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Platelet protein S directly inhibits procoagulant activity on platelets and microparticles

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Summary

Anticoagulant plasma protein S (PS) is essential for maintaining hemostatic balance. About 2.5% of PS is stored in platelets and released upon platelet stimulation. So far, little is known about the functionality and importance of platelet (plt)PS. A platelet-associated protease cleaves plasma-derived (pd)PS and pltPS in the "thrombin-sensitive region", abolishing activated protein C (APC) cofactor activity. However we showed that cleaved PS retains APC-independent anticoagulant activities ("PS-direct"). To investigate whether pltPS or pdPS exert PS-direct on platelets or platelet-shed microparticles, thrombin and factor (F)Xa generation on unstimulated or stimulated washed platelets and microparticles were measured. Western blotting revealed that pltPS and pdPS bound to washed, stimulated platelets and microparticles, and that pltPS had slower electrophoretic mobility than pdPS. Platelet stimulation in the presence of inhibitory anti-PS antibodies resulted in 2.6 ± 1.6-fold ($p<0.0004$, $n=20$) more thrombin generation upon addition of FXa and prothrombin. PltPS exerted PS-direct that was similar to or greater than that of Zn^{2+}-containing pdPS and much greater than that of Zn^{2+}-deficient pdPS. Findings were confirmed using purified pltPS. Platelet-bound pltPS and microparticle-bound pltPS had similar PS-direct. Finally, platelet stimulation in the presence of inhibitory anti-PS antibodies resulted in 1.5 ± 0.2-fold ($p<0.0001$, $n=11$) more FXa generation upon addition of TF/FVIIa and FX. Thus, pltPS inhibits both prothrombinase and extrinsic FXase activities. Neutralizing antibodies against APC and TFPI had no effect on the PS-direct of pltPS or pdPS on platelets. This study indicates that pltPS may be an essential pool of PS that counterbalances procoagulant activities on platelets.

Keywords

activated protein C; protein S; platelets; microparticles; anticoagulant activity

Introduction

Platelets are essential mediators of hemostasis at sites of vascular injury, providing surfaces that accelerate blood coagulation, and promoting formation of mechanical plugs. Most procoagulant reactions are thought to occur on the surface of platelets, where procoagulant activity is increased after stimulation of platelets with agonists such as thrombin, collagen, or Ca^{2+}-ionophore (1). Part of the procoagulant activity generated in platelet suspensions is associated with microparticles that are shed from the platelet membrane upon stimulation (1,

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Conflicts of interests
None declared.

2). It is essential that platelet and microparticle procoagulant activities be counterbalanced by anticoagulant mechanisms, in which protein S (PS) is likely to take part. The importance of circulating PS in hemostatic balance is demonstrated by its heterozygous deficiency that leads to increased risk of venous thromboembolism (3), by embryonic lethality in PS knock-out mice (4, 5), and by its antithrombotic activity in thrombosis models (6).

About 2.5% of PS in blood is found in platelet α-granules and is secreted upon platelet stimulation (2), whereupon it might be well-situated to counterbalance procoagulant activities. Little is known about the physiologic role of secreted platelet PS. Platelets are known to support the anticoagulant cofactor activity of plasma-derived PS toward APC, accelerating APC-mediated inactivation of FVa (1, 7, 8). Both plasma-derived and released platelet PS can overcome the protective effect of FXa toward FVa during inactivation by APC (7). However, much of PS in contact with stimulated platelets is cleaved in the thrombin sensitive region (TSR)(9) by a Ca^{2+}-dependent membrane protease that abolishes PS cofactor activity toward APC (10). *In vitro*, the TSR of PS is cleaved by thrombin at Arg_{49} or by FXa at Arg_{60}, causing complete loss of APC cofactor function (11), but not loss of PS direct anticoagulant activity (also known as "PS-direct")(12). *In vivo*, ~15% of circulating PS is cleaved in the TSR; this percentage increases in patients with intravascular coagulation and hematological malignancies (13, 14). Because of the inactivation of PS cofactor activity for APC by a platelet membrane protease, the direct anticoagulant activity of PS may assume an important role on platelets, but this has not yet been reported.

We and others showed that besides its role in APC-mediated inactivation of FVa/FVIIIa (15, 16), plasma PS also regulates blood coagulation independently of APC and tissue factor pathway inhibitor (TFPI) by *directly* inhibiting FXa, the prothrombinase complex, the extrinsic FXase complex, and the intrinsic pathway (17, 18, 19). The direct anticoagulant activity of PS has been controversial, primarily because it was unrecognized that many PS purification procedures result in loss of intramolecular Zn^{2+} that is essential for the efficient direct anticoagulant activities of PS (18). While Zn^{2+}-deficient PS stimulates inhibition of FXa by TFPI (20, 21), Zn^{2+}-containing PS inhibits FXa and extrinsic FXase independently of TFPI (17).

This study tests the hypothesis that platelet and plasma-derived PS directly down-regulate procoagulant activity on platelets and/or platelet microparticles, analogous to the behavior of native, Zn^{2+}-containing plasma-derived PS in plasma and in purified component systems (17, 18).

Materials and Methods

Reagents

Ca^{2+}-ionophore A23187 and prostaglandin E1 (PGE1) were purchased from Sigma (St. Louis, MO, USA). V-well polypropylene plates and flat bottom Bacti plates were from Thermo-Fisher (Rochester, NY, USA). Neutralizing sheep anti-protein C antiserum was a gift from Prof. Peter Schwarz (Immuno/Baxter, Vienna, Austria), from which antibodies were immunoaffinity-purified. Neutralizing monoclonal antibody T4E2 against TFPI was a gift from Diagnostica Stago (Asnieres, France), and TFPI was a gift from John-Bjarne Hanson (University of Tromso, Norway). Neutralizing rabbit IgG anti-PS antibodies were from Dako (Carpenteria, CA, USA). Immunoaffinity-purified goat anti-human protein S and mouse monoclonal anti-human protein S were prepared in house. Human FX, FXa, FVIIa, and prothrombin were from Enzyme Research Laboratories (South Bend, IN, USA). Tissue factor (TF, Innovin) was from Dade-Behring (Marburg, Germany). Synthetic substrates H-D-CHG-Ala-Arg-pNA32AcOH (Pefachrome TH) and Z-D-Arg-Gly-Arg-pNA32HCl

(Pefachrome Xa 8595) were from Pentapharm (Basel, Switzerland). Equine collagen was from Hormon-Chemie, Munich, Germany.

