form the prothrombinase complex. Complex formation greatly enhances the rate of FXa-mediated conversion of prothrombin (FII) to thrombin. Purified platelets were reconstituted with the minimal coagulation factors necessary to generate thrombin (FII+FXa+AT), such that FV expression by the platelet was limiting. Plasma was not used in this instance as the purpose of the experiment was to confirm the function of the excess FV release by PAR4-AP stimulated platelets as observed in previous experiments.

Platelets were mixed with FII, FXa, and AT and stimulated with either PAR1-AP or PAR4-AP. Unstimulated platelets generated peak thrombin levels of 5.0±1.5 nM, PAR1-AP stimulated platelets generated 32.0±5.5 nM thrombin, and PAR4-AP stimulated platelets generated 48.7±7.9nM thrombin (Fig. 6 A, B). Lag times for PAR4 stimulated platelets were significantly shorter under these conditions (Fig. 6 A, C). These data indicate that PAR4-AP stimulated platelets support greater prothrombinase complex activity and are capable of generating more thrombin than platelets stimulated with PAR1-AP.
Importantly, control studies indicate that the thrombin generated in this assay was dependent upon platelet activation and expression of the cofactor FV on the platelet surface. No significant amount of thrombin was generated in the absence of FXa (II+AT+activated platelets) or when FII, FXa, and AT were incubated with phospholipid vesicles in place of platelets (Fig. 6A). These control studies indicate that 1) FII conversion to thrombin is minimal on the surface of an activated platelet without FXa and 2) FII is activated poorly by FXa in the absence of platelet stimulation and provision of FV. To confirm that activity observed under these conditions was dependent upon FV, platelets were incubated with increasing concentrations of an anti-FV neutralizing antibody before measuring thrombin generation. Supplementary Figure 2A shows that 10 μg/ml of the antibody reduced thrombin generation by 25% and increasing concentrations reduced the thrombin generation peak close to unstimulated values.

An alternative explanation for the enhanced thrombin generation observed in PAR4-AP stimulated platelet samples, is the simultaneous engagement of PAR1 by newly generated thrombin and PAR4 by the PAR4-AP. Simultaneous engagement of PAR1 and PAR4 on PAR1-AP stimulated samples, on the other hand, would not occur until later in the assay after more thrombin has been generated. To investigate the contribution of PAR activation by newly generated thrombin in the thrombin generation assay, we used PAR-directed thrombin cleavage blocking antibodies to inhibit any feed-forward PAR activation by thrombin generated in the system. The cleavage blocking antibodies generated by Ofosu et al (Ofosu et al., 2008), when used in combination, substantially inhibit peak calcium levels in response to up to 2 nM thrombin and completely inhibit the initial rate of intracellular calcium mobilization in response to 10 nM thrombin (Supplementary Figure 2B). Conveniently, both PAR1-AP and PAR4-AP are still able to activate platelets in the presence of the cleavage blocking antibodies (Supplementary Figure 2B). Platelets were incubated with PAR-1 span IgY and PAR-4 span IgY for 30 min prior to
stimulation with PAR1-AP or PAR4-AP. Thrombin generation was conducted under the same conditions as previously indicated. The combination of PAR-1 span IgY and PAR-4 span IgY did not significantly shift the peak or lagtime compared to control conditions (Supplementary Figure 2C). These data indicate that feed-forward activation of PARs by thrombin generated in the system is not significantly contributing to level or rate of thrombin generated. Instead thrombin generation is largely reflective of the initial phenotypes established by PAR1-AP and PAR4-AP alone.

Finally, we confirmed the role of Rho kinase in the presentation of procoagulant phenotypes downstream of PAR4 by pre-incubating platelets with Y-27632 prior to assessing prothrombinase complex activity with the thrombin generation assay. Incubation of platelets with Y-27632 inhibited PAR4 mediated thrombin generation by 25% but had no significant effect on PAR1 mediated thrombin generation (Fig. 6D). These data confirm the role of sustained MLC phosphorylation in enhancing FV release and provide some insight into the function and implications of this signaling pathway in platelet physiology.

