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Research Article

Glycoprotein IIb/IIIa blockade inhibits platelet aminophospholipid exposure by potentiating translocase and attenuating scramblase activity

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Abstract. The present study investigated the mechanisms underlying the inhibition of platelet phosphatidylserine (PS) exposure by GPIIb/IIIa blockade. Platelet PS exposure induced by thrombin stimulation was cell-cell contact dependent. GPIIb/IIIa blockade by c7E3 or SR121566 inhibited thrombin-induced platelet PS exposure. Thrombin stimulation induced mild, while A23187 induced extensive platelet-derived microparticle (PDMP) generation. Thrombin-induced PDMP generation was not inhibited by GPIIb/IIIa blockade. Aminophospholipid translocase activity was reduced upon platelet activation by thrombin. The reduction of non-PS-exposing platelets was attenuated by GPIIb/IIIa blockade, while little translocase activity was seen in PS-exposing platelets. Thrombin increased scramblase activity slightly in non-PS-exposing platelets, which was inhibited by GPIIb/IIIa blockade, and markedly enhanced scramblase activity in PS-exposing platelets. Activation of platelet calpain and caspase-3 or cytosolic calcium mobilization were not altered by GPIIb/IIIa inhibition. Thus, GPIIb/IIIa blockade inhibits platelet PS exposure by enhancing translocase activity and attenuating scramblase activity, but does not inhibit PDMP generation.

Keywords. Platelets, glycoprotein IIb/IIIa inhibitor, platelet-derived microparticles, phosphatidylserine, translocase, scramblase.

Introduction

Negatively charged aminophospholipids (mainly phosphatidylserine, PS, and some phosphatidylethanolamine, PE) are primarily located in the inner leaflet of resting platelets. The asymmetric phospholipid distribution is maintained by the dominating activity of aminophospholipid translocase (selectively and rapidly moving aminophospholipids toward the inner leaflet) over phospholipid scramblase (moving phospholipids unselectively and bidirectionally) [1, 2]. Platelet activation may disrupt asymmetric distribution of aminophospholipids resulting in PS exposure, and vesiculate their plasma membrane to generate platelet-derived microparticles (PDMP), apart from platelet shape change, secretion (including factor Va), and adhesion/aggregation. Platelets may decrypt encrypted tissue factor [3] and receive leukocyte-transferred and blood-born tissue factor [4], which

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acts in concert with FVIIa to activate FX directly. Hence, activated platelets are equipped with all four components of the prothrombinase complex of negatively charged phospholipid surface, FVa, FXa, and calcium, and play critical roles in blood coagulation. Platelet glycoprotein (GP) IIb/IIIa is the principle receptor for fibrinogen that bridges adjunct platelets to form platelet aggregates. The GPIIb/IIIa inhibitors are established anti-platelet drugs used in the management of acute coronary syndromes and percutaneous coronary interventions. There is evidence suggesting that mechanisms other than blockade of fibrinogen-GPIIb/IIIa ligation may contribute to the clinical benefits of GPIIb/IIIa inhibitors. Thus, GPIIb/IIIa inhibition reduces platelet thrombus formation [5] and facilitates thrombolysis of platelet-rich clots [6]. GPIIb/IIIa inhibition attenuates thrombin generation [7, 8], due to the ability to reduce platelet aminophospholipid expression and FV/Va binding [9]. The GPIIb/IIIa inhibitors have also been shown to reduce PDMP generation [10].

Given the importance of platelet aminophospholipid exposure in blood coagulation, we investigated how GPIIb/IIIa inhibition affects changes of asymmetric distribution of aminophospholipids. We hypothesized that GPIIb/IIIa inhibition would alter the balance of translocase and scramblase activities during platelet activation. We also examined the possible influence of GPIIb/IIIa inhibition on platelet vesiculation. Our study demonstrated that platelet activation reduced platelet aminophospholipid translocase activity and increased scramblase activity. The alternation was counteracted by GPIIb/IIIa inhibition, resulting in decreased platelet PS exposure. Our data showed, however, that platelet vesiculation was not influenced by GPIIb/IIIa inhibition.

Methods and materials

Study subjects. Fifteen healthy volunteers (7 males and 8 females, aged 24–63 years) gave informed consent to participate in the study, which was approved by the Ethics Committee of the Karolinska Institute.

Reagents. The platelet GPIIb/IIIa inhibitors used in this study were the non-peptide inhibitor SR121566 (Sanofi Recherche, Toulouse, France) and the chimeric GPIIb/IIIa monoclonal antibody (mAb) 7E3 Fab fragment (c7E3; Centocor B.V., Leiden, The Netherlands).

Human α-thrombin, apyrase, bovine serum albumin (BSA), and the calcium ionophore A23187 were purchased from Sigma (St Louis, MO, USA). Equine collagen was from Nycomed Austria GmbH (Linz, Austria). In flow cytometric analyses, platelets were identified by fluorescein isothiocyanate (FITC)-conjugated GPIX (CD42a) mAb Beb 1 (Becton Dickinson; San Diego, CA, USA). FITC- or R-phycoerythrin (RPE)-conjugated annexin V (Becton Dickinson) was used to monitor platelet PS exposure. The Ca^{2+} tracer Fluo-3 AM was from Molecular Probes Inc. (Eugene, OR, USA). The fluorescent lipid probe 1-Oleoyl-2-{6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phospho-L-serine (NBD-PS) was from Avanti Polar Lipids (Alabaster, AL, USA).

Preparation of washed platelets. Venous blood was collected without stasis using siliconized vacutainer tubes containing 1/10 volume of 3.8% trisodium citrate. Blood was centrifuged at 190 *g* for 10 min at 22°C to obtain platelet-rich plasma (PRP). The PRP was 1:2 mixed with wash buffer (9 mM Na_2EDTA, 26.4 mM Na_2HPO_4, 140 mM NaCl, 0.2 U/mL apyrase; pH 7.4) and centrifuged at 900 *g* for 10 min. The platelet pellet was resuspended in the assay buffer (137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, and 2 mM $CaCl_2$; pH 7.4) and platelet concentration was adjusted to 5×10^8/ml.

