Exposure of plasminogen and the novel plasminogen receptor, Plg-R_{KT}, on activated human and murine platelets

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Key points

- The plasminogen receptor Plg-R_{KT} is expressed on platelets
- Plg-R_{KT} co-localizes with platelet-derived plasminogen retaining plasminogen and facilitates surface-mediated plasminogen activation

Abstract

Plasminogen activation rates are enhanced by binding to cell surfaces. We have previously demonstrated exogenous plasminogen binds to phosphatidylserine-exposing and spread platelets. Platelets reportedly contain plasminogen in their α-granules but secretion of plasminogen from platelets has not been studied. Recently, a novel transmembrane lysine-dependent plasminogen receptor, Plg-R_{KT}, has been described on macrophages. We aimed to determine if platelets express Plg-R_{KT} and examine whether platelets expose and retain a pool of plasminogen. Equal levels of plasminogen were detected in the supernatant from resting and collagen + thrombin stimulated platelets as detected by ELISA. Pre-treatment with the lysine analogue εACA significantly augmented platelet-derived plasminogen (0.33 nmol/10^8 plts *vs.* 0.08 nmol/10^8 plts), indicative of a lysine-dependent mechanism of membrane retention. Lysine-dependent, platelet-derived plasminogen retention on thrombin + convulxin (CVX) activated human and murine platelets was confirmed by flow cytometry. Platelets initiated fibrinolytic activity in fluorescently labelled plasminogen deficient clots and in turbidemtric clot lysis assays. A 17 kDa band consistent with Plg-R_{KT} was detected by Western blotting in the membrane fraction of human platelets. Confocal microscopy of stimulated platelets revealed Plg-R_{KT} co-localized with platelet-derived plasminogen on the activated platelet membrane. Plasminogen exposure was significantly attenuated in thrombin + CVX stimulated platelets from Plg-$R_{KT}^{-/-}$ mice compared to wild type (WT) littermates. Exposure of Plg-R_{KT} exposed on the membrane was not dependent on plasminogen exposure as similar levels were detected in plasminogen$^{-/-}$ mice. These data indicate that Plg-$R_{KT,}$ present in human and murine platelets, functions in retention of platelet-derived plasminogen, potentially enhancing cell-surface mediated plasminogen activation.

Introduction

Platelets are a reservoir for a diverse range of proteins, including multiple that direct the hemostatic response. In addition, they are a focal point of fibrin formation due to their ability to facilitate thrombin generation on the activated platelet. Classically platelets have been described as having anti-fibrinolytic functions, due to the high concentrations of PAI-1 within their α-granules[1], which accounts for the majority of circulating PAI-1. Our work has recently shown that functionally active PAI-1 is retained on the activated platelet membrane[2]. We have also shown that exposure of cellular factor XIII-A (FXIII-A) on the surface of activated platelets facilitates the anti-fibrinolytic function of alpha2-antiplasmin (α2AP) by crosslinking this inhibitor to fibrin[3]. Furthermore, platelets drive the process of clot retraction through fibrinogen binding to the integrin $\alpha IIb\beta_3$. Retraction of clots condenses the crosslinked α2AP[4] and attenuates binding of tissue plasminogen activator (tPA)[5], making them more resistant to lysis than uncompacted clots[6,7].

However, the role of platelets in regulation of fibrinolysis is not clear-cut, as activated platelets also act as a binding site for plasma-derived plasminogen[8,9]. We have demonstrated that plasma-derived plasminogen binds to distinct locations in procoagulant phosphatidylserine (PS) exposing platelets and aggregating spread platelets in both a fibrin-dependent and -independent manner[9]. Under physiological flow conditions exogenously added plasma-derived plasminogen is incorporated into the growing thrombus, binding both directly to the platelet surface and indirectly via platelet-associated fibrinogen, thus facilitating fibrinolysis[9]. The platelet surface acts to promote activation of single chain urokinase (scuPA) by a reciprocal mechanism by activation of membrane-bound plasminogen[10]. Platelets have indirectly been reported to contain their own pool of plasminogen within their α-granules[11,12], however, little is known about this platelet-derived plasminogen.

Plasminogen activation by tPA is enhanced by the binding of fibrin(ogen) to cellular surfaces compared to plasminogen in solution. Binding of plasminogen to fibrin or cells occurs via lysine binding sites in the kringle domains. Furthermore, binding to the cell surface protects plasmin from inhibition by α2AP[13,14]. Multiple plasminogen receptors have been described on different cells types[15]. One common feature of these receptors is the dependence on lysine binding[16]. Recently a novel transmembrane plasminogen receptor has been described

on the surface of macrophages which critically possesses a C-terminal lysine[17]. This receptor has been designated Plg-R_{KT} and has an active role in macrophage migration[18] and recruitment[19].