**Discussion**

We have demonstrated that stimulation of PAR4 on platelets results in the presentation of a procoagulant phenotype exceeding that by stimulation of PAR1 and is characterized by the secretion of more platelet-FV from intracellular stores, the binding of more FV to the platelet surface, and the shedding of dramatically more FV-positive microparticles. The greater procoagulant response results in shorter lagtimes in the initiation of thrombin generation in full plasma, and higher peak thrombin concentrations in a thrombin generation assay measuring activity of the prothrombinase complex. The apparent mechanism through which the PAR4-AP response supersedes the PAR1-AP response is, at least partially, through more sustained phosphorylation of MLC at T18 and S19 downstream of PAR4 activation. The Rho kinase inhibitor Y-27632 reduced PAR4-AP mediated FV release, microparticle production, and
thrombin generation, but had little effect on PAR1-AP mediated events. Moreover, we have demonstrated for the first time a Rho-kinase component to microparticle production downstream of PAR activation in platelets.

Although PAR1 and PAR4 would not be individually engaged endogenously, it is important to understand the full capacity of each receptor to mediate events involved in hemostasis and thrombosis, in order to design safe and effective thrombin receptor antagonists. Our data suggest that PAR1 and PAR4-induced procoagulant phenotypes are not redundant and that PAR4 may be playing a unique, heretofore undescribed role in hemostasis. Currently there are no data on when PAR4 is engaged during hemostasis. However, due to its lower affinity for thrombin, PAR4 should be engaged after PAR1 as the concentration of thrombin at the site of injury rises. This order suggests that the impressive procoagulant response mediated by PAR4 may play a role in supporting continuous thrombin generation during clot formation and propagation. The recently discovered role for RhoA in thrombus stability under flow (Pleines et al., 2012) and the prolonged phosphorylation of MLC downstream of PAR4 are consistent with this hypothesis. Studies are currently being designed to directly address the role of PAR4 in clot formation and stability using dynamic as opposed to static assays that incorporate flow, shear stress, and thrombus formation.

PAR4-AP stimulated platelets caused the release of more FV and a higher density of FV on the platelet membrane. These data indicate that PAR4 stimulation induces a greater release reaction of FV-containing intracellular granules than PAR1. We can’t exclude the possibility that FV binding sites on platelets may be differentially regulated by PAR1 and PAR4. However, it seems more likely that the higher density of FV on PAR4 stimulated platelets is the result of higher concentrations of FV released, particularly in light of the lower level of PS exposure observed with PAR4-AP versus PAR1-AP stimulated platelets.
Our group and several others have presented data illustrating a difference in platelet responses mediated by PAR1 and PAR4 despite being coupled to the same G-proteins, Gαq and Gα12/13. The data presented here in conjunction with work done by Grengard et al (2011) suggest that there is a difference in the regulation of PAR1 and PAR4 initiated signaling pathways instead of an entirely unique signaling pathway initiated by one receptor or the other. We can’t comment on precisely what regulatory element is mediating the enhanced response downstream of PAR4 relative to PAR1, however we present data that both Gq and G12/13 pathways are stronger and more sustained downstream of PAR4 stimulation. Grengard et al recently showed that PAR1-mediated Ca²⁺ mobilization, PKC activity, and granule secretion undergo homologous desensitization, which can be rescued by PAR4 stimulation (Falker et al., 2011). Our data indicate that PKC serine substrate phosphorylation was both stronger and more sustained downstream of PAR4. These data agree with a model of PAR signaling presented by Grengard et al suggesting that PAR1 signaling desensitizes while PAR4 signaling persists. Grengard et al also demonstrated that stimulation of PAR4 could rescue PAR1 signaling desensitization in a Rho-kinase dependent manner. This suggests that the persistence of the PAR4 signal involves regulation of MLC phosphorylation. RhoA, which activates Rho-kinase, has also been shown to be involved in the full granule release response from platelets. Similar to PKC activation, PAR4 stimulated MLC phosphorylation at both sites (T18 and S19) was more robust and more sustained than PAR1.