Flow cytometry. Flow cytometric samples were labeled with fluorescent annexin V or antibodies at room temperature for 20 min in the dark, unless otherwise specified. Flow cytometric analyses using a Beckman Coulter EPICS XL-MCL flow cytometer were performed as described [11]. Platelets and PDMPs were identified by a FITC-GPIX/CD42a mAb. Single platelets and PDMPs were gated according to their characteristic light scattering signals in reference to 10 μM A23187-stimulated platelet samples. The percentages and/or mean fluorescence intensity (MFI) of annexin V-positive and NBD-PS-positive platelets and PDMPs were recorded. Fluorescent beads (Rainbow® particles 3.0–3.4 μm; Becton Dickinson) were used to determine platelet and PDMP counts. Platelet cytosolic calcium ($[Ca^{2+}]_i$) levels are reported as MFI of the Ca^{2+} tracer Fluo-3 AM [11].

Experimental procedure of platelet PS exposure and PDMP generation. Aliquots (500 μl) of platelet suspension (platelet concentrations: 0.5×10^8, 2.5×10^8, or 5×10^8/ml) were added to prewarmed cuvettes and incubated at 37°C for 10 min in the absence or present of GPIIb/IIIa inhibitors without stirring. Vehicle, thrombin (1 U/ml), or A23187 (10 μM) were then added, and the samples were further incubated without stirring for 20 min. Afterwards, the samples were labeled with annexin V-FITC, and the incubation was terminated by adding an equal volume of the binding buffer (10 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$, pH 7.4) containing Rainbow® beads.

Preparation of platelet lysates. To prepare platelet lysates, the above-described platelet stimulation was terminated by adding 1/5 volume of ice-cold lysis buffer (50 mM Tris, 750 mM NaCl, 5 mM EGTA, 5 mM EDTA, 5% NP-40, pH 7.3) containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). The lysates were then aliquoted and stored at –80°C.

Immunoblotting. Platelet lysates containing equal amounts of proteins were subjected to immunoblotting. The samples were boiled in the presence of loading buffer for 5 min. Proteins were separated by SDS-PAGE (4–12% gel; Invitrogen, Carlsbad, CA, USA), and then transferred to a PVDF membrane. The membrane was probed with the anti-calpain mAb B27D8 (1:5000; Abcam, Cambridge, UK) that recognizes both inactivated and activated catalytic subunit of the enzyme [12], and then with polyclonal goat anti-mouse secondary antibodies (Dako, Glostrup, Denmark). The immunoreactive bands were detected by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), and intensity of the calpain bands was measured using a CCD camera (LAS 1000, Fuji Film Co., Tokyo, Japan) set in light transmission mode.

Measurement of aminophospholipid translocase and scramblase activity. Translocase selectively transfers aminophospholipids from the external leaflet to the inner leaflet of the plasma membrane, while scramblase bidirectionally moves all phospholipids. The movement of aminophospholipids by the two enzymes can be tracked by the probes of a fluorescent aminophospholipid analogue that have been loaded in the plasma membrane. Proteins (e.g., BSA) in the incubation medium rapidly extract the lipid probes exposed on the external leaflet but not the ones remaining in the inner leaflet of the plasma membrane. Therefore, using experimental protocols without or with BSA, the fluorescent lipid probes (thus the aminophospholipids) distributed in both leaflets, exposed on the external leaflet, and remaining in the inner leaflet of the plasma membrane can be assessed [13, 14].

In the present study, platelet translocase and scramblase activities were assessed by the distribution of the fluorescent lipid probe NBD-PS over the two leaflets of the plasma membrane using a BSA-back-exchange protocol [13] and flow cytometry [14] with minor modifications. To measure aminophospholipid translocase activity, platelets (0.5×10^8 platelets/ml) were preincubated without or with c7E3 (20 μg/ml) or SR121566 (50 μM) for 5 min at 37°C, and then incubated for 5 min at 37°C without or with thrombin (1 U/ml). Afterwards, NBD-PS (final concentration 0.5 μM, amounting to approximately 2 % of the endogenous phospholipid content) was added, and sample aliquots were taken at different time points (before and 2, 4, 6, or 8 min after adding NBD-PS), mixed with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose; pH 7.4) with or without BSA (final concentration 1 %) to monitor the amount of NBD-PS present in the inner leaflet and the total amount present in both leaflets, respectively. After an additional 5-min incubation to allow BSA-back extraction of NBD-PS on the outer leaflet, the samples were labeled with RPE-annexin V to detect surface-exposed endogenous PS. After this time, the samples were analyzed by flow cytometry.

To measure scramblase activity, platelets (5×10^8 platelets/ml) were preloaded at 37°C for 30 min with 2.5 μM NBD-PS (amounting to approximately 1 % of the endogenous phospholipid concentration), and the GPIIb/IIIa inhibitors c7E3 (20 μg/ml) or SR121566 (50 μM) were added at the last 5 min. Subsequently, the NBD-PS loaded platelets at the concentrations of 2.5×10^8/ml were further incubated without or with thrombin (1 U/ml) or A23187 (1 μM) at 37°C for 5 min. Thereafter, sample aliquots containing 2.5×10^6 platelets were transferred to HEPES buffer without or with BSA (final volume 100 μl, 1 % BSA) and further processed as described in translocase assay.

Caspase activity measurement. Caspase-3-like activity was measured using the 7-amino-methylcoumarin-derived substrate DEVD-AMC (Peptide Institute, Osaka, Japan). Proteolytic cleavage of DEVD-AMC by caspase-3 produces a bright blue fluorescent product and allows the measurement of caspase-3 activity using a modified fluorometric assay [15]. Washed platelets were incubated without or with thrombin or A23187 for 20 min in the absence or presence of 20 μg/ml c7E3. The incubation was terminated by adding a cell lysis buffer supplied (BioSource International, Camarillo, CA, USA). After centrifugation, the lysate aliquots (8×10^7 platelets/well) were added to a black 96-well microplate. Caspase activity assay was initiated by adding DEVD-AMC and reaction buffer (100 mM HEPES, 10 % sucrose, 5 mM dithiothreitol (DTT), 0.0001 % NP40, and 0.1 % CHAPS, pH 7.25). The fluorescence was measured during a 120-min incubation at 37°C (excitation wavelength: 340–360 nm, emission wavelength: 440–460 nm) using a FL600 microplate fluorescence reader (Labsystem, Stockholm, Sweden), and the data were expressed as caspase-3 activity units per million cells.