Here we demonstrate that platelet-derived plasminogen is exposed and retained on the surface of activated platelets. Platelets promote plasminogen activation on their surface and are functional in fibrinolysis. We show for the first time the presence of the novel transmembrane receptor, Plg-R_{KT}, on the platelet membrane which functions to retain platelet-derived plasminogen.

Methods

Collection of human blood and preparation of platelets

Blood was drawn from healthy volunteers according to the declaration of Helsinki. Peripheral blood was collected in acid citrate dextrose (ACD) A vacutainers (Greiner Bio-one LTD). Platelets were isolated by centrifugation at 260 *x g* for 15 min to collect platelet-rich plasma (PRP). PRP was centrifuged at 870 *x g* for 15 min and then washed by centrifugation at 870 *x g* for 15 min in Hepes wash buffer (10 mM Hepes pH 6.6, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA) containing 0.1 U/ml apyrase (Sigma-Aldrich) and ACD (80 mM trisodium citrate, 52 mM citric acid and 183 mM glucose). Pelleted platelets were resuspended in Hepes buffer (10 mM Hepes pH 7.45, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA) containing 0.1 U/ml apyrase. Platelet counts were performed on a Siemens ADVIA 2120i Hematology System by the Haematology department, Aberdeen Royal Infirmary or on Sysmex XP-300 Hematology analyzer.

Preparation of mouse platelets

Wild type (WT) mice of C57Bl/6J background, Plg-R_{KT} knock out (Plg-$R_{KT}^{-/-}$) mice generated as described[19] or plasminogen knock out mice (Plg $^{-/-}$) were anaesthetized with CO_2 prior to performing cardiac puncture. Blood was collected by a trained professional into 3.8 % trisodium citrate. Blood was pooled from three age and sex-matched mice and the final volume adjusted to 7 ml using modified Tyrode's buffer (10 mM HEPES pH 6.5, 135 mM NaCl, 2.9 mM KCl, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 11.9 mM $NaHCO_3$). PRP was obtained by

centrifugation at 230 x *g* for 7 min at ambient temperature (IEC PR-7000 Centrifuge, International Equipment Company, Massachusetts, USA). PRP was centrifuged at 500 x *g* for 13 min in modified Tyrode's buffer (pH 6.5) to obtain platelet pellets. The pellet was resuspended in modified Tyrode's buffer (10 mM HEPES pH 7.4, 135 mM NaCl, 2.9 mM KCl, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 11.9 mM $NaHCO_3$) containing 0.1 U/ml apyrase. Platelets counts were obtained on a IDEXX ProCytoDx^{TM} Hematology analyzer.

Detection of plasminogen by ELISA

Platelets (5 x 10^8 platelets/ml) were isolated ± εACA (200 mM) and stimulated with collagen (20 µg/ml) and thrombin (100 nM) for 30 min. Plasminogen was detected in the supernatant using an ELISA, as described previously[20] with the exception that the detection antibody was a goat-anti human plasminogen polyclonal antibody (Enzyme Research Laboratories).

Flow cytometry

Washed platelets (2 x 10^8 platelets/ml) in Hepes buffer pH 7.45 (human) or modified Tyrodes buffer (mouse) were left unstimulated or stimulated in the presence of 2 mM $CaCl_2$ with 100 ng/ml convulxin (CVX; Enzo Life Sciences) ± 20 µM thrombin receptor activator peptide-6 (TRAP-6) or 100 nM thrombin (Sigma-Aldrich) in the absence or presence of εACA. Annexin Alexa fluor 488 (AF488) or Alexa fluor 568 (AF633) (1/20, Life technologies) was added after 45 min. Platelet-derived plasminogen was detected using a mouse monoclonal antibody labelled in house with DyLight 633 or 488 (DL633 or DL488) labelling kit (Life Technologies). This antibody preferentially recognizes receptor induced binding sites which are latent on Glu-plasminogen but become available upon binding of Glu-plasminogen to cell surfaces[21] . Plg-R_{KT} was detected with a mAb (mAb 7H1 [17] labelled in house with DyLight 550 or 633 (DL550 or DL633) labelling kit (Life Technologies). A minimum of 10,000 events were collected using a Fortessa flow cytometer (Becton Dickinson). Data analysis was performed using FlowJo software (Tree Star Inc.).