As an additional measure of procoagulant potential we compared PAR1 and PAR4 induced microparticle formation. Recently, Connor et al. demonstrated that Annexin V binding to platelet derived microparticles relies on the type of buffer and the Ca²⁺ concentration used to analyze platelet poor plasma samples (Connor et al., 2010). Since our goal was to compare the efficacy of PAR1 and PAR4 in the production of microparticles we relied on CD41 and CD62P staining to confirm that the microparticles were derived from the platelet membrane. Our study...
represents the first documentation of microparticle formation in response to selective PAR1 or PAR4 activation, and demonstrate that the majority of microparticles generated in response to PAR activation are mediated through PAR4 stimulation thus revealing an unappreciated role for PAR4 in microparticle generation. Given the link between circulating microparticles and disease states, this data also has implications for PAR4 as the proper target choice for a thrombin receptor antagonist. The signaling events leading to microparticle formation are not well understood. Elevations in intracellular Ca\(^{2+}\) and activation of the Ca\(^{2+}\)-dependent protease calpain have been implicated in microparticle formation, in addition to acute decreases in PIP\(_2\) membrane composition but do not appear to account for all pathways (Flaumenhaft et al., 2009, Flaumenhaft et al., 2010, O'Connell et al., 2005). When we preincubated platelets with Y-27632, PAR4-mediated microparticle production was reduced to PAR1-mediated levels. These data indicate, for the first time, that the Rho pathway and MLC phosphorylation contribute to microparticle production from human platelets. This is plausible given that RhoA activity has been implicated in membrane blebbing during apoptosis in other cell lines (Coleman et al., 2001). We also demonstrated that PKC inhibition reduced PAR4-mediated microparticle production but drastically enhanced PAR1-mediated microparticle production. Given the enhancing effect that BIM-1 has on PAR1-mediated calcium mobilization as documented by Poole et al (Harper and Poole, 2011), and the established role of intracellular calcium in microparticle production, the differential effect of the PKC inhibitor should be anticipated. These data indicate that in addition to calcium and acute decreases in PIP\(_2\) membrane composition, Rho-kinase is an important signaling component in microparticle production downstream of PAR stimulation. Moreover, due to the role of Myosin IIa in platelet function, the importance of Rho-kinase signaling suggest that platelet contraction may be a precursor to or may enhance microparticle production.
Finally, we demonstrated that PAR4 induced more prothrombinase complex activity than PAR1 stimulated platelet using a thrombin generation assay consisting of purified components. Vretenbrant et al demonstrate that PAR4 is involved in the initiation of thrombin generation and the development of clot elasticity using thrombin generation assays in plasma and PAR inhibitors (Vretenbrant et al., 2007). Our data illustrating prolonged signaling and more robust FV secretion point to a role for PAR4 in continuous thrombin generation during clot development which would be consistent with a role for PAR4 in regulating clot dynamics. We can’t comment on which receptor would be involved in initiating thrombin generation as we did not use antagonists to explore their relative roles, due to a lack of a potent small molecule antagonist for PAR4. Vretenbrant et al also demonstrated a more robust response for PAR4 versus PAR1 when measuring fibrinogen binding, paralleling our observations of more FV release and more microparticle production.

Our data establish that PAR4 stimulation is more efficacious than PAR1 stimulation in the induction of procoagulant phenotypes on platelets. Recently, clinical trials of the PAR1 antagonist, Vorapaxar, were curtailed due to a high number of intracranial hemorrhagic events (Tricoci et al., 2011). Given the high affinity of PAR1 for thrombin, and its engagement by newly generated thrombin early in the process of hemostasis, its inhibition may suppress the platelet’s ability to respond to newly generated thrombin and amplify thrombin generation, a critical process in hemostasis. Therefore, unwanted bleeding side effects are an anticipated complication of therapy targeting PAR1. Since thrombin has a lower affinity for PAR4 than PAR1, PAR4 would be engaged only after higher concentrations of thrombin are reached at a vascular site of injury. Specific inhibition of PAR4 would leave PAR1 signaling intact, allowing platelets to respond to low concentrations of thrombin, perhaps facilitating the initial amplification of thrombin generation, and preserving hemostasis.
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Conducted experiments: Duvernay, Young

Contributed new reagents or analytical tools:

Performed data analysis: Duvernay, Young

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References


the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 101: 841-3.