Statistics. Data are presented as mean ± SEM, if not otherwise specified. Effects of GPIIb/IIIa blockade were analyzed by paired Student's *t*-test and/or repeated measurements ANOVA. $p<0.05$ was considered statistically significant.

Results

Effect of GPIIb/IIIa inhibition on PS exposure. Figure 1a shows that thrombin stimulation (1 U/ml, 37°C, 20 min, no stirring) elevated annexin V binding levels in washed platelets (5×10^8/ml), from 3.5±0.9 % to 53.2±3.6 % in platelets and from 51.1±7.0 % to 77.1±5.5 % in PDMPs. The calcium ionophore A23187 increased annexin V binding of platelets and PDMPs even more markedly. The GPIIb/IIIa antagonists c7E3 (20 μg/ml) and SR121566 (50 μM) markedly reduced the percentages of annexin V-binding positive cells of thrombin-activated platelets, but slightly increased annexin V binding in PDMPs.

Thrombin stimulation also elevated MFIs of the FITC-annexin V-positive platelets (Fig. 1b). The enhancement was abolished by c7E3 or even reversed by SR121566. Thrombin stimulation did not increase FITC-annexin V MFI of PDMPs, whereas the latter was slightly elevated by GPIIb/IIIa blockade (Fig. 1b).

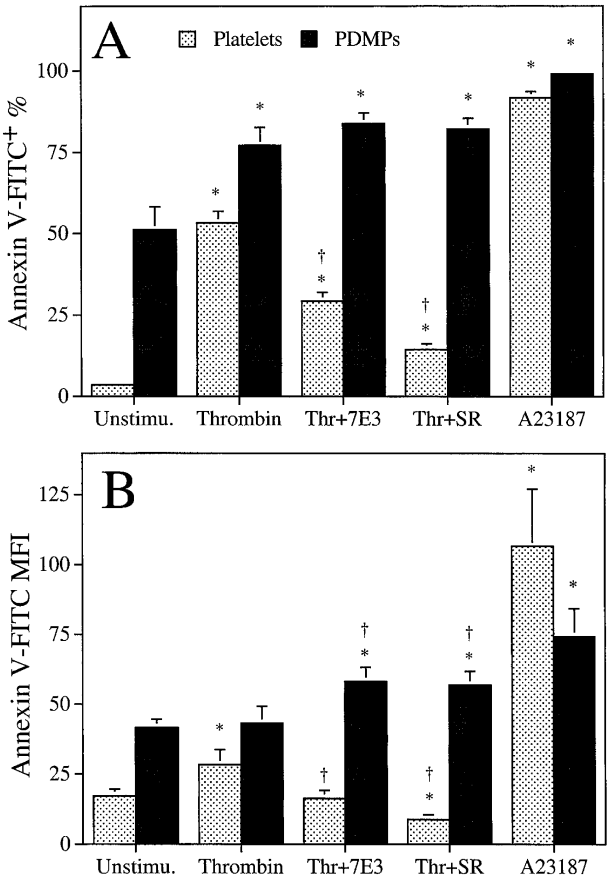

Figure 1. Effect of glycoprotein (GP) IIb/IIIa blockade on platelet phosphatidylserine (PS) exposure. Washed platelets (5×10^8/ml) were preincubated without or with the GPIIb/IIIa inhibitors c7E3 (20 μg/ml) or SR121566 (50 μM) at 37°C for 10 min. The samples were further incubated in the absence or presence of thrombin (1 U/ml) or A23187 (10 μM) for 20 min without stirring. Afterwards, the samples were labeled with FITC-annexin V, and annexin V binding/PS exposure of platelets and platelet-derived microparticles (PDMPs) were measured by flow cytometry. Mean ± SEM of annexin V-positive percentages (*a*) and mean fluorescence intensity (MFI) (*b*) are plotted; $n=6$. * $p<0.05$, compared to unstimulated samples; † $p<0.05$ compared to corresponding thrombin-stimulated samples.

Effect of GPIIb/IIIa antagonists on dynamic exposure of PS. Platelet suspension (10^6/ml) containing FITC-annexin V was stimulated with thrombin or A23187, and platelet annexin V binding was continuously monitored by flow cytometry before and after stim-