Fluorescent confocal Microscopy

Washed platelets at 0.5 x 10^8 platelets/ml in Hepes buffer, pH 7.45 (1% BSA) were adhered to µ-Ibidi VI$^{0.4}$ coated with 0.6 µg equine tendon type I collagen (American Biochemical Pharmaceuticals) ± 3 pmol thrombin or 0.45 nmol TRAP-6. In some case mAb to plasminogen-DL633 or mAb to Plg-R_{KT}-DL550 were included during stimulation. After stimulation annexin A5-AF488 (1/20 dilution) (Life Technologies) and 2 mM $CaCl_2$ were included. For time course analysis platelets were added directly to the coated surface with annexin A5-AF568 (1/20 dilution) (Life Technologies), mAb to plasminogen-DL633, P-selectin AF488 (Biolegend) and 2 mM $CaCl_2$. Images were recorded on Zeiss 710 laser scanning confocal microscope with a 63 x 1.40 oil immersion objective using Zeiss Zen 2012 software.

Turbidimetric fibrinolysis assays

Purified human plasminogen-free fibrinogen (2.4 µM, Enzyme Research Laboratories), ± glu-plasminogen (0.24 µM, Enzyme Research Laboratories) ± 2.5 x 10^8 washed platelets/ml, in hepes buffer pH 7.45 was added in triplicate to 96-well polystyrene plates. Clotting was initiated by thrombin (0.25 U/ml) and $CaCl_2$ (5 mM), and turbidity was monitored every min at 340 nm for 30 min at 37 °C in a FLX-800 plate reader (Biotek Instruments). After 30 min polymerisation 1 nM tPA (Genentech) was overlaid on to each clot and turbidity monitored for 5 h.

Fluorescent imaging of clot lysis

Clots were prepared as above with the inclusion of plasminogen-free fibrinogen labelled with DyLight 488 (0.25 µM). The clots were formed in µ-Ibidi VI$^{0.4}$ chamber slides and incubated at 37°C for 30 min. After this time 75 nM tPA was added to the edge of the clots and the lysis front monitored on a UVP Biospectrum 810 imaging system taking an image every 15 min for 18 h.

Western blotting

Human platelets (1 x 10^7) were lysed (L) and separated by ultracentrifugation into soluble protein (S) and membrane (M) fractions. The fractions were run on 4-12% NuPage gels followed by Western blotting with an antibody (7H1) to Plg-R_{KT}.

Statistical analysis

Statistical analysis was performed in GraphPad Prism® 5.04 using one-way analysis of variance with Bonferroni post-hoc test or an unpaired student t test. $P < 0.05$ was considered to be significant. Results are represented by the mean ± standard error of the mean (SEM).

Results

Platelet-derived plasminogen is retained on the activated membrane

To determine the pool of plasminogen in platelets, we quantified antigen levels in the supernatant of stimulated platelets by ELISA. The supernatant from unstimulated and collagen + thrombin stimulated platelets contained similar levels of plasminogen (0.08 *vs.* 0.07 nmol/10^8 platelets, Figure 1A). Blocking lysine dependent plasminogen binding to the platelet surface, by isolating platelets in the presence of the lysine analogue εACA, resulted in a 4.7-fold increase to 0.33 nmol/10^8 platelets in the supernatant fraction (Figure 1A, $p < 0.05$). These data indicate that platelet-derived plasminogen is released from platelets upon stimulation but is retained on the activated membrane. Similarly, flow cytometry revealed a 3.7-fold increase upon stimulation with thrombin + CVX, compared to unstimulated platelets (Figure 1B & C, $p < 0.01$). Thrombin alone resulted in the same level of exposure as to that of thrombin + CVX (MFI 602.8 ± 162.9 *vs.* 605.0 ± 102.1). Inclusion of εACA during platelet stimulation with thrombin + CVX significantly reduced plasminogen exposure (MFI 159.0 ± 58.8 *vs.* 605.0 ± 102.1, $p > 0.01$). Plasminogen exposure was apparent in both PS positive and PS negative platelets (Figure 1D).

