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Trypsin causes platelet activation independently of known protease-activated receptors

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Abstract

To identify a physiological agonist of PAR3, we used PAR4 null murine platelets, which were known to express only PAR3. In this study, we tested several proteases and found that trypsin, but not heat-inactivated trypsin, activated PAR4 null murine platelets. Even at high concentrations, trypsin caused shape change without increasing intracellular calcium levels in PAR4 null murine platelets. Consistent with this result, the G_q inhibitor YM-254890 had no effect on trypsin-induced shape change. However, trypsin-induced platelet shape change was abolished by either p160ROCK inhibitor, Y27632 or H1152. Furthermore, trypsin caused phosphorylation of myosin light chain (Thr18), but not Akt or Erk. Surprisingly, trypsin caused a similar shape change in PAR4-desensitized PAR3 null murine platelets as in PAR4 null murine platelets, indicating that trypsin did not activate PAR3 to cause shape change. More interestingly, the Src family kinase (SFK) inhibitor PP2 abolished trypsin-induced, but not AYPGKF-induced, shape change. Hence, trypsin activated a novel signaling pathway through RhoA/p160ROCK and was regulated by SFKs. In conclusion, our study demonstrates a novel protease signaling pathway in platelets that is independent of PARs. This protease-induced novel signaling pathway regulates platelet shape change through SFKs and p160ROCK.

Keywords

Trypsin; Protease-activated receptor; Platelet shape change

Introduction

Platelets, which are derived from megakaryocytes, play a central role in hemostatic processes including detection of vascular lesions, adherence at injury sites, recruitment of additional platelets and formation of a haemostatic plug (1, 2). Thrombin is one of most potent agonists and mediates platelet function primarily through a family of G protein-

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Conflict of interest:

The authors declare that there is no conflict of interest.

coupled protease-activated receptors (PARs). To date, four PARs have been discovered. PAR1 and PAR4 are activated by thrombin and play a major role in activation of human platelets (3). PAR3 acts as a co-receptor and allows murine platelets to be activated through PAR4 by lower concentrations of thrombin (4). Different from these PARs, PAR2, which is not expressed in platelets, is activated by trypsin, mast cell tryptase and coagulation factors VIIa and Xa, but not by thrombin (5–7).

Agonist stimulation of PARs induces activation of $G_{12/13}$ and G_q pathways. Whereas thrombin activates Rho A and p160ROCK kinases through $G_{12/13}$ pathways, stimulation of G_q pathway results in the activation of phospholipase $PLC\beta_2$ ($PLC\beta_2$). Activated $PLC\beta_2$ hydrolyzes membrane bound phosphatidylinositol-4, 5-trisphosphate (PIP2) to inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes calcium and DAG activates protein kinase C (PKC). Platelets, thus activated by thrombin, undergo shape change, aggregate and secrete their dense granular contents.

Among these four PARs, murine PAR3 only functions as a co-receptor for PAR4 (8). To date, PAR3's physiological agonist has not been identified. In heterologous systems, human PAR3 activation by thrombin was shown to result in phosphoinositide hydrolysis which was significantly enhanced by co-transfection with $G\alpha_{16}$ (4). However, thrombin failed to activate PAR3 and cause signaling events in PAR4 null murine platelets (9–11). PAR3 was also cleaved by elastase or cathepsin G in murine platelets. Elastase or cathepsin G can disrupt PAR3's co-receptor function in heterologous systems without activating PAR3 (12). Whether or not these proteases would interfere with the physiological stimulation of PAR3 by other proteases is not known.

The studies on PARs showed that in addition to major agonists (PAR1 and PAR4 agonist thrombin or PAR2 agonist tryptase), PARs also could be activated by other proteases. For example, TF-FVIIa-FXa complex could activate PAR1 and PAR2 (6). PAR4 was also found to be activated by neutrophil-secreted cathepsin G (13) and plasmin (14, 15). Among the couple of proteases tested, Factor Xa, Factor VIIa, cathepsin G, plasmin, tissue plasminogen activator, chymotrypsin, and elastase, caused little or no activation of PAR3 in heterologous systems (4).

In this study, we found that trypsin causes platelet shape change in PAR4 null platelets. Our investigation on the mechanism of trypsin-induced PAR4-independent platelet activation showed that this platelet shape change was regulated by SFKs that is RhoA-dependent but not G_q -calcium-dependent. Our results also show that trypsin-induced shape change was independent of PAR3 or other known protease-activated receptors, which would lead us to find a novel pathway of protease-mediated platelet activation.