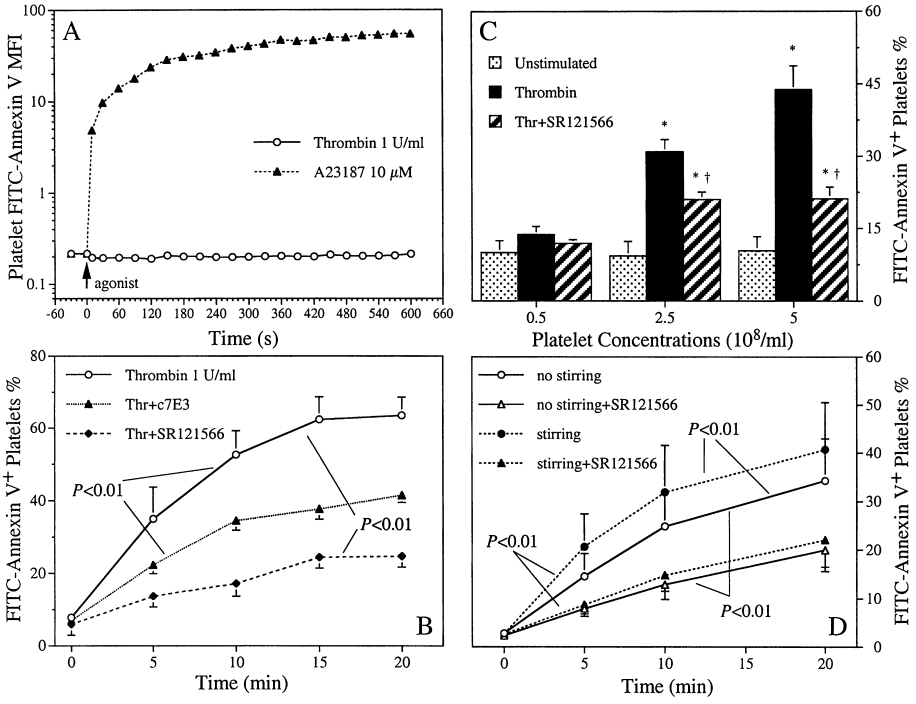

Figure 2. Influence of cell-cell contact and GPIIb/IIIa inhibition on platelet PS exposure. (*a*) Washed platelets were suspended in binding buffer at the concentration of 10^6/ml and in the presence of FITC-annexin V. Platelet annexin V binding was continuously monitored before and after thrombin (1 U/ml) or A23187 (10 μM) stimulation using flow cytometry. (*b*) Washed platelets (5×10^8/ml) were preincubated without or with c7E3 (20 μg/ml) or SR121566 (50 μM) (37°C, 10 min). The samples were stimulated with 1 U/ml thrombin, and aliquots were taken at 0, 5, 10, 15, and 20 min and labeled for flow cytometric measurement of platelet annexin V binding. *p* values are from repeated measures ANOVA analysis; $n=6$. (*c*) Platelet suspensions with different cell concentrations (0.5, 2.5, and 5×10^8/ml) were preincubated at 37°C for 10 min without or with 50 μM SR121566, and further incubated in the absence or presence of thrombin (1 U/ml) for 20 min. Samples were then labeled with FITC-annexin V and analyzed with flow cytometry. * $p<0.05$ compared with unstimulated samples; † $p<0.05$ compared with corresponding thrombin-stimulated samples without SR121566; $n=4$–7. (*d*) Washed platelets (2.5×10^8/ml) were preincubated without or with 50 μM SR121566, and then stimulated by 1 U/ml thrombin without or with stirring (1000 rpm). Platelet PS exposure was monitored by FITC-annexin V using flow cytometry. *p* values are from repeated measures ANOVA analysis; $n=4$.

ulation. Figure 2a shows that A23187 stimulation boosted platelet annexin V binding immediately, whereas thrombin stimulation hardly changed platelet annexin V binding during the 10 min of continuous measurement. The results suggested that mechanisms underlying thrombin- and A23187-induced platelet PS exposure may be different, and that low platelet concentration (*i.e.*, low level of direct cell-cell contact) may be the factor limiting PS exposure in thrombin-stimulated samples. Subsequently, PS exposure was investigated in platelet suspension incubated at higher concentration (5×10^8/ml), and 1 U/ml thrombin-stimulated platelet FITC-annexin V binding was monitored at 5-min interval for 20 min in the absence or presence of GPIIb/IIIa inhibitors (Fig. 2b). Thrombin stimulation gradually enhanced platelet PS exposure, which peaked at approximately 15 min. GPIIb/IIIa blockade significantly reduced the speed and the level of platelet PS exposure ($p<0.01$), in which c7E3 and SR121566 reduced platelet annexin V binding by approximately 30 % and 60 % at 20 min, respectively.

Further investigations on the impacts of GPIIb/IIIa blockade and direct cell-cell contact on platelet PS exposure were also performed in platelet suspension with different cell concentrations and with stirring. Thrombin stimulation did not significantly enhance platelet annexin V binding in the samples with a platelet concentration of 0.5×10^8/ml. However, the same stimulus markedly enhanced platelet annexin V binding in the samples with platelet concentrations of 2.5×10^8/ml and particularly 5.0×10^8/ml (Fig. 2c), and the enhancement was attenuated by GPIIb/IIIa blockade with SR121566. Figure 2d shows that thrombin-induced platelet annexin V binding was more rapid and more marked in stirred samples (*i.e.*, with increased cell-cell contact) than in unstirred samples (platelet concentrations: 2.5×10^8/ml for both). Stirring/contact-dependent enhancement on thrombin-elevated platelet annexin V binding was observed already at 5 min and was persistent throughout the observation. The enhancement was, however, almost abolished in the presence of GPIIb/IIIa blockade with SR121566.

Influence of GPIIb/IIIa blockade on PDMP generation. Thrombin-induced PDMP generation was investigated in platelet suspensions with different cell concentrations (0.5×10^8, 2.5×10^8, and 5×10^8/ml). Figure 3a shows that platelet counts were considerably decreased (40–50 %) during incubation (37°C, 20 min) even without stirring or stimulation, presumably due to cell adhesion to the cuvette wall and/or to each other to form micro-aggregates. Thrombin stimulation (1 U/ml) reduced single platelet counts further in the platelet suspensions with high cell concentrations (by 68 % at 2.5×10^8/ml and by 90 % at 5×10^8/ml). Thrombin-induced reductions of single platelets were attenuated by GPIIb/IIIa blockade with c7E3 and SR121566. In contrast, thrombin stimulation or GPIIb/IIIa inhibition influenced single platelet counts little in 0.5×10^8/ml platelet suspension. The calcium ionophore A23187 (10 μM) reduced single platelet counts by more than 95 % (Fig. 3a).

Thrombin stimulation elevated PDMP numbers in the platelet suspensions with the platelet concentrations at 0.5×10^8 and 2.5×10^8/ml (Fig. 3b). The elevations were not inhibited but slightly enhanced by GPIIb/IIIa blockade. PDMP counts in 5×10^8/ml platelet suspension with thrombin stimulation were not elevated, probably because the PDMPs were integrated into platelet aggregates, but were slightly elevated by GPIIb/IIIa blockade. A23187 markedly increased PDMP counts, from 79.5±12.6 without stimulation to 651.9±35.6×10^3 per million incubated platelets, and platelet vesiculation was not influenced by GPIIb/IIIa inhibition (data not shown). Collagen-induced PDMP generation was also investigated. Collagen or its combination with thrombin induced more PDMP generation than thrombin alone, which was not inhibited by GPIIb/IIIa blockade (data not shown).

The effect of stirring on PDMP generation was compared in 2.5×10^8/ml platelet suspensions. Figure 3c shows that stirring slightly increased PDMP numbers in unstimulated platelet suspensions. Thrombin stimulation enhanced PDMP generation in unstirred samples, which was further elevated by GPIIb/IIIa blockade. Without GPIIb/IIIa blockade, thrombin stimulus did not increased PDMP numbers in stirred platelet suspensions. With GPIIb/IIIa blockade, however, thrombin stimulation markedly increased PDMP counts, which were significantly higher than corresponding samples without stirring.