Confocal microscopy was utilized to investigate the localization of platelet-derived plasminogen on the platelet surface. In PS-exposing platelets (Annexin A5 positive) plasminogen was detected in the 'cap' or platelet body (Figure 2A). Plasminogen was located centrally over the granulomere in spread platelets. This is consistent with our previous observation of the localization of binding of plasma-derived plasminogen in both phenotypes.[9] P-selectin is commonly used as a marker of α-granule release; using confocal microscopy the timing of release of plasminogen and P-selectin was investigated. P-selectin and platelet-derived plasminogen were simultaneously detected on the surface of platelets on a thrombin and collagen coated surface (Figure 2B). Interestingly, this was prior to detectable PS exposure, suggesting that PS is not required for plasminogen release. Simultaneous exposure with P-selectin is consistent with plasminogen being an α-granule protein.

Platelet-derived plasminogen is functional in fibrinolysis

Previously we have demonstrated plasmin generation on the platelet surface[9]. To determine whether the plasmin generated is sufficient to drive fibrinolysis we formed purified plasminogen-free fibrinogen clots in the absence and presence of platelets. Incorporation of fluorescently labelled fibrinogen allowed the progression of lysis to be monitored following addition of tPA to the top of the chamber. A distinct lysis front was visible in a control sample containing purified plasminogen (Figure 3A, Supplementary Video 1). In the absence of platelets, no fibrinolytic activity was observed, however, upon inclusion of platelets fibrinolysis was observed. Platelets (2.5×10^8 platelets/ml) lysed 32.8 ± 3.6 % of the distance lysed by the control sample containing plasminogen (set as 100 %) (Figure 3A & B, Supplementary Video 1). Consistent with these observations turbidimetric analysis of clot lysis was observed upon inclusion of platelets compared to the no plasminogen control (Figure 3C & D). The addition of purified plasminogen (0.24 μM) induced significantly faster lysis compared to clots containing platelets (time to 50 % lysis, 93 ±15.5 *vs.* 233.3 ±57.2 min respectively, $p > 0.05$). The plasminogen concentration in 2.5×10^8 platelets is estimated to be 0.83 μM based on the ELISA data (Figure 1A), despite this the rate of lysis was 3.6-fold slower than the plasminogen control (0.24 μM).

Plg-R_{KT} is exposed on PS-exposing and spread platelets

The transmembrane receptor Plg-R_{KT} has been shown to be responsible for plasminogen retention on the surface of macrophages[17,18]. Western blots were performed on platelet fractions and a monoclonal antibody with a specificity for the 9 C-terminal amino acids of Plg-R_{KT}[18]. A 17 kDa band, corresponding to the transmembrane receptor was identified in total platelet lysates and in the membrane fraction, but not the secretome (Figure 4A). Flow cytometry analysis confirmed the presence of Plg-R_{KT} on the platelet membrane, with a similar number of positive platelets observed pre- and post-stimulation (Figure 4B). In contrast the MFI value increased 4.8-fold upon stimulation with thrombin + CVX (Figure 4C), indicative of the release of Plg-R_{KT} from platelet pools. The localization of Plg-R_{KT} on the platelet surface was determined by confocal microscopy. The receptor localized to the center of spread PS-negative platelets and in the cap region of PS-exposing platelets (Figure 4D).

Both platelet-derived plasminogen and Plg-R_{KT} could be detected in the same location, however, the antibody raised against Plg-R_{KT} is targeted to the C-terminal lysine residue of the receptor, therefore direct competition between the antibody and plasminogen is occurring.

Plg-R_{KT} functions to retain plasminogen on the activated platelet membrane

To study the role of Plg-R_{KT} in retention of plasminogen on the platelet surface, platelets were isolated from Plg-$R_{KT}^{-/-}$ mice and plasminogen retention quantified by flow cytometry. Consistent with the human data, murine Plg-R_{KT} $^{(+/+)}$ platelets were found to retain platelet-derived plasminogen on their surface, which was increased 12.2-fold upon stimulation with thrombin + CVX. Plasminogen retention was significantly attenuated in mice deficient in Plg-R_{KT} $^{(-/-)}$, (Figure 5A), while no difference was noted in the unstimulated platelets. This indicates that approximately half of the pool of plasminogen that is exposed upon stimulation is retained via Plg-R_{KT}. Exposure of Plg-R_{KT} was increased upon stimulation in platelets isolated from WT mice as observed with human platelets (Figure 5B). Deficiency of plasminogen did not significantly alter the level of Plg-R_{KT} indicating that plasminogen is not required to induce exposure of this receptor (Figure 5B).