Footnotes

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**Figure Legends**

**Figure 1. Thrombin generation supported by platelet stimulation with PAR agonists** Gel filtered platelets were stimulated with increasing concentrations of PAR1-AP and PAR4-AP for 5 min before the addition of an equal volume of human plasma. Thrombin generation is initiated as described in materials and methods. A. Representative tracings of two independent experiments. B. Decreases in lagtime as a function of PAR1-AP and PAR4-AP concentration. Depicted is the average of two separate experiments with SEM. A non-paired t-test comparing the lowest values for each agonist demonstrated statistically significant differences. *p-value = 0.0054

**Figure 2. Expression of FV on platelets in response to PAR activation.** A. Immunocytochemical (ICC) analysis of FV surface expression in response to PAR stimulation. Intact, unpermeabilized platelets were stimulated with either 20 μM PAR1-AP or 200 μM PAR4-AP or vehicle control for 15 min and stained with 10 μg/ml anti-factor V sheep polyclonal antibody and Alexa488 conjugated secondary. Scale bar = 10 μm. Inset = 3x zoom. B. Flow cytometry analysis of FV surface expression on the platelet surface in response to PAR1-AP and PAR4-AP. Shown is the average with SEM, (n=4). A paired t-test comparing maximum values for PAR1-AP and PAR4-AP from each individual non-linear regression curve (sigmoidal dose response) indicated statistically significant differences. p-value = 0.021. Raw max MFI values±SEM: unstimulated 4.6±0.6, PAR1-AP 18.2±3.9, PAR4-AP 27.5±5.8. A paired t-test comparing PAR1-AP and PAR4-AP MFI values indicated statistical significance, p-value = 0.018. C. Histogram representation of FV staining on platelets stimulated with 20 μM PAR1-AP or 200 μM PAR4-AP. Grey unfilled, IgG control (overlaps with unstimulated). Grey filled, unstimulated. Grey unfilled, PAR1-AP and PAR4-AP (as indicated with arrows). D. Percent platelets positive for FV staining. Platelets were stimulated with 20 μM PAR1-AP or 200 μM PAR4-AP. E. Annexin V binding in response to stimulation with 20 μM PAR1-AP, 200 μM PAR4-AP, or
vehicle control. Shown are representative histograms from three repeats. Grey lines, unstimulated platelets. Black lines, stimulated platelets. F. FV concentrations in the supernatants of platelets stimulated with increasing doses of PAR1-AP and PAR4-AP as determined by sandwich ELISA. Shown are the averages with SEM, (n=3). A paired t-test comparing maximum values for PAR1-AP and PAR4-AP from each individual non-linear regression curve (sigmoidal dose response) generated with PAR1-AP and PAR4-AP indicated statistically significant differences, p-value=0.0062. G. P-selectin surface expression on platelets stimulated with increasing doses of PAR1-AP and PAR4-AP as determined by flow cytometry. Shown are the averages with SEM, (n=3). A paired t-test comparing maximum values for PAR1-AP and PAR4-AP from each individual non-linear regression curve (sigmoidal dose response) generated with PAR1-AP (max, 10.1±0.8 fold) and PAR4-AP (max, 12.7±0.8 fold) indicated statistically significant differences. p-value=0.0049. Maximal MFI values: unstim, 24.7±4.7; PAR1-AP, 243.4±28; PAR4-AP, 307.2±38.6.