Platelet preparation

Blood was obtained from healthy consenting donors by venipuncture, after institutional review. Six volumes of blood were collected into 1 vol anticoagulant (ACD; acid citrate-dextrose, final pH 6.5) and centrifuged for 15 min, 250g at 23°C. Platelet-rich plasma was collected and centrifuged 10 min, 170g to remove remaining erythrocytes and leukocytes. Platelet-rich plasma was then centrifuged for 15 min, 600g, and the platelet pellet was resuspended in 9 vol modified HEPES-Tyrode's buffer consisting of 20 mM HEPES (pH 6.5), 136 mM NaCI, 2.68 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, and 1 vol ACD. Platelets were washed twice using this procedure and platelet concentrations were determined with a Hemavet counter (Drew Scientific, Dallas, TX, USA). Platelets were resuspended in modified HEPES-Tyrode's buffer (pH 7.4) and used within 6 hours of blood drawing. Where noted, platelets were prepared in presence of 0.5 μg/mL PGE1 to inhibit activation.

Human plasma-derived and platelet protein S

Zn^{2+}-containing PS was purified from citrated plasma by barium adsorption, elution with ammonium sulfate and dialysis. After 5% PEG precipitation to remove PS complexed with C4b-binding protein, PS was immunoaffinity-purified on monoclonal antibody S7-Sepharose (18), dialyzed in HEPES-buffered saline, (HBS, 0.02 M HEPES, 0.15 M NaCl, pH 7.4), and stored at −80°C. Zn^{2+}-deficient PS was obtained from Enzyme Research Laboratories. Zn^{2+} analyses of PS were performed as described (18). Platelet PS was purified from freshly washed platelets from 500 ml of blood. After 5 min Ca^{2+}-ionophore stimulation, 2 mM EDTA was added to elute platelet-bound platelet PS. After 1 min platelets were removed by centrifugation at 1,000g. An inhibitor cocktail (complete, Roche Diagnostics, Mannheim, Germany) was added, and PS in the supernatant was immunoaffinity-purified on monoclonal antibody S7-Sepharose (18). Platelet PS was concentrated using a centrifugal filter (Amicon 10,000 MWCO, Millipore, Billerica, MA, USA), dialyzed against TBS-1mM sodium citrate, and frozen in aliquots.

Testing of neutralizing antibodies

Affinity-purified sheep anti-protein C was tested in plasma in the presence and absence of Protac C, a protein C activator (American Diagnostica, Stamford, CT, USA). An amount of antibody that neutralized all APC generated was chosen for use in assays for PS direct anticoagulant activity to exclude possible PS cofactor activity for any APC generated in platelet experiments (6). Rabbit anti-PS was tested in purified component prothrombinase assays and in plasma assays (6). An amount that neutralized all PS direct anticoagulant activity was chosen for platelet experiments. Monoclonal antibody against TFPI was tested in purified component extrinsic FXase assays with and without purified TFPI. An amount that neutralized the inhibitory effect of 1 nM TFPI was chosen for platelet experiments. Plasma contains ~0.25 nM active TFPI (21).

Thrombin generation on platelets and microparticles

Prothrombinase assays for PS direct activity in platelet suspensions or releasate were modified from those described (6), using 50 pM FXa, 2×10^8 platelets/mL unless otherwise noted, 0.6 μM prothrombin, 0.5% BSA, HEPES-buffered saline, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 2.68 mM KCl, and 5 mM dextrose, pH 7.4. Prothrombinase activity was amplified by released platelet FVa. For the anticoagulant activity of plasma-derived PS, platelets were preincubated 8 min (23°C) with 5 μM Ca^{2+}-ionophore and gentle agitation in the presence and absence of plasma-derived PS and presence of antibodies against TFPI (20 μg/mL), and

APC (13 μg/mL) before addition of FXa and prothrombin. For neutralization of the anticoagulant activity of platelet PS, 0.3 mg/mL anti-PS antibodies were used, and plasma-derived PS was omitted. Aliquots were removed at 0.5 min intervals from the reactions in v-wells and quenched in buffer containing 20 mM EDTA and 0.5% BSA in the wells of Bacti plates. Thrombin chromogenic substrate Pefachrome TH was added to 0.2 mM, and the amount of thrombin generated was determined using a kinetic plate reader. The rate of thrombin generation was determined from the slope of thrombin generated over time. In some experiments, platelets were stimulated with 2 μg/mL collagen for 11 min instead of with Ca^{2+}-ionophore, and thrombin generation was measured 2 min after addition of prothrombin.

For the anticoagulant activity of platelet-bound platelet PS, platelets were Ca^{2+}-ionophore-stimulated in the presence of 1 mM $CaCl_2$, washed twice with modified HEPES-Tyrode's buffer containing 1 mM $CaCl_2$, pH 7.4 (700g, 1 min, 23°C), adjusted to 1×10^9/mL, and diluted in prothrombinase reaction mixture. The anticoagulant activity of platelet PS in platelet-free releasate (from 1×10^9 platelets/mL, centrifuged 3 min 1000g) was tested with and without anti-PS, directly or after removal of microparticles by ultracentrifugation (229,000g, 90 min, 4°C). Releasate or washed platelets (typically 20 μL) were tested in 100 μL of prothrombinase assay mixture.

FXa generation on platelets and microparticles

Assays were conducted in a similar manner as thrombin generation assays, where 100 pM FVIIa replaced FXa and 0.16 μM FX replaced prothrombin. Platelets were stimulated in the presence of 5 pM tissue factor and blocking antibodies against APC and TFPI. Pefachrome FXa was used to measure FXa generation.

Protein S binding to platelets and microparticles

For the detection of platelet- and microparticle-bound plasma-derived PS or platelet PS, washed platelets (200 μL, 1×10^9/mL) supplemented with 1 mM $CaCl_2$ were stimulated by Ca^{2+}-ionophore (5 μM, 37°C) in the presence or absence of 400 nM biotinylated Zn^{2+}-containing plasma-derived PS (Biotin-Zn^{2+}-PS). At different times, platelets were centrifuged (700g, 1.5 min, 23°C), washed twice by gentle resuspension and recentrifugation in 500 μL modified HEPES-Tyrode's buffer (pH 7.4), 1 mM $CaCl_2$, and then incubated in 60 μL modified HEPES-Tyrode's buffer, 10 mM EDTA (60 μL, 1 min, 23°C). Platelet eluate, releasate, and aliquots of the second wash fractions were added to SDS-PAGE loading buffer (LI-COR Biosciences, Lincoln, NE, USA). Microparticle-free releasate was prepared by ultracentrifugation (229,000g, 90 min, 4°C). The microparticle pellet was washed twice without resuspension with modified HEPES-Tyrode's buffer, 1 mM $CaCl_2$, and resuspended in SDS-PAGE loading buffer equal to the starting volume of releasate. For Western blots, Biotin-Zn^{2+}-PS (10 μL) or platelet PS (30 μL) were transferred from 4–12% SDS gels (Invitrogen, Carlsbad, CA, USA) to PVDF membranes. Platelet PS was detected with biotinylated rabbit anti-PS and Streptavidin-IRDye680 (LI-COR Biosciences). Biotin-Zn^{2+}-PS was detected with Streptavidin-IRDye680. Blots were analyzed using an Odyssey Infrared Imager (LI-COR).