Methods and Materials

Animals and Materials

Heterozygous PAR3 mice were purchased from MMRRC (Mutant Mouse Regional Resource Centers, North Carolina). The mice were used for physiologic measurements by using the protocol approved by the Institutional Animal Care and Use Committee of Temple

University. The animals used for the experiment were subject to very minimal distress and killed with an overdose of pentobarbital before decapitation at the end of experimentation. Hexapeptide SFLLRN was obtained from New England Peptide (Gardner, MA, USA). AYPGKF was purchased from GenScript Corp. (Piscataway, NJ, USA). Trypsin from porcine pancreas was purchased from both Sigma Chemical Co. (St. Louis, MO, USA) and Princeton Separations, INC. (Adelphia, NJ, USA). FVIIa, Plasmin, FXa were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Plasma kallikrein and FXIIa were purchased from Enzyme Research Laboratories (South Bend, IN, USA). FXIIa, elastase and α -chymotrypsin were obtained from Dr. Peter Walsh (Temple University, PA, USA). BMS-200261 was obtained as a generous gift from Dr. Steven Seiler (Bristol-Myers Squibb). Luciferin-luciferase reagent was purchased from Chrono-Log. YM-254890 was a gift from Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki, Japan). PP2, PP3 and ROCK inhibitor H1152 were purchased from Biomol (Plymouth Meeting, PA, USA). ROCK inhibitor Y-27632 was from Calbiochem (San Diego, CA, USA). Fura-2 acetoxymethyl ester (Fura-2 AM) was from Molecular Probes (Eugene, OR, USA). The anti-mouse CD41-FITC and IgG isotype control antibodies were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). The anti-mouse GPVI-FITC, GPIIb α -FITC, JON/A-PE and IgG isotype control antibodies were obtained from Emfret Analytics (Wuerzburg, Germany). Phospho-44/42 MAPK (Erk1/2), Phospho-Akt (Ser473), total Erk, Phospho-Src Family (Tyr416), Phospho-MLC (Thr18/Ser19) were obtained from Cell Signaling (Beverly, MA, USA) and phospho MLC (Thr18), horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (IgG) were from Santa Cruz (Santa Cruz, CA, USA). Unless specifically mentioned, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of washed murine platelets

Murine blood was collected from anesthetized mice by cardiac puncture into syringes containing 3.8% sodium citrate as anticoagulant and centrifuged at 100g for 10 minutes to get the platelet rich plasma (PRP). Prostaglandin E1 (PGE1, 1 μ M) was added to PRP and PRP was centrifuged at 400g for 10 minutes. The platelet pellet was resuspended in Tyrode's buffer containing 0.1 unit/mL apyrase.

Platelet aggregation

The platelet count was adjusted to 1.5×10^8 cells/mL and aggregation of 0.25 mL washed platelets was analyzed using a P.I.C.A. Lumi-aggregometer (Chrono-log, Havertown, PA). Aggregation was measured by light transmission under stirring conditions (900 rpm) at 37°C. In experiments where inhibitors were used, the platelet sample was incubated with the inhibitors for 10 minutes at 37°C prior to the addition of agonists. Indomethacin (1 μ M) was added before adding agonists. The aggregation data are represented in the form of actual tracings. For platelet desensitization, washed platelets were incubated with 500 μ M AYPGKF at 37°C for one hour before adding agonists.

Cytosolic Calcium Measurements

Intracellular calcium mobilization was measured as described previously (16). Briefly, murine blood, mixed with 4 volumes of piperazine-1, 4-bis-2-ethanesulfonic acid-buffered

Tyrode's solution (pH 6.5) containing 500 μ M ethyleneglycoltetraacetic acid (EGTA), 10 μ M indomethacin, and 1 μ M PGE1, was centrifuged at 100g for 15 minutes at room temperature. The PRP was diluted with the above buffer, and centrifuged at 800g for 15 minutes at room temperature to isolate the pellet. The platelet pellet was resuspended in the above buffer in the presence of 5 μ M FURA-2 AM for 45 minutes at room temperature. The platelets were centrifuged at 800g for 10 minutes at room temperature and finally resuspended in Tyrode's buffer containing 0.1 unit/mL apyrase. 0.5 mL sample was analyzed and fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz et al (17), where F_{min} and F_{max} were determined with each respective platelets preparation.