**Figure 3. Platelet microparticle production in response to PAR activation.** A. Representative controls for gating of microparticles against whole platelets. Left panel, forward and side scatter dot plots of 1 μm beads. Right panel, forward and side scatter dot plots of platelet sample stimulated with 20 μM PAR1. B. Forward and side scatter dot plots of microparticle enriched samples prepared from unstimulated platelets and platelets stimulated with 20 μM PAR1-AP and 200 μM PAR4-AP illustrating the depletion of whole platelets from microparticle enriched samples. C. Microparticle production in response to increasing concentrations of PAR1-AP and PAR4-AP. Shown is the average fold increase in CD62P (P-selectin) and CD41 (integrin α-IIb) positive particles under 1μm. n=2. A t-test comparing maximal values from each dose response curve (PAR1-AP, 4.3±0.3 fold; PAR4-AP, 10.3±0.0 fold) indicates statistically significant differences. p-value=0.0142.
Figure 4. Regulation of Myosin Light Chain Phosphorylation by PAR stimulated platelets.
A. PAR1 and PAR4 mediated PKC (s) substrate phosphorylation and myosin light chain phosphorylation at S19 (pMLC S19) and T18 (pMLC T18). Platelets were stimulated with 20 μM PAR1-AP and 200 μM PAR4-AP for the indicated time periods. Reactions were stopped and samples prepped for western blot analysis as indicated in methods section. Top panel, PKC (s)-substrate; middle panels, pMLC S19 and T18; lower panel p44/p42 ERK loading controls. B. Quantification of pMLC S19 and T18. Data were collected from three independent experiments and analyzed with Image J. Data are expressed as fold increase relative to the density of bands in unstimulated lanes. Asterisks indicate statistically significant differences between PAR1-AP and PAR4-AP stimulated MLC phosphorylation at the indicated time point using a paired t-test. p-values (S19, 1 min=0.0173, 2 min=0.008, 5 min=0.0133, 10 min=0.0222, 15 min=0.017, 30 min=0.0038; T18, 10 min, 0.0049, 15 min=0.0471, 30 min=0.0498)

Figure 5. The effect of PKC inhibition and Rho kinase inhibition on platelet secretion and microparticle production. A. The effect of Y-27632 on S19 and T18 pMLC. Platelets were preincubated with 10 μM Y-27632 or H2O for 10 minutes prior to stimulation with 20 μM PAR1-AP or 200 μM PAR4-AP for the indicated time periods. B. The effect of Rho-kinase inhibitor on PAR-mediated FV release. Platelets were pre-incubated with 10 μM Y-27632 for 10 minutes before stimulation with 20 μM PAR1-AP or 200 μM PAR4-AP for the indicated time periods and supernatants were prepared for sandwich ELISA as indicated in methods. Shown are the averages and SEMs (n=3). Paired t-test *PAR4-AP vs. PAR4-AP + Y-27632, #PAR4-AP vs. PAR1-AP, **PAR4-AP vs. PAR1-AP + Y-27632. 1 min *p-value=0.0327; 2 min *p-value=0.017, **p-value=0.0117; 5 min *p-value=0.0123, **p-value=0.0463; 10 min *p-value=0.048, #p-value=0.0145, **p-value=0.0072; 15 min *p-value=0.0002, #p-value=0.0203, **p-value=0.0031. C. The effect of PKC inhibitor on PAR mediated FV secretion. Platelets were preincubated with 10 μM BIM-1 or DMSO for 10 min prior to stimulation with 20 μM PAR1-AP or 200 μM PAR4-AP.
for 15 minutes. Shown are the averages and SEMs (n=4). Paired t-test, PAR1-AP vs. PAR1-AP + BIM-1 *p-value= 0.0312, PAR4-AP vs. PAR4-AP + BIM-1 **p-value=0.016. Raw values for FV concentration: unstimulated, 22.7±4.2 ng/ml; unstimulated+BIM-1, 21.4±3.8 ng/ml; PAR1-AP, 68.9±8.1 ng/ml; PAR1-AP+BIM-1, 34.7±12.5 ng/ml; PAR4-AP, 93.3±7.8 ng/ml; PAR4-AP+BIM-1, 30.1±6.3. D. The effect of Rho kinase inhibitor on PAR mediated microparticle production. Platelets were preincubated with 10 μM Y-27632 for 10 minutes prior to stimulation with 20 μM PAR1-AP or 200 μM PAR4-AP for 30 minutes. Microparticle enriched supernatants were prepared as indicated in methods. Shown are the averages with SEM, (n=3). A paired t-test comparing PAR4-AP to PAR4-AP+Y-27632 demonstrated statistically significant differences. *p-value=0.0024. A paired t-test comparing PAR1-AP to PAR1-AP+Y-27632 did not demonstrate statistically significant differences, p-value=0.117. A paired t-test comparing PAR1-AP to PAR4-AP demonstrated statistically significant differences, p-value=0.002. Raw values for microparticle production (dual positive particles): unstimulated, 79.5±18.4; unstimulated+Y-27632 77.6±15.6; PAR1-AP, 164.9±31.3; PAR1-AP+Y-27632, 269.4±57.8; PAR4-AP, 769.0±151.2; PAR4-AP+Y27632, 216.8±29.9. E, The effect of PKC inhibitor on PAR-mediated microparticle production. Platelets were preincubated with 10 μM BIM-1 for 10 min prior to stimulation with 20 μM PAR1-AP or 200 μM PAR4-AP for 30 minutes. Microparticle enriched platelet supernatants were prepared as indicated in methods. Shown are the averages with SEM, (n=4). Raw values for microparticle production (dual positive particles): unstimulated, 120.1±15.5; unstimulated+BIM-1, 76.1±8.6; PAR1-AP, 300.6±71.9; PAR1-AP+BIM-1, 5092.2±1662.1; PAR4-AP, 821.5±332.5; PAR4-AP+BIM-1, 139.3±33.6.