Effect of GPIIb/IIIa inhibition on aminophospholipid translocase activity. In the absence of BSA, approximately 99 % of unstimulated platelets became NBD-PS positive within 2 min of adding NBD-PS to the samples, suggesting a rapid NBD-PS integration in almost all platelets. The NBD-PS integration re-

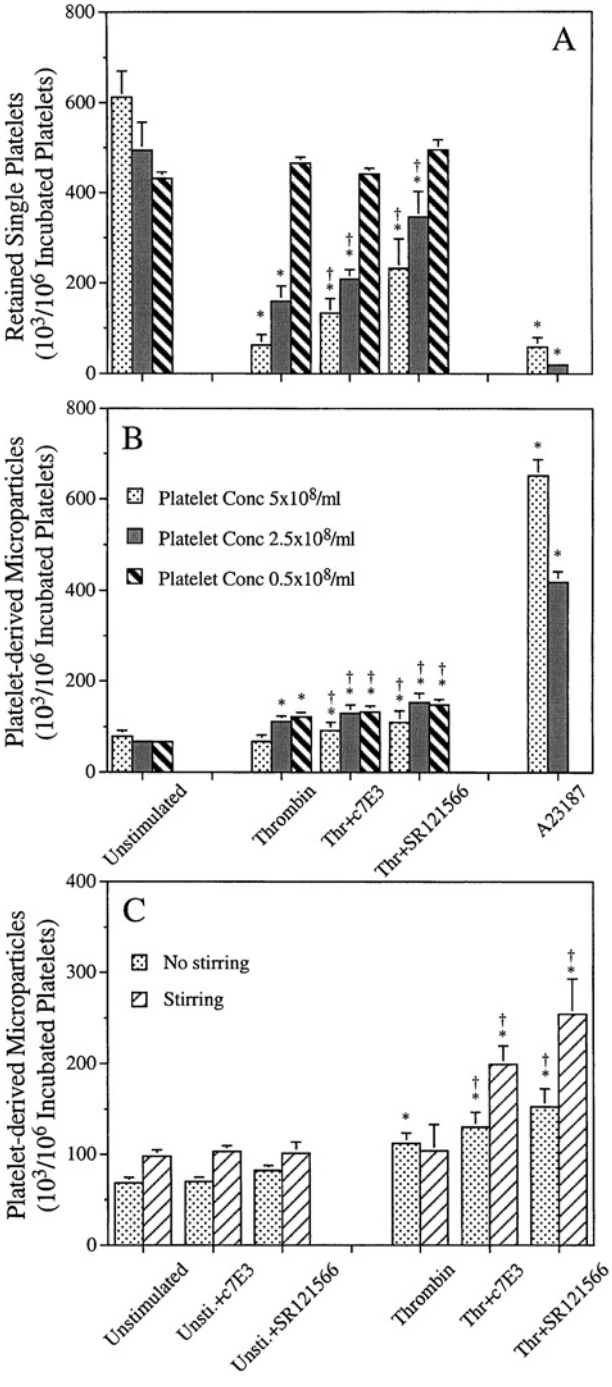

Figure 3. Influence of cell-cell contact and GPIIb/IIIa blockade on PDMP generation. Washed platelets with different cell concentrations (0.5, 2.5, and 5×10^8/ml for *a* and *b*, 2.5×10^8/ml for *c*; n=7 for all) were preincubated without or with 20 μg/ml c7E3 or 50 μM SR121566 at 37°C for 10 min. The samples were then incubated in the absence or presence of thrombin (1 U/ml) or A23187 (10 μM) for 20 min. The counts of single platelets and PDMPs were measured by flow cytometry using fluorescent beads. * $p<0.05$ compared with unstimulated samples, † $p<0.05$ compared with corresponding thrombin-stimulated samples without GPIIb/IIIa blockade.

mained unchanged during the prolonged incubation, and was not influenced by GPIIb/IIIa blockade by c7E3 or SR121566. In thrombin-stimulated samples, however, only 32.9±3.5 % of platelets were NBD-PS positive at 2 min. The percentage gradually increased over time, but reached only 70.8±5.5 % at 8 min. These data suggest that platelet activation reduces the efficiency of phospholipid integration, and that phospholipid integration of platelets may not be a simple chemical process. GPIIb/IIIa blockade by c7E3 or SR121566 elevated NBD-PS levels in thrombin-stimulated platelets. For instance, SR121566 increased the percentages to 40.0±8.7 % at 2 min and to 87.2±4.4 % at 8 min, respectively.

BSA, which extracts NBD-PS on the outer leaflet of phospholipids bilayers, did not markedly decrease NBD-PS-positive platelets (>95 % after platelets were incubated with NBD-PS for more than 2 min) in unstimulated samples (Fig. 4a), indicating that integrated NBD-PS was rapidly transferred into the inner leaflet of the plasma membrane, *i.e.*, high translocase activity. Thrombin stimulation markedly reduced NBD-PS-positive platelets in the samples (Fig. 4a), which were only 10.6±1.6 % at 2 min and 54.3±5.7 % at 8 min, and suggest reduction of translocase activity by thrombin stimulation. GPIIb/IIIa blockade did not influence the percentages of NBD-PS-positive unstimulated platelets, but elevated the percentages of thrombin-stimulated platelets (Fig. 4a). Analyses also showed that PDMPs hardly had any translocase activity (2–3 % NBD-PS positive at 8 min).

Platelets were also separated according to their characteristics of annexin V binding (annexin V negative, *i.e.*, non-PS-exposing, Fig. 4b; annexin V positive, PS-exposing, Fig. 4c). High translocase activity was seen in unstimulated non-PS exposed platelets; the enzyme activity was reduced by thrombin stimulation, and the reduction was attenuated by GPIIb/IIIa blockade with c7E3 and especially with SR121566 (Fig. 4b). Translocase activity was limited in thrombin-stimulated PS-exposing platelets, and was slightly, albeit significantly, elevated by GPIIb/IIIa blockade with SR121566 (Fig. 4c). PS-exposing platelets in unstimulated samples were rare and insufficient to generate reliable data, and the results were thus not presented in Figure 4c (the same in Fig. 4f).