Chromogenic assay for testing proteolytic activity of trypsin

Trypsin was incubated with or without BMS200261 or soybean trypsin inhibitor for 5 minutes at 37 °C and the volume was adjusted to 50 μ l by using Tyrode's buffer. 50 μ l of the chromogenic substrate S-2222 (final concentration 0.4 mM) was added and incubated for 3 minutes at 37 °C. 50 μ l of 20% acetic acid was added to stop the reaction and the proteolytic activity of trypsin was determined by recording the increase in absorbance at 405 nm.

Western blotting

Platelets were stimulated with agonists in the presence or absence of inhibitors for the appropriate time, and the reaction was stopped by the addition of 3 $\times$ SDS Laemmli's buffer. Platelet samples were boiled for 10 minutes and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked with 5% non-fat milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20) for 60 minutes at room temperature, and probed overnight at 4°C with the primary antibody (1:1000 dilution in TBST with 5% BSA) with gentle agitation. After three washes for 5 minutes each with TBST, the membranes were incubated with an HRP-conjugated secondary antibody (1:5000 dilution in TBST with 5% BSA) for 30 minutes at room temperature. After additional washing steps, membranes were then incubated with chemiluminescent HRP substrate (Tropix, Bedford, MA) for 5 minutes at room temperature and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH, Japan).

Flow cytometry

Washed murine platelets were analyzed to measure surface expression of GPVI receptor by anti-mouse GPVI-FITC antibody, expression of GPIIb by anti-mouse CD41-FITC antibody, surface expression of GPIb α by anti-mouse GPIb α -FITC antibody and the level of activated $\alpha_{IIb}\beta_3$ receptors on murine platelets by JON/A-PE antibody. Washed murine platelet aliquots (20 μ l) was incubated with agonists and 5 μ l antibody or non-immune IgG isotype control for 15 minutes at room temperature in the dark in non-stirring condition. Reaction was stopped by adding 400 μ l PBS. The sample was analyzed within 30 minutes by using on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). A total of 10000 platelet

events were acquired per sample, and the mean fluorescence intensity (MFI) of positive platelets was analyzed.

Results

Trypsin causes shape change in PAR4 null murine platelets

Among the four known PARs, the physiological agonist of PAR3 has not been identified to date. To identify a physiological agonist to PAR3, we used PAR4 null murine platelets, which are known to express only PAR3 (18). We tested several proteases (FXa, FVIIa, FXIIf, Elastase, Plasma kallikrein, α -chymotrypsin, Plasmin, Thrombin, Trypsin) and none of the proteases (25 nM) except trypsin activated PAR4 null murine platelets (19). Trypsin was found to activate PAR4 null murine platelets in a concentration-dependent manner (Fig. 1A). Results showed that trypsin started causing platelet shape change from 500 nM to 50 μ M. However, no α IIb β 3 activation was detected at 50 μ M trypsin (Fig. 1B), suggesting that trypsin failed to cause fibrinogen receptor activation in PAR4 null murine platelets even at high concentrations. When we inactivated trypsin by heating at 95°C for 30 min or inhibited trypsin with soybean trypsin inhibitor (Fig. 1C), trypsin lost its ability to activate PAR4 null murine platelet (Fig. 1D), indicating that the enzyme activity of trypsin is required for causing platelet shape change. The results were confirmed by using trypsin from a different source.

Trypsin-induced PAR4 null murine platelet shape change is independent of PAR3

Since only PAR3 was expressed in PAR4 null murine platelets, we hypothesized that trypsin activated PAR4 null murine platelets through PAR3. To investigate this possibility, PAR3 null murine platelets were used. PAR4 was normally expressed in PAR3 null murine platelets (8, 20), which also could be activated by trypsin. We desensitized PAR4 receptors in both wild type and PAR3 null murine platelets by incubating platelets with 500 μ M AYPGKF at 37°C for 1 hour. The successful desensitization was confirmed by failure of platelet response to either AYPGKF or thrombin (Fig 2). Other platelet receptors, such as ADP receptors, responded normally after desensitization (data not shown). Our data showed that PAR4 desensitized wild type or PAR3 null murine platelets still could be activated by trypsin (Fig 2), which was similar to trypsin-induced shape change in PAR4 null murine platelets. All these data indicated that PAR3 is not required for trypsin-induced shape change in PAR4 null murine platelets.