Figure 6. Assembly of the prothrombinase complex on PAR stimulated platelets

A. Thrombin generation on platelets simulated with 20 μM PAR1-AP or 200 μM PAR4-AP. Stimulated platelets are mixed with coagulation factors: FII (1.4 μM), FXa (100 nM), and AT (100 μg/ml). Shown is a representative tracing from three separate experiments. B. Peak
thrombin generation, shown is the average with SEM (n=3). A paired t-test comparing peak thrombin generation in response to PAR1-AP and PAR4-AP indicated statistically significant differences. *p=0.0129. C. Lagtime, time it takes to reach 10 nM thrombin, shown is the average with SEM (n=3). A paired t-test comparing lag times for PAR1-AP and PAR4-AP stimulated platelets indicated statistically significant differences. *p=0.0206. D. Effect of Rho kinase inhibitor on PAR induced thrombin generation. Platelet were preincubated with 10 μM Y-27632 or H2O vehicle control for 10 min prior to stimulation with 20 μM PAR1 or 200 μM PAR4-AP. Thrombin generation was conducted as outlined above and in methods section. Shown is the average with SEM (n=3). A paired t-test comparing peak thrombin levels for PAR4-AP and PAR4-AP+Y-27632 indicated statistically significant differences. *p=0.0021.
Figure 1.

A. Thrombin (nM) vs. Time (min) for different concentrations of PAR1-AP and PAR4-AP.

B. Lag time (min) vs. Log [PAR1-AP] M and Log [PAR4-AP] M.
Figure 2

A. unstim | PAR1-AP | PAR4-AP

B. Fold increase FV (MFI)

C. Number

D. % positive FV

E. Number

F. FV (ng/ml)

G. Fold increase (CD62P MFI)
Figure 3.

A.

B.

Unstim

PAR1-AP

PAR4-AP

C.

Fold increase (relative to unstimulated)

Fold increase (relative to unstimulated)

log [PAR1-AP] M

log [PAR4-AP] M
Figure 4.

A. Phospho-serine PKC substrate

B. Fold increase pMLC S19 (relative to unstim) vs. time (min)

Fold increase pMLC T18 (relative to unstim) vs. time (min)
Figure 5.

A. Western blots showing the phosphorylation of pMLC T18, pMLC S19, and ERK p44/p42 in response to PAR1-AP and PAR4-AP stimulation with Y-27632 inhibition.

B. Time course of FV production in response to PAR1-AP and PAR4-AP stimulation with Y-27632 inhibition.

C. Fold increase in FV release in control and BIM-1 treated samples.

D. Fold increase in MP production in control and Y-27632 treated samples.

E. Fold increase in MP production in control and BIM-1 treated samples.