Other methods

Samples of platelet releasate or 1% Triton X-100 lysate were diluted into 5% BSA and PS antigen was measured by ELISA (22), using capture antibodies of rabbit anti-PS or monoclonal antibody S7, and detecting antibodies of biotin rabbit anti-PS or biotin affinity-purified goat anti-PS. PS and selected antibodies were biotinylated using EZ-link sulfo-NHS-LC-biotin according to manufacturer's instructions (Pierce, Rockford, IL, USA). Statistical comparisons were made using 2-tailed *t* tests with software from GraphPad, San

Diego, CA, USA. Values of $p < 0.05$ were considered significant. Curve fitting was performed with GraphPad software.

Results

Stimulated platelets support the direct anticoagulant activity of plasma-derived PS

Since stimulated platelets are known to provide active surfaces for hemostatic reactions, we first investigated the effect of two types of purified plasma-derived PS on thrombin generation in the presence of stimulated washed platelets (Fig. 1). After addition of prothrombin and FXa to Ca^{2+}-ionophore stimulated platelets, Zn^{2+}-containing PS exhibited dose-dependent down-regulation of thrombin generation, while Zn^{2+}-deficient PS had a weaker effect, similar to results in studies using synthetic phospholipid vesicles (18). To exclude that the apparent effect of PS was due to impurities of APC or TFPI, experiments were performed in the presence and absence of inhibitory antibodies against APCand TFPI, but no differences were observed (Fig. 1A). Thus, any possible traces of TFPI or APC had no effect on the amounts of thrombin generated or on the direct anticoagulant effects exhibited by PS. Separate experiments were performed with collagen-stimulated platelets, using the same types of controls. Although lower amounts of thrombin were generated with collagen-stimulated platelets, PS showed similar anticoagulant effects (Fig. 1B).

Plasma-derived PS binds to stimulated platelets and microparticles

We examined whether stimulated platelets and platelet-shed microparticles can bind plasma-derived PS. To discriminate plasma-derived PS from platelet PS, Zn^{2+}-containing plasma-derived PS was coupled to biotin (Biotin-Zn^{2+}-PS), and dye-labeled streptavidin was used for its detection. The direct anticoagulant activity of Zn^{2+}-PS was unchanged after biotinylation. When electrophoresed under non-reduced conditions, Biotin-Zn^{2+}-PS in the supernatant of unstimulated and stimulated platelets was detected on blots as a single band of ~68 kDa (Fig. 2A, left panels), similar to the starting material. Consistent with a previous report (23), ultracentrifugation of Biotin-Zn^{2+}-PS-containing stimulated platelet supernatant showed that plasma-derived PS bound to platelet-shed microparticles (Fig. 2A, right panel). In contrast to the previous report, however, Biotin-Zn^{2+}-PS bound to the stimulated platelet surface as well (Fig. 2A, left panels). The low or non-detectable PS in the wash fractions and in the eluate from unstimulated platelets served as non-specific controls. Thus, plasma-derived PS binds specifically to stimulated platelets and to their shed microparticles.

Platelet PS binds to both stimulated platelets and microparticles

We investigated whether platelet membranes also support binding of secreted platelet PS. Non-reduced releasate from platelets stimulated by Ca^{2+}-ionophore exhibited two bands of platelet PS after Western blotting, with apparent MW of ~72 kDa and ~82 kDa. The lower band was more abundant (Fig. 2B, upper left), and both bands were slower in mobility than plasma-derived PS. After washing the stimulated platelets and then eluting them with EDTA-containing buffer, a similar doublet of non-reduced platelet PS was detected in the eluate, but with a more prominent upper band compared to platelet PS in releasate (Fig. 2B, lower left). After ultracentrifugation of releasate, most platelet PS was located on pelleted microparticles, rather than in the supernatant (Fig. 2B, right). Because only the lower band of the previous doublet was detected, additional proteolysis likely occurred during sample preparation. The low or non-detectable PS in the wash fractions and in the eluate from unstimulated platelets served as non-specific controls. These data demonstrate that released platelet PS binds specifically in a Ca^{2+}-dependent manner to stimulated platelets and to their shed microparticles.

Platelet PS has direct anticoagulant activity

When washed platelets were stimulated in the presence and absence of neutralizing anti-PS antibodies (Fig. 3A), as much as 5.7-fold more thrombin was generated after addition of FXa and prothrombin in the presence of anti-PS antibodies. Thus, platelet PS must have had an anticoagulant effect in the absence of neutralizing anti-PS antibodies. There were variations in thrombin generation, and probably in individual donors and degree of platelet PS degradation, therefore data from 20 experiments were normalized in Fig. 3B. The mean increase in thrombin generation in the presence of neutralizing anti-PS antibodies ± standard deviation was 2.6 ± 1.6-fold, $p \leq 0.0004$. For comparison, 40 nM plasma-derived PS reduced thrombin generation to 38 ± 7 % of control values (mean ± standard deviation, $p \leq 0.004$, n =3) (Fig. 3B). Some experiments were performed with collagen-stimulated rather than ionophore-stimulated platelets (Fig. 3B, striped bar). In these experiments, blocking PS with anti-PS antibodies almost doubled the thrombin generation after addition of FXa and prothrombin.

When FXa and prothrombin were added to unstimulated platelets, platelets were modestly stimulated, leading to low levels of thrombin generation. The low rate of thrombin generation was not linear, but rather increased over time, probably due to small amounts of FV/Va that were released (Fig. 3C). This effect was most evident in the presence of neutralizing anti-PS antibodies, suggesting that gradually released platelet PS was down-regulating thrombin generation in the absence of anti-PS antibodies. Exogenous purified Zn^{2+}-containing PS potently down-regulated thrombin generation. When unstimulated, washed platelets were PGE1-treated to inhibit activation, neutralizing anti-PS antibodies had no effect (data not shown), indicating that the anti-PS antibodies did not have a non-specific procoagulant effect, and that there was little release of PS from the PGE1-treated platelets.

Both platelets and microparticles promote platelet PS anticoagulant activity

As platelet-bound platelet PS might differ from that bound to microparticles, their direct anticoagulant activities were studied separately. In the presence of neutralizing anti-PS antibodies, significantly more thrombin was generated after addition of FXa and prothrombin in the presence of 20 μL and 30 μL platelet-free releasate containing microparticles, respectively (mean ± standard deviation: 1.8 ± 0.1-fold, $p \leq 0.05$ and 3.2 ± 0.3-fold, $p \leq 0.01$, n=3) (Fig. 4A). In experiments such as shown in Fig. 4A, less thrombin generation was observed with larger volumes versus smaller volumes of releasate in the absence of anti-PS. This is presumably because the larger amount of releasate contained more platelet PS. After ultracentrifugation of the platelet-free releasate, no significant anticoagulant or procoagulant activity was detected in the microparticle-free releasate in the absence or presence of synthetic phospholipid vesicles. This suggests that little or no free PS was present and also that little or no platelet FVa was present to act as a cofactor for the low concentration of FXa added (data not shown).