Discussion

We have definitively demonstrated that platelets harbor a discrete pool of plasminogen that is released upon activation. Platelet-derived plasminogen was retained in a lysine-dependent manner. Platelets express the transmembrane receptor, Plg-$R_{KT,}$ which possesses a c-terminal lysine and functions to retain plasminogen on their surface. Binding via Plg-R_{KT} accounts for approximately half of the platelet pool of plasminogen. In the absence of an exogenous source of plasminogen, platelets were able to actively direct fibrinolysis.

Multiple receptors for plasminogen have been described in various cell types, but not all cell types express the same receptors[15]. One feature many of these receptors have in common is that they bind plasminogen via its lysine binding sites within the kringle domains[22]. Due to the closed nature of native glu-plasminogen crystallography studies indicate that only the binding domain in kringle one is available for initial binding[23]. Binding to lysine induces a more open easily activated conformation[24], thus binding to cell surfaces enhances activation. Furthermore, binding of plasminogen to cellular surfaces protects plasmin generated on the surface from inhibition by α2AP[13]. Inclusion of the lysine analogue to block lysine dependent binding completely abolished the augmented retention of platelet derived plasminogen, indicating a lysine-dependent mechanism is responsible. Plg-R_{KT} is the first described integral transmembrane receptor that possess a c-terminal lysine. Plg-R_{KT} has been shown to promote plasminogen activation, cell migration, recruitment and polarization of macrophages[18,25]. Here, we establish that this receptor is exposed upon stimulation and around 50 % of the plasminogen that is exposed and retained is bound via this receptor. Similar to our observations for exogenous plasminogen, stimulation with TRAP-6 did not induce plasminogen retention to the same level as that observed when thrombin was used. This is consistent with a fibrin dependent mechanism as TRAP-6 activates PAR-1 but does not cleave fibrinogen to fibrin.

In a system free from exogenous plasminogen platelets were able to drive fibrinolysis. Despite the plasminogen content of the clots being higher upon the addition of platelets (0.83 μM) the rate of lysis was slower compared to the plasminogen control (0.24 μM). Platelets

contain multiple antifibrinolytics proteins, primarily active PAI-1 which is present in high concentrations[1,2,26], but also TAFI[27,28], α2AP[29] and FXIII-A[30]. The slower rate of lysis with platelet-derived plasminogen likely reflects that these inhibitors partially counteracts this pool of plasminogen highlighting the complexities of the balance of the fibrinolytic system. Furthermore, the platelet membrane enhances activation of scuPA in a mechanism of reciprocal plasminogen activation[10]. We recently demonstrated that plasminogen activation by factor XIIa (FXIIa) is enhanced in the presence of platelet-derived polyphosphate (polyP)[31]. Along with plasminogen, FXIIa and platelet-derived polyP accumulate on the activated platelet membrane[31]. The fibrinolytic capacity of the platelet membrane is clearly dependent on the balance and the assembly of these fibrinolytic proteins on the surface and the surrounding milieu.

Targeting the fibrinolytic potential of the platelet surface is therefore an interesting potential for thrombolytic therapies. Indeed, scuPA has been targeted directly to the platelet surface via $\alpha_{IIb}\beta_3$ has been shown to effectively prevent occlusion in a ferric chloride model in a plasminogen dependent manner[32]. Importantly this fusion protein did not prolong bleeding times. Previously we demonstrated that plasminogen accumulated at the base or core of thrombi in a whole blood model of thrombus formation and lysis. This was apparent both prior to and after visible fibrin formation[9]. The thrombus architecture is heterogeneous consisting of a core of fully activated, densely packed platelets and an outer 'shell' of less-activated, loosely associated platelets[33-37]. The microenvironment of the tightly packed core dictates solute transport, with restricted protein diffusion evident[33,35,38]. Therefore, our observation that plasminogen accumulates at the thrombus core suggests that platelet-derived or associated plasminogen may have importance in driving remodeling of the thrombus.

We conclusively demonstrate that platelets contain a pool of intracellular plasminogen that is retained on the surface of activated platelets. Almost half of this pool of plasminogen is anchored via the transmembrane receptor Plg-R_{KT}. The platelet surface acts as scaffold housing both pro- and anti-fibrinolytic factors, the balance of which has important

implications in driving fibrinolysis. Harnessing the bodies endogenous potential for plasminogen activation could provide a new avenue for novel thrombolytics.