Trypsin causes calcium-independent shape change in PAR4 null murine platelets

In platelets, agonists-induced shape change occurs through both G_q – mediated calcium-dependent and $G_{12/13}$ -mediated calcium-independent pathways (21). To investigate the mechanism of trypsin-induced shape change in PAR4 null murine platelets, we tested whether trypsin could cause calcium mobilization from intracellular stores. Our data shows that, even at high concentrations, trypsin failed to cause calcium mobilization in PAR4 null murine platelets, indicating that trypsin-induced shape change was not through G_q – calcium dependent pathway (Fig. 3A). To further confirm this result, the G_q inhibitor YM-254890 was used. YM-254890 completely inhibited 2MeSADP-induced platelet calcium mobilization and aggregation (data not shown), but it had no effect on trypsin-induced shape

change in PAR4 null murine platelets (Fig. 3B). On the other hand, when PAR4 null murine platelets treated with the p160ROCK inhibitor Y27632 (20 μ M) or H1152 (10 μ M), trypsin-induced platelet shape change was abolished (Fig. 3C), suggesting that trypsin-induced PAR4 null murine platelet shape change was mediated by RhoA – p160ROCK pathway, but not by G_q-calcium pathway.

Trypsin causes myosin light chain (MLC) phosphorylation

Since trypsin caused platelet shape change in PAR4 null murine platelets, we further investigated trypsin-induced downstream signaling events in platelets. Our results show that trypsin did not cause platelet Akt and Erk phosphorylation, however, it caused MLC (Thr)18 phosphorylation (Fig 4A and 4B). Previous study from our group showed that G_{12/13}- Rho A/p160ROCK signaling was essential for myosin light chain (Thr18) phosphorylation (22). Our data was consistent with this finding and further confirmed that trypsin-mediated platelet activation is through Rho A- p160ROCK pathway.

Trypsin-induced PAR4 null murine platelet shape change is abolished by Src family kinase inhibitor

Src family kinases (SFKs) are non-receptor tyrosine kinases that mediate both inside-out signaling (23) and integrin-dependent outside-in signaling in platelets (24). It is known that AYPGKF-induced shape change (a PAR4 agonist) is not inhibited by the SFK inhibitor PP2. Interestingly, our results showed that PP2, but not the inactive analog PP3, inhibited trypsin-induced PAR4 null murine platelet shape change (Fig. 5A). When SFKs were inhibited by PP2, 1 μ M of trypsin-induced MLC phosphorylation was also dramatically but not completely inhibited (Fig. 5B). These data suggest that trypsin activates a novel signaling pathway, which regulates platelet shape change through both SFK dependent and independent pathways.

PAR1 antagonist BMS200261 inhibits trypsin-induced PAR4-null murine platelet shape change

Although murine platelets do not express PAR1 (9), as shown in Fig. 5C and 5D, the PAR1 antagonist BMS200261 (40 μ M) inhibited trypsin-induced PAR4 null murine platelet shape change in a concentration-dependent manner without inhibiting the enzyme activity of trypsin. However, BMS200261 had no effect on PAR4 agonist AYPGKF-induced murine platelet activation (data not shown). In addition, BMS200261 alone had no effect on VASP phosphorylation (data not shown), suggesting that BMS200261 did not affect platelet reaction by increasing cAMP levels. It is possible that BMS200261 caused inhibition of trypsin-induced shape change because it non-specifically inhibits other receptors, which were activated by trypsin.

The effect of trypsin on platelet surface glycoproteins

Since trypsin is an active serine protease of low specificity that could cleave most proteins at the carboxyl side of lysine or arginine, we tested its effect on the expression level of three major platelet surface glycoproteins (Fig. 6). Our data showed that trypsin did not change

the expression of GPVI and GPIIb on platelet surface. However, 50 μ M of trypsin decreased GPIb expression level, which is consistent with previous study (25).