As much as 4-fold more thrombin was generated on washed stimulated platelets in the presence versus absence of anti-PS antibodies (mean ± standard deviation: 3.1 ± 0.9-fold (Fig. 4B). Neither adding FVa (Fig. 4B) nor increasing the number of platelets (data not shown) significantly increased thrombin generation, suggesting that sufficient functional platelet FVa was present for the FXa added. Together, these data show that both platelet- and microparticle-bound platelet PS exhibit direct anticoagulant activity.

Purified platelet PS and platelet PS in platelet suspensions have similar or greater direct anticoagulant activity than Zn^{2+}-containing plasma-derived PS

To confirm that platelet PS has direct anticoagulant activity, platelet PS was purified (see Methods). The purified platelet PS had slower electrophoretic mobility than plasma-derived

PS (Fig. 5A), in agreement with Fig. 2B Western blots. Only the lower band of the doublet as seen in Fig. 2B was observed, suggesting that the platelet PS may have been degraded during purification. When tested in purified component prothrombinase assays with synthetic vesicles, it had direct anticoagulant activity similar to that of a plasma-derived Zn^{2+}-containing PS preparation (Fig. 5B). PS concentrations at which thrombin formation was inhibited by 50% (IC50) were 129 ± 14 nM for purified platelet PS and 91 ± 10 nM for Zn^{2+}-containing plasma-derived PS. Both activities are in ranges found for Zn^{2+}-containing PS in earlier studies (18, 24).

Based on antigen measurements using three different antibodies, prothrombinase reaction mixtures containing stimulated platelets at physiologic concentrations contained 3.0 – 4.3 nM platelet PS, in agreement with earlier studies (2). These reactions displayed less than half the thrombin generation in the absence of neutralizing anti-PS antibodies (i.e., in the presence of platelet PS) than they did in the presence of anti-PS antibodies. This suggests an IC50 for this direct anticoagulant activity that is less than the amount of PS present (IC50 < 4 nM), which is considerably more anticoagulant than plasma-derived Zn^{2+}-containing PS (IC50 = 20 – 30 nM) in these experiments. Since platelet PS in platelet suspensions exhibited greater direct anticoagulant than purified platelet PS, it might indicate that platelet PS lost activity due to degradation during purification, or that something in suspensions of stimulated platelets enhanced the activity of platelet PS, or that newly released platelet PS at the platelet surface is more advantageously located than purified platelet PS added to a bulk solution.

Platelet and plasma-derived PS down-regulate FXa generation on platelets

We also investigated whether platelet PS and plasma-derived PS can directly inhibit extrinsic FXase on platelets, analogous to the behavior of native, Zn^{2+}-containing plasma-derived PS in plasma and in purified systems with synthetic phospholipid vesicles (17, 18). Washed platelets were stimulated in the presence of tissue factor. FVIIa and FX were then added and FXa generation was measured (Fig. 6). No FXa was generated unless tissue factor was added and platelets were present. Up to 1.7-fold more FXa was generated in the presence versus absence of neutralizing anti-PS antibodies (mean ± standard deviation: 1.5 ± 0.2 fold, n =11, $p<0.0001$). Exogenous purified Zn^{2+}-containing PS down-regulated FXa generation in a dose-dependent manner, while Zn^{2+}-deficient PS had little effect (Fig. 6 and data not shown). Neutralizing anti-PS antibodies had a similar effect when platelet-free releasate was used in place of platelet suspensions, indicating that platelet and plasma PS can down-regulate extrinsic FXase on microparticles as well as platelets (not shown). Since PS can modestly inhibit FXa amidolytic activity, Western blotting was performed as described (17) to confirm that larger amounts of FXa were indeed generated by extrinsic FXase in the presence of neutralizing anti-PS antibodies (data not shown).

Since platelets contain TFPI (25), two experiments were performed in the presence and absence of anti-TFPI. 5–34% more FXa was generated in the presence of anti-TFPI antibodies. However, the antibodies had no effect on inhibition by Zn^{2+}-containing PS or on the effect of anti-PS antibodies, where 1.5-fold more FXa was generated whether or not anti-TFPI was present (data not shown).

Discussion

This study shows that PS released from stimulated platelets binds to platelets and microparticles and directly inhibits procoagulant activity on both surfaces, thus possibly serving as a necessary counterbalance to procoagulant reactions. The direct anticoagulant activity of platelet PS was evident whether the agonist was Ca^{2+}-ionophore, collagen, or the thrombin formed upon addition of prothrombin and pM levels of FXa. Thus, at physiologic

platelet levels, platelet PS was directly anticoagulant at high, moderate, or very low rates of thrombin generation. Plasma-derived PS also inhibited procoagulant activity on stimulated platelets in an APC- and TFPI-independent manner. In agreement with our previous studies using synthetic phospholipid vesicles (18), Zn^{2+}-containing plasma-derived PS was more potent than its Zn^{2+}-deficient counterpart in the presence of platelets. In the present studies, the direct activity of platelet PS was more similar to that of Zn^{2+}-containing plasma-derived PS than to that of Zn^{2+}-deficient PS.

In the prothrombinase studies, platelet PS may have inhibited FXa or FVa, or competed with FXa or FVa for binding to phospholipids, since these have been reported as mechanisms of PS direct anticoagulant activity (18, 24, 26). Certainly, FXa was inhibited, because the effect of platelet PS was overcome if higher concentrations of FXa were used (≥ 4 nM, data not shown). It is unlikely that platelet PS was competing with FXa or platelet FVa for binding to platelet membranes, since platelets were used at a high enough concentration that lipids would not have been limiting. We cannot be certain that platelet PS inhibited platelet FVa, but the amounts of thrombin generated in most cases were such that platelet FVa must have amplified the activity of the low concentrations of FXa used, and this thrombin generation was inhibited by platelet PS.

Since most of our experiments involved unpurified, unmanipulated platelet PS, this argues for the validity of the direct anticoagulant activity of PS, especially at crucial sites on activated platelets. Earlier investigators may not have observed this activity because they were focused on the APC cofactor activity of PS. In our earlier platelet experiments, we observed no effects of goat anti-PS antibodies (1), but we have since found that these antibodies do not neutralize the direct anticoagulant activity of PS.