Effect of GPIIb/IIIa inhibition on scramblase activity. Figure 4d shows that the percentages of NBD-PS-positive cells in NBD-PS-loaded unstimulated platelets were only slightly lower in the presence of BSA as compared to those without BSA (indicating limited scramblase activity), and that GPIIb/IIIa blockade by c7E3 influenced NBD-PS-positive platelets little. NBD-PS-positive platelets were reduced by thrombin stimulation, and further reduced in the presence of BSA, suggesting scramblase activation. GPIIb/IIIa blockade by either c7E3 or SR121566 attenuated the reductions, suggesting some inhibition of thrombin-induced scramblase activity.

When annexin V-negative platelets were analyzed (Fig. 4e), >95 % platelets were NBD-PS positive in unstimulated samples and were not affected by the addition of BSA, indicating that there was little scramblase activity in those platelets. Thrombin slightly reduced the percentage of NBD-PS-positive platelets, which suggested mild activation of scramblase. The changes were counteracted by GPIIb/IIIa blockade.

Figure 4f shows that the percentages of NBD-PS-positive platelets in thrombin-stimulated, annexin V-positive platelets were low, as compared to those of annexin V-negative platelets (Fig. 4e). The percentages were significantly reduced in the presence of BSA, indicating increased scramblase activity. These alterations were not influenced by the GPIIb/IIIa inhibitors.

NBD-PS-positive platelets were decreased by A23187 stimulation, and were further decreased in the presence of BSA (Fig. 4d–f). These data indicate that A23187 induced marked scramblase activation.

Impact of GPIIb/IIIa inhibition on platelet intracellular calcium mobilization. Thrombin stimulation evoked a spiked elevation of $[Ca^{2+}]_i$ in the absence of extracellular calcium, reflecting platelet intracellular calcium mobilization. Thrombin induced a more prominent and sustained $[Ca^{2+}]_i$ elevation in the presence of extracellular calcium, indicating platelet calcium influx. Neither platelet intracellular calcium mobilization nor calcium influx was influenced by GPIIb/IIIa blockade with c7E3 or SR121566 (data not shown).

Effect of GPIIb/IIIa antagonists on calpain activation. To confirm the present finding of no effect of GPIIb/IIIa blockade on PDMP generation, the enzyme activities of calpain and caspase 3, which are thought to be involved in PDMP generation [16], were also investigated. Using the calpain-specific mAb B27D8 that recognizes both native (inactive) and degraded (active) catalytic subunit of the enzyme [12], an 85-kDa immunoreactive band (inactive calpain) was detected in unstimulated samples, and an 81 kDa band (degraded calpain) was detected in thrombin-stimulated samples (Fig. 5a). The bands were not influenced in the presence of c7E3 or SR121566. In A23187-stimulated samples, two degraded forms (81 kDa and predominantly 78 kDa) of calpain catalytic subunit were detected.