Di Meglio et al Neurology 2019 – acute ischaemic stroke thrombi have an outer shell that impairs fibrinolysis

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Figure legends

Figure 1 – Activated platelets release plasminogen and retain it on the activated membrane. (A) Isolated human platelets were stimulated with collagen (20 μg/ml) and thrombin (100 nM) ± εACA (200 mM) for 30 min. Plasminogen was detected in the supernatant by ELISA. (B-D) Platelets (2 x 10^8 platelets/ml) were stimulated ± CVX (100 ng/ml) with TRAP-6 (15 μM) or thrombin (100 nM) ± εACA. Annexin-AF488, anti-plasminogen antibody-DL633 were included to detected PS exposure and platelet-derived plasminogen respectively. (B) Representative flow cytometry curves. Data are presented as mean ± SEM for (C) median fluorescence intensity (MFI) for platelet-derived plasminogen exposure (D) PS exposure in plasminogen positive platelets. * P <0.05 and ** P <0.01. Data are expressed as mean ± SEM, n ≥3.

Figure 2 – Platelet-derived plasminogen is retained in a distinct pattern on activated platelets. Platelets (0.5 x 10^8 platelets/ml) stimulated on a collagen (0.6 μg) and thrombin (3 pmol) coated surface stained with (A) Annexin-AF488 and anti-plasminogen antibody-DL633. (B) Time course of platelet activation labelled with Annexin-AF568, AF488 P-selectin antibody and anti-plasminogen antibody-DL633. Scale bars represent 5 μm, representative images of n ≥3.

Figure 3 – Platelets can drive fibrinolysis via a plasmin-mediated mechanism. (A - B) Purified clots were formed from plasminogen-free fibrinogen (2.4 μM) and DL488 plasminogen-free fibrinogen (0.25 μM) ± glu-plasminogen (0.24 μM) ± 2.5 x 10^8, 1 x 10^8 or 0.5 x 10^8 platelets/ml. Clotting was initiated by thrombin (0.25 U/ml) and $CaCl_2$ (5 mM) and allowed to form for 30 min prior to the addition of 75 nM tPA to the edge of the clots. Lysis was monitored by imaging every 15 min for 18 h. (B) The distance lysed as a percentage of the plasminogen control (set to 100 %). (C - D) Purified clots were formed as above with or without 2.5 x 10^8 platelets/ml in the absence of DL488-fibrinogen. Turbidity monitored every min at 340 nm for 30 min at 37 °C in a FLX-800 plate reader (Biotek Instruments). After 30 min polymerisation 1 nM tPA

(Genentech) was overlaid on to each clot and turbidity monitored for 5 h. (D) 50 % lysis times * *P* <0.05 and **** *P* <0.0001 compared with plasminogen controls clots. Data are expressed as mean ± SEM, *n* ≥3.

Figure 4 – Platelets express the transmembrane Plg-R_{KT}. Platelets (1 x 10^7) fractions were separated by SDS-PAGE b before Western blotting for Plg-R_{KT}. Lysate (L), soluble protein (S) and membrane (M) fractions. The fractions were run on 4-12% NuPAge gels followed by Western blotting with an antibody (7H1) to Plg-R_{KT}. Representative image of *n*=4. (B - C) Platelets (2 x 10^8 platelets/ml) were stimulated ± CVX (100 ng/ml) with thrombin (100 nM) in the presense of anti-plg-R_{KT} antibody-DL550. Data are presented as mean ± SEM for (B) percentage positive for Plg-R_{KT} and (C) median fluorescence intensity (MFI). * *P* <0.05 vs. unstimulated, *n* = 3. (D) Platelets (0.5 x 10^8 platelets/ml) stimulated on a collagen (0.6 μg) and thrombin (3 pmol) coated surface for 45 min stained with Annexin-AF488 and anti-plasminogen antibody-DL633, anti-plg-R_{KT} antibody-DL550. Scale bars represent 5 μm, representative images of *n* = 4.

Figure 5 – Plg-$R_{KT}^{-/-}$ platelets retain reduced membrane bound plasminogen. Washed platelets (2 x 10^8 platelets/ml) in modified Tyrodes buffer pH 7.45 from WT or Plg-R_{KT} mice were left unstimulated or stimulated in the presence of 2 mM $CaCl_2$ with 100 ng/ml convulxin (CVX; Enzo Life Sciences) + 100 nM thrombin (Sigma-Aldrich). Annexin A5 AF633 with 2 mM $CaCl_2$ was added after 45 min. Platelet-derived plasminogen was detected using anti-plasminogen DL488 antibody. A minimum of 10000 events were collected using a Fortessa flow cytometer (Becton Dickinson). * *P* <0.05 compared with WT stimulated platelets. Data are expressed as mean ± SEM, *n* ≥ 4.