The apparent molecular weight of platelet PS was higher than that of plasma-derived PS, consistent with an earlier report (27). After non-reducing electrophoresis, plasma-derived PS displayed a single band, but platelet PS appeared as a doublet. Plasma PS is partially cleaved at Arg60 in its TSR by a platelet-bound protease, resulting in a doublet only under reducing conditions (2, 10, 12). This suggests that either platelet PS is cleaved differently than its plasma counterpart, or that there are two forms of platelet PS. For FV/Va, another plasma cofactor protein found in platelets, differences in proteolytic susceptibility were reported (28), and in contrast to plasma-derived FV, platelet FV expresses substantial cofactor activity upon platelet secretion (28, 29). Further studies are needed to find whether platelet PS is a single form before secretion, whether it is a substrate for the same platelet-associated protease as described for plasma PS (10), and whether possible proteolysis affects its anticoagulant activity. Since the direct anticoagulant activity of platelet PS in platelet suspensions diminishes by 6 hr after phlebotomy and is seldom detectable after 24 hr (data not shown), proteolysis or loss of favorable modification over time is likely. The time required for purification and dialysis of purified platelet PS may allow for such proteolysis or modification loss, such that purified platelet PS is not more active than a good preparation of plasma-derived PS, while PS in fresh platelet suspensions appears to have substantially enhanced direct anticoagulant activity. We cannot exclude the possibility that a PS cofactor exists in releasate.

Differences in molecular weight between platelet PS and plasma PS might be due to differences in posttranslational modifications upon secretion and/or exposure to platelet enzymes. For example, in contrast to plasma FV, the heavy chain of platelet-derived FV/Va was found totally resistant to phosphorylation by a platelet-associated kinase (30), whereas it is uniquely modified at Thr402 with N-acetylglucosamine or N-acetylgalactosamine (31). We recently reported that kinases released from stimulated platelets phosphorylate plasma PS (32), and these studies will be extended.

Consistent with a report by Dahlbäck et al. (23), we demonstrated that plasma-derived PS binds to stimulated platelet microparticles. However, that study did not detect binding of PS to platelets themselves, while our results indicated Ca^{2+}-dependent binding of plasma-derived PS to stimulated platelets but not to unstimulated platelets. Explanations for this discrepancy might be that we used higher concentrations of biotin-labeled PS, more concentrated platelets, and analysis by immunoblotting, whereas Dahlbäck et al. used fluorescent-labeled antibody and flow cytometry for detection of PS in dilute platelet samples. In support of PS and platelet PS binding to platelets, controls such as in Fig. 2 showed nondetectable or barely detectable PS in the wash fractions of stimulated platelets, yet PS was found in the EDTA eluates of the stimulated, washed platelets. Thus, it is concluded that these forms bound to the stimulated, washed platelets. This was confirmed in activity assays such as in Fig. 4, where platelet PS on both the microparticles (A) and the stimulated, washed platelets (B) exerted direct anticoagulant activity. After washing the stimulated platelets, any fraction of microparticles present would be expected to be too small to account for the amount or activity of PS observed.

Several studies indicated that plasma-derived PS binds to unstimulated platelets as well. Mitchell et al. (10) reported that unstimulated human platelets provide 1,100 binding sites per platelet for PS, independent of APC or FVa, whereas we reported that unstimulated platelets provide ~190 binding sites for ^{125}I-human PS, while stimulated platelets provide 400 sites, independent of APC (2). Notably, it is difficult to prevent platelets from partial activation during platelet preparation or incubations (Fig. 3C). Also, we cannot exclude that Zn^{2+}-containing PS behaves differently from Zn^{2+}-deficient PS in binding studies. Many PS preparations used earlier were purified using anion exchange in the presence of EDTA, which causes loss of Zn^{2+}.

Overall, our data show that plasma-derived and platelet PS bind to stimulated platelets and their microparticles and that platelet PS is thus well-positioned to down-regulate procoagulant reactions, independently of APC or TFPI. Zn^{2+}-containing plasma-derived PS directly down-regulates thrombin and FXa generation on stimulated platelets, and behaves similarly to platelet PS in these experiments. Yet, possible differences in potency, apparent molecular weight, and proteolytic susceptibility to cleavage between plasma-derived PS and platelet PS suggest that platelet PS forms a unique pool. Further studies are needed to map the specific differences between platelet PS and plasma PS and the physiologic consequences thereof.

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References

1. Tans G, Rosing J, Thomassen MC, Heeb MJ, et al. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood. 1991; 77:2641–2648. [PubMed: 2043766]
2. Schwarz HP, Heeb MJ, Wencel-Drake JD, et al. Identification and quantitation of protein S in human platelets. Blood. 1985; 66:1452–1455. [PubMed: 2933098]
3. Schwarz HP, Fischer M, Hopmeier P, et al. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984; 64:1297–1300. [PubMed: 6238642]

4. Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. J Clin Invest. 2009; 119:2942–2953. [PubMed: 19729839]
5. Saller F, Brisset AC, Tchaikovski SN, et al. Generation and phenotypic analysis of protein S-deficient mice. Blood. 2009; 114:2307–2314. [PubMed: 19567881]
6. Heeb MJ, Marzec U, Gruber A, et al. Antithrombotic activity of protein S infused without APC in a baboon thrombosis model. Thromb Haemost. 2012; 107:690–698. [PubMed: 22370911]
7. Solymoss S, Tucker MM, Tracy PB. Kinetics of inactivation of membrane-bound FVa by APC. Protein S modulates FXa protection. J Biol Chem. 1988; 263:14884–14890. [PubMed: 2971660]
8. Harris KW, Esmon CT. Protein S is required for bovine platelets to support APC binding and activity. J Biol Chem. 1985; 260:2007–2010. [PubMed: 3156128]
9. Lundwall A, Dackowski W, Cohen E, et al. Isolation and sequence of the cDNA for protein S, a regulator of blood coagulation. Proc Natl Acad Sci USA. 1986; 83:6716–6720. [PubMed: 2944113]
10. Mitchell CA, Salem HH. Cleavage of protein S by a platelet membrane protease. J Clin Invest. 1987; 79:374–379. [PubMed: 2948968]
11. Long GL, Lu D, Xie RL, et al. Human protein S cleavage and inactivation by coagulation FXa. J Biol Chem. 1998; 273:11521–11526. [PubMed: 9565566]
12. Heeb MJ, Griffin JH. APC-dependent and -independent anticoagulant activities of protein S have different structural requirements. Blood Cells Mol Dis. 2002; 29:190–199. [PubMed: 12490286]
13. Brinkman HJ, Mertens K, van Mourik JA. Proteolytic cleavage of protein S during the hemostatic response. J Thromb Haemost. 2005; 3:2712–2720. [PubMed: 16359509]
14. Heeb MJ, Mosher D, Griffin JH. Activation and complexation of protein C and cleavage and decrease of protein S in plasma of patients with intravascular coagulation. Blood. 1989; 73:455–461. [PubMed: 2521800]
15. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of FV and FVa by APC. J Biol Chem. 1994; 269:31869–31880. [PubMed: 7989361]
16. O'Brien LM, Mastri M, Fay PJ. Regulation of FVIIIa by APC and protein S: Inactivation of cofactor in the intrinsic FXase. Blood. 2000; 95:1714–1720. [PubMed: 10688829]
17. Fernandes N, Mosnier LO, Tonnu L, et al. Zn^{2+}-containing protein S inhibits extrinsic FX-activating complex independently of TFPI. J Thromb Haemost. 2010; 8:1976–1985. [PubMed: 20492471]
18. Heeb MJ, Prashun D, Griffin JH, et al. Plasma protein S contains zinc essential for efficient APC-independent anticoagulant activity and binding to FXa, but not for efficient binding to TFPI. FASEB J. 2009; 23:2244–2253. [PubMed: 19244162]
19. Chattopadhyay R, Sengupta T, Majumder R. Inhibition of intrinsic Xase by protein S. Arterioscler Thromb Vasc Biol. 2012; 32:2387–2393. [PubMed: 22904276]
20. Ndonwi M, Broze G Jr. Protein S enhances the TFPI inhibition of FXa but not its inhibition of FVIIa-tissue factor. J Thromb Haemost. 2008; 6:1044–1046. [PubMed: 18419747]
21. Hackeng TM, Sere KM, Tans G, et al. Protein S stimulates inhibition of the tissue factor pathway by TFPI. Proc Natl Acad Sci USA. 2006; 103:3106–3111. [PubMed: 16488980]
22. Heeb MJ, Koenen RR, Fernandez JA, et al. Direct anticoagulant activity of protein S-C4b binding protein complex in Heerlen heterozygotes and normals. J Thromb Haemost. 2004; 2:1766–1773. [PubMed: 15456488]
23. Dahlbäck B, Wiedmer T, Sims PJ. Binding of anticoagulant vitamin K-dependent protein S to platelet-derived microparticles. Biochem. 1992; 31:12769–12777. [PubMed: 1463747]
24. Heeb MJ, Schuck P, Xu X. Protein S multimers and monomers each have direct anticoagulant activity. J Thromb Haemost. 2006; 4:385–391. [PubMed: 16420570]
25. Niemetz J, Fallon JT, Harrington E, et al. Rapid generation of thrombin by atheroma and platelets. J Thromb Haemost. 2004; 2:321–326. [PubMed: 14995996]
26. Heeb MJ, Mesters R, Tans G, et al. Binding of protein S to FVa associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993; 268:2872–2877. [PubMed: 8428962]
27. Ozawa H, Kobayashi I, Marumo M. is platelet protein S different from plasma protein S in human being? Japan J Clin Med. 1992; 50:292–296.