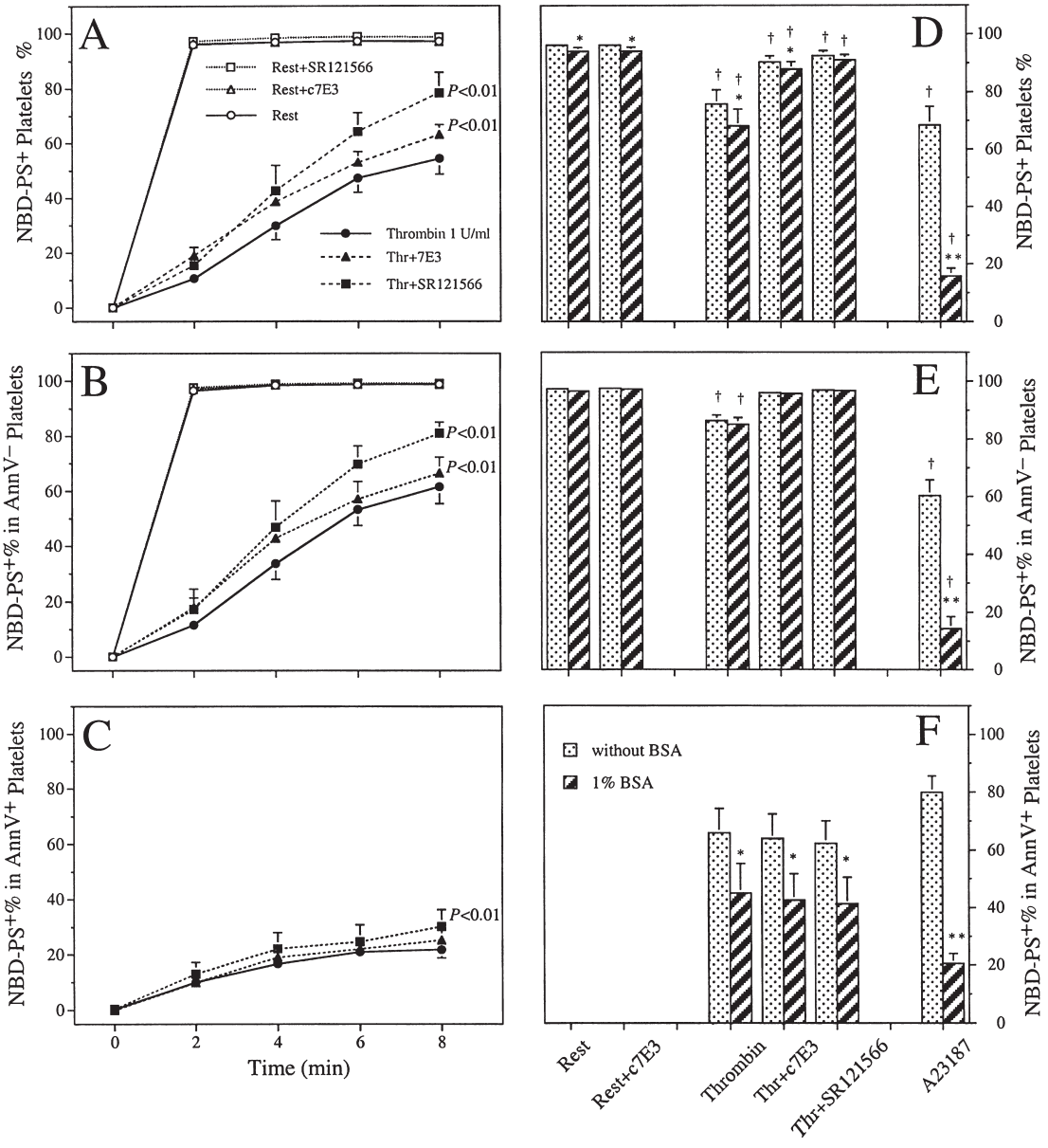

Figure 4. Effect of GPIIb/IIIa blockade on platelet aminophospholipid translocase and phospholipid scramblase activity. The protocols of translocase and scramblase activity measurement using the fluorescent lipid probe NBD-PS and BSA-back extraction were detailed in Methods and materials. NBD-PS integration and translocation were monitored by flow cytometry. Inward transportation of NBD-PS by translocase was monitored after BSA-back extraction and are shown in total platelets (*a*), non-PS-exposing (*b*), and PS-exposing (*c*; the events in unstimulated samples were rare, and the data were thus not shown) platelets. *p* values shown are from repeated measures ANOVA (with *versus* without GPIIb/IIIa blockade); $n=5$. Scramblase activity was elucidated by the difference of NBP-PS-positive platelet percentages between the samples without (dotted bars) and with NBD-PS back extraction by BSA (stripped bars). Mean ± SEM of the percentages of NBD-PS-positive cells in all platelets (*d*), non-PS-exposing (*e*), and PS-exposing (*f*; similar to *c*, the data were only from stimulated samples) platelets were plotted. * $p<0.05$, ** $p<0.01$, compared with corresponding samples without BSA, † $p<0.01$ compared with unstimulated samples without BSA; $n=5$.

Influence of GPIIb/IIIa antagonists on platelet caspase activation. Figure 5b shows that converted substrate of platelet caspase-3 accumulated over time, and that thrombin stimulation did not enhance platelet caspase-3 activity as compared to unstimulated platelets. Furthermore, platelet capase-3 activity was not affected by GPIIb/IIIa blockade with c7E3. Platelet caspase-3 activity was, however, enhanced by A23187 stimulation (Fig. 5b). As a positive control, A23187-stimulation induced a marked increase of caspase-3 activity in isolated lymphocytes, which was at least 1000-fold higher than that in platelets (data not shown).

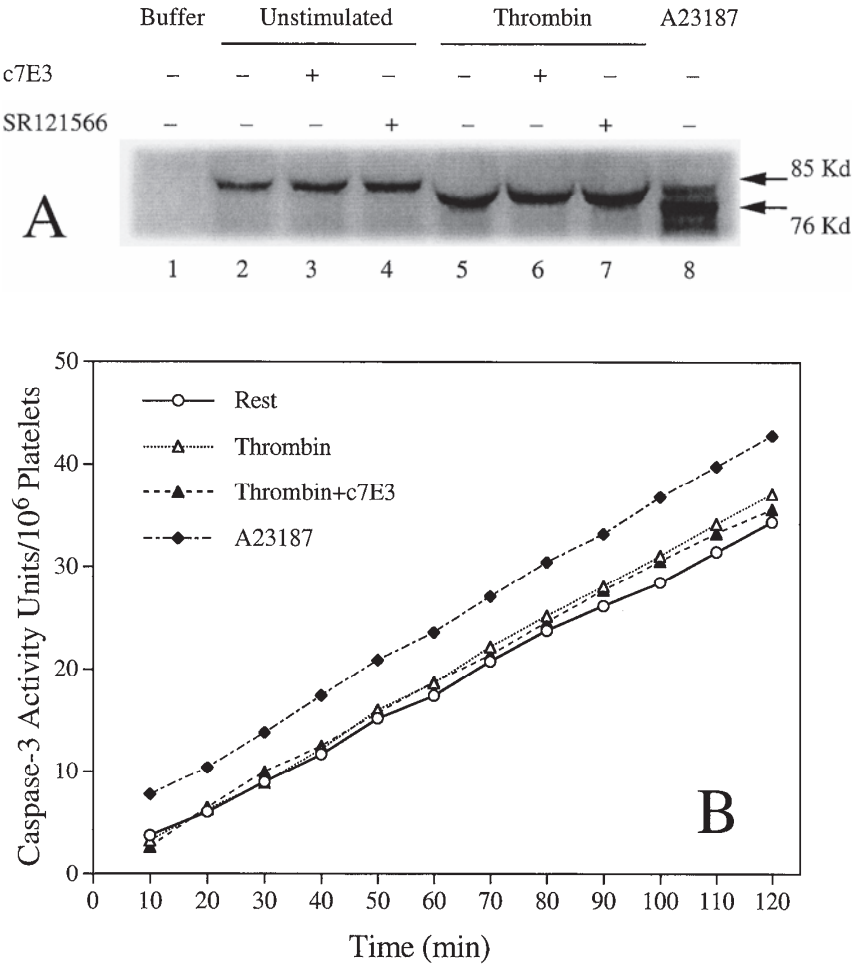

Figure 5. Influence of GPIIb/IIIa inhibition on platelet calpain and caspase-3 activation. (*a*) Washed platelets (5×10^8/ml) were preincubated without or with c7E3 (20 μg/ml) or SR121566 (50 μM) at 37°C for 10 min, and then further incubated without or with 1 U/ml thrombin or 10 μM A23187 for 5 min. The reaction was stopped by adding 5× ice-cold lysis buffer. Lysis buffer was used as the negative control (lane 1). Platelet calpain activation was probed with the calpain mAb B27D8. Results are a representative from four experiments. (*b*) Washed platelets were incubated without or with 20 μg/ml c7E3 and in the absence or presence of thrombin (1 U/ml) or A23187 (10 μM). The incubation was followed by platelet lysis. Caspase-3 activity of platelet lysates were monitored using the caspase-3 fluorescent substrate DEVD-AMC in a FL600 microplate fluorescence reader during a 120-min incubation at 37°C. Means from six experiments are plotted.