Discussion

The original aim of this study was to find the possible PAR3 physiological agonist by using PAR4 null murine platelets. Among tested proteases, we found that trypsin caused PAR4 null murine platelet shape change. It is possible that the protease could also activate the receptor through non-proteolytic mechanisms such as direct binding to the receptor. However, our data indicated that trypsin induced this shape change through proteolytic cleavage of certain receptors, as heat-inactivated trypsin was without effect. Furthermore, as trypsin caused shape change in PAR4-desensitized PAR3 null platelets, we ruled out PAR3 as the receptor activated by trypsin. In addition, the intact GPVI and GPIIb expression on the platelet surface suggested that trypsin-induced shape change is not because of the non specifically cleavage of surface glycoproteins. Although trypsin was showed to cleave GPIb (25), there is no evidence to show that cleaving GPIb could cause platelet shape change. Recent study also showed that thrombin could induce platelet activation in dependent of PAR1 and PAR4 and GPIIb α . PAR1 and PAR4 inactivation and GPIIb α proteolysis only minimally affected thrombin-induced platelet actin polymerization and cytoskeleton reorganization (26). It is possible that trypsin cleaved some other receptor instead of PAR3 to cause shape change in PAR4 null murine platelets.

Trypsin is a serine protease and is known to activate PAR2 (5, 27) as well as PAR1 (28) and PAR4 (29). Soslaw et al. showed that trypsin had a similar affinity/catalytic site as thrombin and hence could activate PAR4 in platelets (30). In their study, low concentrations (0.5 nM) of trypsin-induced platelet activation was completely abolished when PAR4 was desensitized. We found that when we increased the trypsin concentration, it caused platelet shape change even in the absence of PAR4. It is possible that low concentrations of trypsin are sufficient to activate PAR4 and higher concentrations of trypsin could activate other receptors in addition to PAR4. Since trypsin and thrombin have a similar affinity/catalytic site, we speculate that the unknown receptor which trypsin activates has a similar sequence as thrombin receptors (PARs).

Trypsin was also found to activate matrix metalloproteinases (MMPs). MMPs are extensively studied in cancer. Trypsin activated a number of procollagenases, including MMP-1, MMP-8, MMP-13 (31), MMP-7 (32), MMP-2 and MMP-9 (33). Trypsin might initiate proteolytic cascades further enhanced by MMPs. In platelets, studies showed that MMP-1, 2, 3 and 14 were present in platelets (34–37). Previous studies showed that MMP-2 could be secreted upon platelet activation (37, 38). Recent studies showed that exposure of human platelets to collagen could cause MMP-1 activation on the platelet surface, leading to cleavage and activation of PAR1 independent of thrombin. MMP-1 cleaved PAR1 at a different site as thrombin cleaved, which strongly stimulated G_{12/13} – Rho – dependent pathways (39). This activation was inhibited by PAR1 antagonist (39). Interestingly, the PAR1 antagonist BMS200261 completely abolished trypsin-caused shape change, although PAR1 is not expressed in murine platelets.

Murine platelets express MMP-2 but not MMP-1. MMP-2 has been shown to contribute to platelet activation by acting on an unidentified receptor on platelets that activates signaling pathways similar to PARs (40). Deficiency of MMP-2 could protect platelets from arterial thrombosis (41). In addition, MMP-2 was shown to be activated by trypsin (42). Hence, it is possible that in platelets, trypsin will also activate MMP-2 and, subsequently, activate a receptor having a similar sequence as PAR1 and cause Rho A – p160ROCK dependent shape change.

It has been shown that the membrane Na-K ATPase was involved in trypsin-induced human platelet activation (43) and, in other cell systems, Na-K ATPases can activate Rho and enhance MLC phosphorylation (44). The study showed that blockade of ATPase with ouabain blocked trypsin-induced human platelet aggregation without blocking platelet shape change (43). Consistent with this result, we also find that ouabain could not inhibit trypsin-induced shape change in PAR4 null murine platelets (data not shown), suggesting that Na-K ATPase is not important in trypsin-induced PAR4 null murine platelet shape change.

One of the interesting findings in our study is that trypsin-induced PAR4 null murine platelet shape change was inhibited by the SFK inhibitor PP2. As PAR-mediated G12/13-dependent shape change was not inhibited by the pan-SFK inhibitor PP2 (45, 46), suggesting that trypsin-induced shape change might be regulated by different pathways than PARs. Over the years, studies have shown crosstalk between integrins, SFKs and Rho-family GTPase at adhesion signaling in bone remodeling, cell migration, mechanotransduction and angiogenesis (47). In platelets, Src was activated following fibrinogen binding to integrin and there was evidence showing that Rho A activity was required to maintain stable interactions of integrin α IIB β 3 with the ECM under conditions of shear stress; however, the role of the integrin – α IIB β 3 – Src complex in regulating RhoA activity was not clear (48). Here our study shows that trypsin-induced shape change is inhibited by both SFK and RhoA – p160Rock inhibitors. Furthermore, at high concentration of trypsin, PP2 inhibited most but not all of MLC phosphorylation suggesting that trypsin-induced shape change is through both SFKs dependent and independent pathways.