28. Monkovic DD, Tracy PB. Functional characterization of platelet-released FV and its activation by FXa and thrombin. J Biol Chem. 1990; 265:17132–17140. [PubMed: 2211616]

29. Kane WH, Mruk JS, Majerus PW. Activation of coagulation FV by a platelet protease. J Clin Invest. 1982; 70:1092–1100. [PubMed: 6752201]

30. Rand MD, Kalafatis M, Mann KG. Platelet coagulation FVa: The major secretory platelet phosphoprotein. Blood. 1994; 83:2180–2190. [PubMed: 8161784]

31. Gould WR, Silveira JR, Tracy PB. Unique in vivo modifications of coagulation FV produce a physically and functionally distinct platelet-derived cofactor: Characterization of purified platelet-derived FV/Va. J Biol Chem. 2004; 279:2383–2393. [PubMed: 14594814]

32. Stavenuiter F, Heeb MJ. Phosphorylation of protein S by platelet kinases enhances its direct anticoagulant activity. J Thromb Haemost. 2011; 9 [abstr].

What is known about this topic?

- Most procoagulant reactions take place on the surface of platelets.
- ~2.5% of the anticoagulant protein S (PS) in blood is contained in platelets and is released upon stimulation.
- PS is partially cleaved in its thrombin-sensitive region by a platelet membrane protease, and is thus impaired in its ability to serve as a cofactor to activated protein C (APC).
- Plasma PS has both APC cofactor activity and activity that is independent of APC (PS- direct). PS cleaved in the thrombin-sensitive region retains PS-direct.

What does this article add?

- Both platelet PS and Zn^{2+}-containing plasma-derived PS bind to washed, stimulated platelets and to their microparticles and exert PS-direct, independent of APC and TFPI.
- Platelet PS has slower electrophoretic mobility than plasma PS, and has equal or greater PS-direct than Zn^{2+}-containing plasma-derived PS.
- When platelet PS is neutralized, a mean of 2.6-fold more thrombin and 1.5-fold more FXa are generated in the presence of 2×10^8/mL washed stimulated platelets.
- Platelet PS may constitute an essential pool of PS that provides a local increase in PS levels to counterbalance procoagulant activities on stimulated platelets.

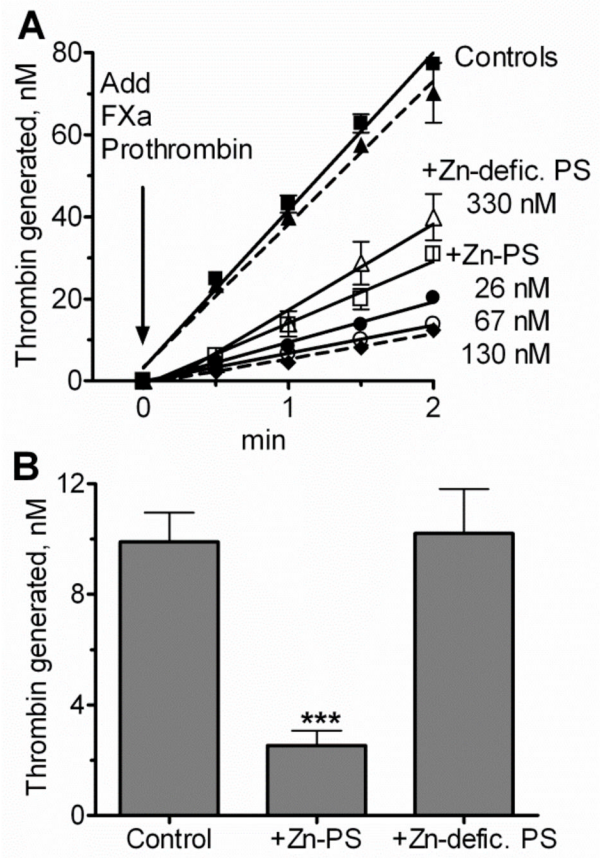

Figure 1. Stimulated platelets support the direct anticoagulant activity of plasma-derived PS

A) Washed platelets were stimulated with Ca^{2+}-ionophore (see Methods). Activations were performed in the absence and presence of Zn^{2+}-deficient plasma-derived PS or several concentrations of Zn^{2+}-containing plasma-derived PS (Zn-PS) and in the presence of neutralizing antibodies against APC and TFPI. As controls, similar incubations were performed in the absence of antibodies against APC and TFPI (dashed lines) for no PS and for 130 nM PS. After 8 min, FXa and prothrombin were added to initiate thrombin generation by FXa and platelet FVa. Aliquots of each mixture were removed at intervals and quenched, and thrombin formation was quantified. Data are from two representative experiments. B) Similar to A, except that platelets were stimulated with collagen, and 40 nM Zn-PS was used. (***, p=0.0002, n=5).