Discussion

The present study investigated the effects of GPIIb/IIIa blockade on platelet aminophospholipid exposure and PDMP generation. We found that GPIIb/IIIa blockade reduced PS exposure in thrombin-stimulated platelets but not in PDMPs, and that GPIIb/IIIa blockade achieved the effect by attenuating the reduction of aminophospholipid translocase activity and by inhibiting scramblase activity in thrombin-activated platelets. Thrombin stimulation induced mild PDMP generation. GPIIb/IIIa blockade did not inhibit thrombin-induced PDMP generation, platelet calcium mobilization or calpain activation.

Asymmetric distribution of negatively charged phospholipids is maintained by a predominant aminophospholipid translocase activity compared to scramblase activity in platelets [1]. Our results show that translocase rapidly transferred NBD-PS, the tracer of aminophospholipid transportation, to inner leaflet of the plasma membrane upon NBD-PS addition to unstimulated platelets, indicating high efficiency of the enzyme. When platelets were activated by thrombin, platelet translocase activity was reduced, and the percentage of platelets positive for internalized NBD-PS was only about 10% at 2 min after NBD-PS addition, compared to >95% in unstimulated platelets. The reduction was markedly attenuated by the GPIIb/IIIa inhibitors, which led to the inhibition of platelet PS exposure by those inhibitors. In agreement with a recent report by Wolfs et al. [14], we show that translocase activity was limited in PS-exposing thrombin-stimulated platelets. The present study shows that thrombin stimulation also resulted in a reduction of translocase activity in non-PS-exposing platelets.

The reductions the percentages of NBD-PS-positive cells by BSA in NBD-PS loaded platelets were small in non-PS-exposing platelets, and marked in PS-exposing platelets. Our data support the concept that scramblase activity is mainly observed in PS-exposing platelets [14]. Thrombin stimulation induced a mild decrease of NBD-PS-positive platelets, *i.e.*, scramblase activation in some non-PS-exposing platelets, and the change was virtually abolished by GPIIb/IIIa blockade. It should also be noted that thrombin and A23187 stimulation reduced the number of NBD-PS-

positive platelets in the absence of BSA. It appears that internalized NBD-PS of some platelets was totally transferred to the cell surface by activated scramblase, and that the exposed NBD-PS was then disintegrated from the cells and/or competitively taken up by adjacent cells.

Taken together, platelet activation reduces aminophospholipid translocase activity and hampers inward transport of aminophospholipids; platelet activation also enhances scramblase activity and increases outward transport of aminophospholipids. These two actions thus collaborate to elevate platelet PS exposure [14]. GPIIb/IIIa blockade attenuates the reduction of translocase activity and inhibits scramblase activity in activated platelets, and subsequently reduces platelet aminophospholipid exposure. The latter likely works in concert with GPIIb/IIIa blockade-reduced platelet FV/FVa binding [9] to constitute the mechanism underlying GPIIb/IIIa blockade-dependent inhibition of thrombin generation [7, 8]. This anticoagulant property of GPIIb/IIIa inhibitors may contribute importantly to their therapeutic effects. However, it should also be noted that GPIIb/IIIa inhibitors have been shown to increase the production of FVa-expressing COAT (collagen and thrombin-activated) platelets [17], and that GPIb is involved in thrombin-induced platelet procoagulant activity [18]. Furthermore, the present study supported the notion that direct cell-cell contact is important for platelet PS exposure [18], as thrombin-induced platelet PS exposure was enhanced at higher platelet concentrations and by stirring that increased cell-cell contact. For this reason, platelet suspensions with higher cell concentrations were used in the present study as most of our experiments did not involve sample stirring. Higher platelet concentrations increased platelet aggregation during thrombin stimulation (Fig. 3a). Thrombin-induced platelet aggregation is fibrinogen-GPIIb/IIIa and fibrin-GPIb [19, 20] dependent cell-cell adhesion, and is unlikely to recruit non-PS-expressing or PS-expressing platelets into platelet aggregates selectively, as thrombin (1 U/ml)-stimulated platelets are all positive for fibrinogen binding. Thus, we assume that measurement of the fraction of PS-expressing platelets (*i.e.*, percentages of annexin V-positive platelets) in suspension is presumably unaltered by the aggregation.

The present data show that GPIIb/IIIa blockade did not inhibit PDMP generation. Our data are, however, less than consistent with some previous reports [10, 21] showing that GPIIb/IIIa blockade reduced agonist- and shear-induced PDMP generation. It should be noted that PDMP generation is expressed as PDMPs generated per million incubated platelets in the present study, whereas PDMP generation was estimated according to the ratio of PDMPs over single platelets in those two studies. Agonist stimulation and shear stress induce platelet adhesion and microaggregation, and therefore reduce single platelet counts. The reduction of single platelets will increase PDMP/single platelet ratio even with an unchanged PDMP numbers. GPIIb/IIIa blockade inhibits agonist- or shear-induced platelet adhesion/aggregation and subsequently increases single platelet numbers, which will decrease the ratio and may give a false impression that GPIIb/IIIa blockade inhibits PDMP generation. Our results actually show that PDMP numbers were slightly elevated by GPIIb/IIIa blockade. This is likely because the GPIIb/IIIa inhibitors reduced adhesion/integration of PDMPs to platelets/platelet clot and subsequently elevated PDMP counts. Hence, the present results do not support previous observations [10, 21] that GPIIb/IIIa blockade inhibited PDMP formation. Furthermore, activation of calpain and caspase-3 and elevation of cytosolic calcium are involved in platelet vesiculation [16]. The present study showed that thrombin-induced platelet calpain activation was not altered by GPIIb/IIIa blockade, and that platelet caspase-3 activity was little enhanced by thrombin stimulation as previously shown by Wolf et al. [15]. Elevation of $[Ca^{2+}]_i$ levels was not influenced by GPIIb/IIIa blockade as we have demonstrated before [11]. Therefore, these lines of evidence support that GPIIb/IIIa blockade does not inhibit PDMP generation.

In conclusion, GPIIb/IIIa blockade attenuates the reduction of translocase activity and inhibits scramblase activity in activated platelets, and subsequently inhibits platelet aminophospholipid exposure. The present study indicates that platelet aminophospholipid exposure is a direct cell-cell contact-dependent event, and that GPIIb/IIIa blockade does not inhibit PDMP generation.

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