In conclusion, our study shows a novel protease-induced platelet activation mechanism. Trypsin caused RhoA – p160ROCK dependent platelet shape change, which is independent of known protease-activated receptors, and this activation is regulated by SFKs. Although the concentration of trypsin we used in this study is much higher than in physiological conditions, this study still could be used as a method to identify a new platelet receptor and its physiological agonist ex vivo which would enhance our knowledge of the platelet physiology.

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Figure 1. Trypsin causes PAR4 null murine platelet shape change

(A) Washed PAR4 null murine platelets were stimulated with trypsin (0.5–50 μ M) or 2MeSADP (100 nM) under stirring condition at 37 °C. (B) PAR4 null murine platelets were stimulated with trypsin or convulxin and the level of activated α IIB β 3 receptors was measured by JON/A-PE antibody binding. (C, D) Trypsin activity was inhibited by soybean trypsin inhibitor or heat inactivation at 95 °C for 30 minutes and the activity of trypsin was determined by reading the 405 nm absorbance. PAR4 null murine platelets were stimulated with heat-inactivated or soybean trypsin inhibitor treated trypsin. Platelet aggregation was

determined by measuring light transmittance. Tracings are representative of at least three separate experiments.

Figure 2. Trypsin – induced PAR4 null murine platelet shape change is independent of PAR3
 Washed wild-type and PAR3 null murine platelets were incubated with 500 μ M AYPGKF at 37 °C for 1 hour under non-stirring condition and then stimulated with AYPGKF (500 μ M), thrombin (1 U/ml) or trypsin (5 μ M). Tracings are representative of at least three separate experiments.

Figure 3. Trypsin causes G_{12/13} – Rho A calcium independent shape change in PAR4 null murine platelets

(A) Calcium mobilization was tested in washed PAR4 null murine platelets stimulated with 2MeSADP (100 nM), AYPGKF (500 μ M) or trypsin (1–50 μ M). (B, C) Washed PAR4 null murine platelets were stimulated with trypsin in the absence or presence of G_q inhibitor YM-254890 (100 nM), p160 ROCK inhibitor Y27632 (20 μ M) or H1152 (10 μ M). Platelet aggregation was determined by measuring light transmittance. Tracings are representative of at least three separate experiments.

Figure 4. Trypsin causes MLC18 but not Akt and Erk Phosphorylation in PAR4 null murine platelets

Washed PAR4 null murine platelets were stimulated with 1 μ M of trypsin or 100 nM of 2MeSADP under stirring condition at 37 °C. Akt, Erk and MLC 18 phosphorylation were measured by western blot. The data are representative of experiments done using platelets from at least three different experiments.

C **D**

Figure 5. Trypsin –induced PAR4 null murine platelet shape change is inhibited by SFK inhibitor PP2 or PAR1 antagonist BMS200261

Washed PAR4 null murine platelets were stimulated with trypsin in the absence or presence of (A) SFK inhibitor PP2 (10 μ M), negative control of SFK inhibitor PP3 (10 μ M), or (C) PAR1 antagonist BMS200261 (0–40 μ M) under stirring condition at 37 °C. (B) 1 μ M of trypsin induced SFK and MLC phosphorylation were measured by western blot in the presence of PP2 (10 μ M) or PP3 (10 μ M). (D) The activity of trypsin in the presence of BMS200261 was determined by reading the 405 nm absorbance. Tracings are representative of at least three separate experiments.

Figure 6. The effect of trypsin on platelet surface glycoproteins

The expression of GPVI, GPIIb and GPIb α on the surface of PAR4 null murine platelets was measured in the presence or absence of trypsin (50 μ M) by incubating with anti-mouse GPVI-FITC, CD41-FITC, GPIb α -FITC antibodies for 15 minutes at room temperature in the dark in non-stirring condition. Non-immune IgG isotype was used as negative control. Reaction was stopped by adding 400 μ l PBS and the surface expression of glycoproteins was analyzed by flow cytometry.