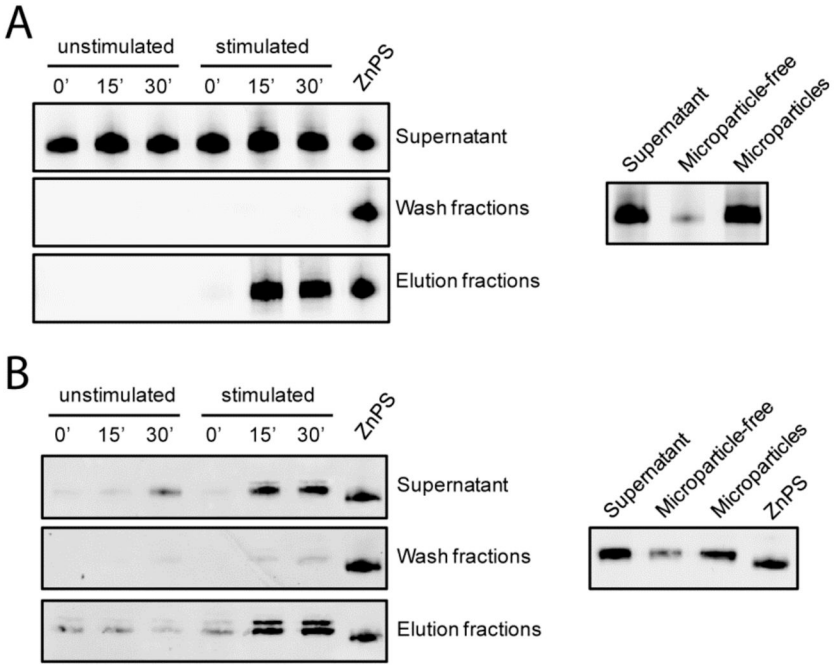

Figure 2. Plasma-derived and platelet PS bind to stimulated platelets and microparticles

A) *Left:* Washed platelets were stimulated with Ca^{2+}-ionophore in the presence of Ca^{2+} and Biotin-plasma-derived Zn^{2+}-PS. At times indicated, aliquots of platelets were gently washed, then PS was eluted with EDTA (see Methods). Unstimulated platelets were taken as controls. Non-reduced aliquots (10 μL) were subjected to SDS-PAGE and membrane transfer. Biotin-Zn^{2+}-PS in releasate, wash fractions, and EDTA eluate as well as in the standard biotin-ZnPS was detected using streptavidin-IRDye. A) *Right:* Biotin-Zn^{2+}-PS in supernatant (a), microparticle-free supernatant (b), and on washed microparticles (c). B) *Left:* Washed platelets were stimulated with Ca^{2+}-ionophore in the presence of Ca^{2+}. At times indicated, platelets were gently washed, then platelet PS was eluted with EDTA. Unstimulated platelets were taken as controls. Non-reduced aliquots (25 μL) were subjected to SDS-PAGE and immunoblotting using biotin-anti-PS. B) *Right:* Platelet PS in supernatant (a), microparticle-free supernatant (b), and on microparticles (c). Data were confirmed by other immunoblots. "Zn-PS" in panel B was non-biotinylated plasma-derived PS.

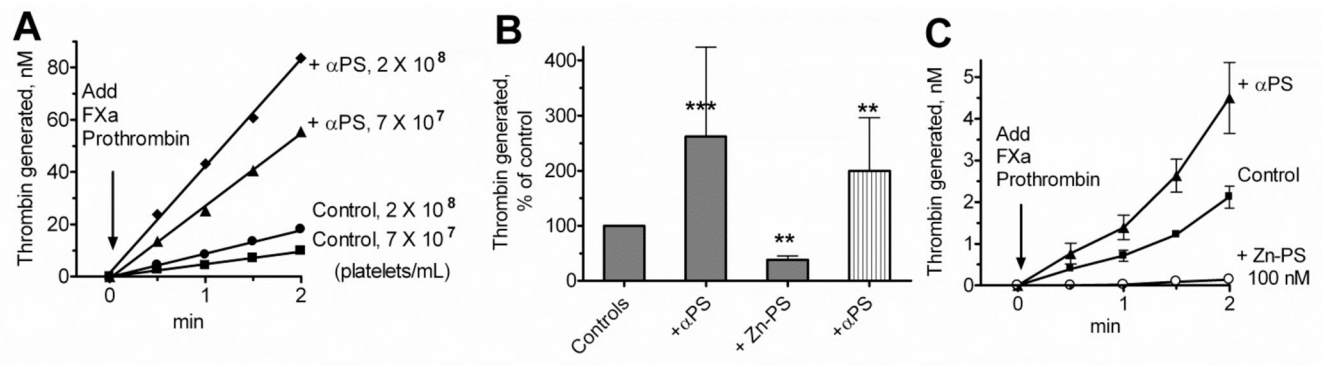

Figure 3. Platelet PS has direct anticoagulant activity

A) Washed platelets at two concentrations were stimulated with Ca^{2+}-ionophore in the presence of neutralizing antibodies against APC and TFPI, and in the presence and absence of neutralizing anti-PS antibodies. B) Washed platelets were stimulated with Ca^{2+}-ionophore (grey bars), or collagen (striped bar) in presence of neutralizing antibodies against APC and TFPI, and in the absence and presence of neutralizing anti-PS antibodies or in the presence of 40 nM plasma-derived Zn^{2+}-PS, as indicated. C) Unstimulated washed platelets were incubated in the presence of neutralizing antibodies against APC and TFPI, and in the absence or presence of anti-PS antibodies or of plasma-derived Zn^{2+}-PS. After agonist (in A–B), FXa and prothrombin were added to initiate thrombin generation. Aliquots of each mixture were removed and quenched at intervals, and thrombin formation was quantified (see Methods). Panel B shows percentage thrombin generation in the presence of anti-PS antibodies compared to that in controls (set to 100%) without anti-PS antibodies. In panel C, unstimulated platelets were gradually stimulated after addition of procoagulants. Means ± SD are shown. (***$p \leq 0.0004$, n=20; ** $p \leq 0.004$, n=4).

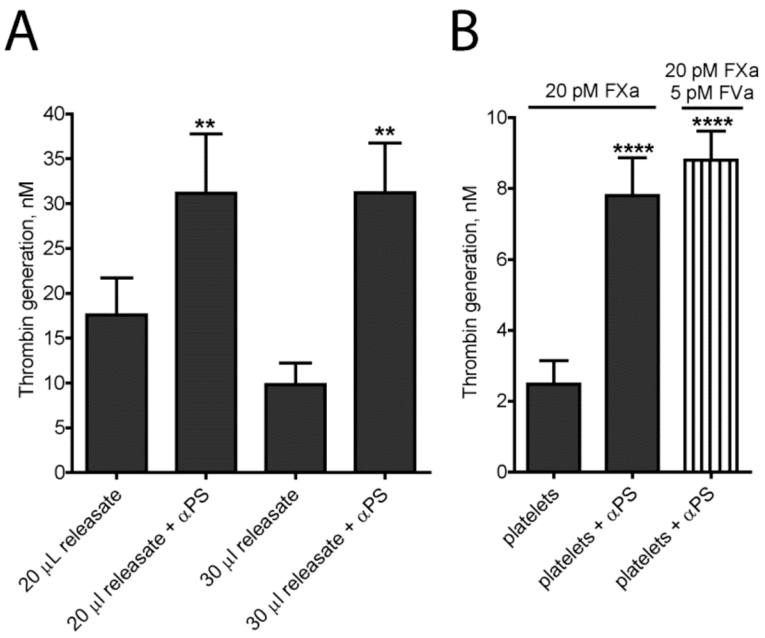

Figure 4. Both platelet- and microparticle-bound platelet PS exert direct anticoagulant activity

A) Releasate or B) washed stimulated platelets were incubated 5 min in the absence and presence of anti-PS antibodies (see Methods), followed by incubation with 20 pM FXa without (grey bars) or with (striped bar) 5 pM FVa added. After 5 min, prothrombin was added. At intervals, aliquots of each mixture were removed and quenched, and thrombin formation was quantified at 2 min. Means ± SD are shown. (**p <0.01, ****p <0.0001)

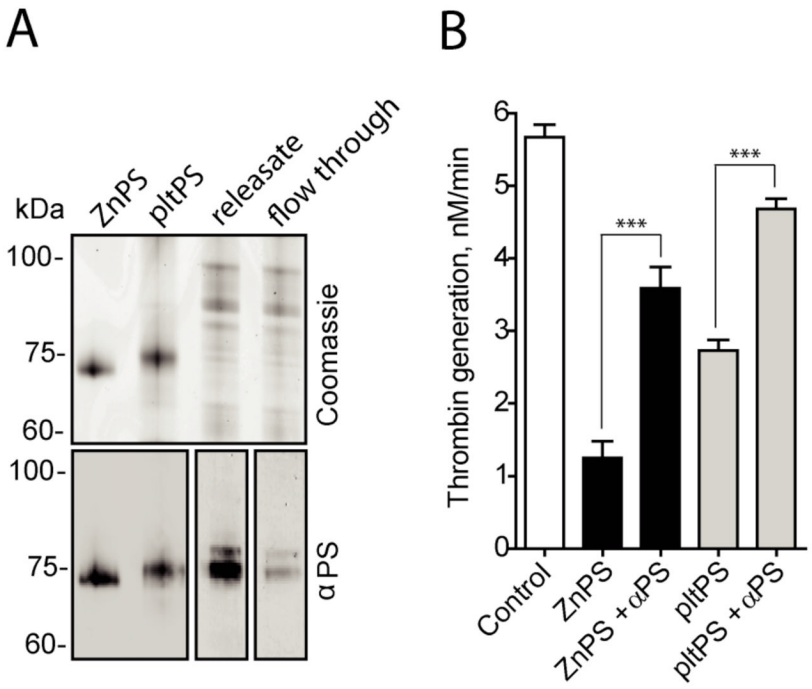

Figure 5. Purified platelet PS has direct anticoagulant activity and slower electrophoretic mobility than plasma-derived PS

A) Nonreduced purified Zn^{2+}-containing plasma-derived PS (ZnPS) and platelet PS (pltPS) (1 μg/lane), and releasate before and after immuno-precipitation (25 μL/lane) were analyzed on 4–12% gels. Protein was visualized by Coomassie staining (upper panel) or after Western blotting (lower panel). Blots were scanned at sensitivities 0.5, 4, and 7 for purified PS (left two lanes) and releasate (middle and right lanes), respectively. B) Purified ZnPS (black bars) and pltPS (gray bars) (140 nM) were incubated 8 min in the absence and the presence of anti-PS antibodies, then incubated with 20 pM FXa, 50 pM FVa, and 25 μM PC/PS (80/20%) lipid vesicles. After 5 min, prothrombin was added to initiate thrombin generation. After 2 additional min, aliquots were removed and quenched, and thrombin formation was quantified (see Methods) and compared to thrombin formation in the absence of neutralizing anti-PS antibodies. White bar represents thrombin generation in the absence of PS and anti-PS. Data are given as mean ± SD. (***$p<0.001$, n=5).

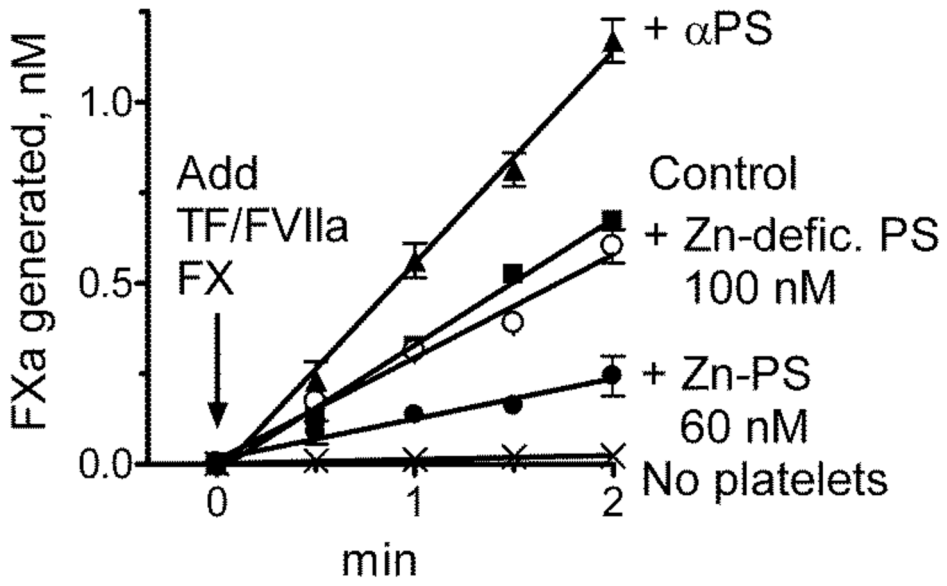

Figure 6. Platelet and plasma-derived PS inhibit FXa generation on stimulated platelets

Washed platelets in FXase assay buffer were stimulated with Ca^{2+}-ionophore in the presence of tissue factor in the presence of neutralizing antibodies against APC and TFPI, and in the absence or presence of neutralizing anti-PS antibodies, or PS, or platelets. After 8 min, extrinsic FXase was initiated by addition of procoagulants. Aliquots were quenched at intervals, and generation of FXa was measured. Data are from two representative experiments.