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Platelet α-granule secretion and its modification by SC-57101A, a GPIIb/IIIa antagonist

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Abstract

Platelet–agonist interaction results in aggregatory and secretory responses. While the activation of glycoprotein (GP) IIb/IIIa plays an essential role in platelet aggregation, its role in granule secretion is not clear. The present study was performed to examine the effect of 3-[[[[1-[4-(aminoiminomethyl) phenyl]-2-oxo-3*S*-pyrrolidinyl]amino]carbonyl]amino]-propanoate monohydrochloride salt (SC-57101A), a GPIIb/IIIa antagonist, on platelet α-granule secretion responses to collagen, ADP, and thrombin receptor activating peptide (TRAP). Both SC-57101A and prostaglandin E_1 (PGE_1) inhibited collagen-, ADP-, and TRAP-induced platelet aggregation in a concentration-dependent manner. SC-57101A inhibited the collagen- and ADP-induced release of platelet-derived growth factor (PDGF) and β-thromboglobulin (β-TG) from platelets, but not TRAP-induced secretion of these granule contents. On the other hand, PGE_1 inhibited the release of PDGF and β-TG from platelets activated with all the agonists used. ADP and TRAP elicited P-selectin expression in the absence of platelet aggregation, while collagen produced no such reaction. SC-57101A only moderately inhibited P-selectin expression induced by ADP and had no inhibitory effect on that induced by TRAP. The inhibition of ADP-induced secretion of α-granule contents by SC-57101A was abolished when platelets were pretreated with aspirin. These results suggest that GPIIb/IIIa activation plays a minor role, if any, in α-granule secretion in human platelets.

Keywords: GPIIb/IIIa; SC-57101A; Orbofiban; Platelets; α-Granule; Secretion

1. Introduction

Platelet activation at injured vessel walls is essential to hemostasis and arterial thrombosis [1–3]. Platelets are activated by a number of agonists such as collagen, ADP, and thrombin. Platelet–agonist interaction results in shape change, aggregation, and secretion of granule contents. ADP and 5-hydroxytryptamine released from the dense granules are potent activators of platelet aggregation and vasoconstriction. PDGF, β-TG, and P-selectin released from the α-granules are potent activators of smooth muscle cell proliferation and platelet–leukocyte adhesion [4–6]. While this clearly supports an important role of granule secretion in hemostasis and thrombosis, the mechanisms underlying the secretion of granule contents are not completely understood.

GPIIb/IIIa on the platelet membrane surface acts as a fibrinogen receptor and mediates the final common pathway for aggregation. GPIIb/IIIa antagonists inhibit cross-linking between active conformers of GPIIb/IIIa and fibrinogen. Indeed, GPIIb/IIIa antagonists have been shown to inhibit platelet aggregation induced by various kinds of agonists [7–9]. In contrast to these antiaggregatory effects, the antisecretory effects of GPIIb/IIIa have not been fully investigated. In the present study, we examined the effect of SC-57101A, the active metabolite of orbofiban [10], on platelet α-granule secretion responses to collagen, ADP, and TRAP. We also compared SC-57101A with PGE_1, an adenylyl cyclase activator. Our data suggest that the antisecretory effect of SC-57101A is associated with the inhibition of aggregation-dependent TXA_2 formation but not with its direct GPIIb/IIIa antagonistic activity.

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Abbreviations: PDGF, platelet-derived growth factor; β-TG, β-thromboglobulin; GPIIb/IIIa, glycoprotein IIb/IIIa; TRAP, thrombin receptor activating peptide; PGE_1, prostaglandin E_1; TXA_2, thromboxane A_2; FITC, fluorescein isothiocyanate; MoAb, monoclonal antibody; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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2. Materials and methods

2.1. Reagents and antibodies

SC-57101A (3-[[[[1-[4-(aminoiminomethyl) phenyl]-2-oxo-3*S*-pyrrolidinyl]amino] carbonyl]amino]-propanoate monohydrochloride salt) was supplied by G.D. Searle. PGE_1 was purchased from Cayman, and the RGDS peptide from Peptide Institute. ADP and aspirin were from Sigma Chemical Co., and TRAP (SFLLRN) was from Bachem. The activation-dependent anti-GPIIb/IIIa MoAb PAC-1 [11] conjugated with FITC was purchased from Becton Dickinson. The anti-P-selectin (anti-CD62P) MoAb (CLB-Thromb/6) conjugated with FITC was purchased from Immunotech.

2.2. Platelet aggregation study

Blood from healthy volunteers who had indicated that they had not used any medication for the previous 7 days was collected by venipuncture with a 21-gauge butterfly needle into plastic tubes containing 1/9 volume of 3.8% (w/v) sodium citrate. PRP was obtained by centrifugation at 100 *g* at room temperature for 15 min. The remaining blood was centrifuged further at 2,000 *g* for 10 min to obtain PPP. Platelet counts in PRP were adjusted to 3×10^8 platelets/mL using PPP. Platelet aggregation was determined according to the method of Born and Cross [12] on an aggregometer (PAM-8C; Mebanix). After preincubating the PRP (230 μL) at 37° for 1.5 min with stirring, the PRP was stimulated by 10 μL of collagen, ADP, or TRAP. Changes in light transmission were recorded after stimulation. Aliquots (10 μL) of inhibitor, or saline as a control, were added to the PRP 1.5 min before addition of the agonist. The extent of aggregation was estimated by the percent of maximum increase in light transmission, with the PPP representing 100% transmittance.

2.3. Enzyme immunoassay (EIA)

Aggregation was terminated by the addition of an ice-cold EDTA (10 mM)–indomethacin (100 μM) solution 6 min after stimulation with the agonist. The mixture was centrifuged at 10,000 *g* for 3 min at 4°. The supernatant was isolated and stored at −20° for PDGF and β-TG assay. PDGF-AB and β-TG levels in samples diluted with assay buffer (5- to 80-fold for PDGF and 50- to 800-fold for β-TG) were determined by EIA kits purchased from Amersham and Boehringer Mannheim, respectively. All samples were assayed in duplicate. The limit of detection of EIA for PDGF and β-TG was 0.1 ng/mL and 5 IU/mL, respectively.

2.4. Ligand binding study with a flow cytometer

PRP was diluted in 10 parts of modified Tyrode's buffer [137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM HEPES, 0.4 mM Na_2HPO_4, 5.5 mM glucose, 0.35% (w/v) BSA, pH 7.4]. The diluted PRP (460 μL) was incubated with 20 μL of SC-57101A or PGE_1 or RGDS peptide at room temperature for 10 min and stimulated for 5 min with 20 μL of agonists. An aliquot (100 μL) was further incubated at room temperature for 15 min with 20 μL of saturating concentrations of the FITC-conjugated antibodies. Platelets were then fixed immediately with 1.5 mL of 1% (v/v) formaldehyde for 15 min at room temperature.

The samples were analyzed by a flow cytometer (Epics XL, Coulter) at 488 nm excitation, set to detect FITC fluorescence with band-pass filters of 525 nm. Platelet populations were gated according to their forward and side light scatter. Histograms were generated using 5,000 counts. The levels of P-selectin expression and PAC-1 binding were expressed as the percentages of cells positive for anti-CD62P and PAC-1, respectively. The negative cut-off for each antibody was set using resting platelets that gave less than 4% of positive results. In some experiments, the PRP was incubated with 1 mM aspirin to prevent thromboxane formation.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. Data were analyzed using Dunnett's test for multiple comparison. The SAS software package (SAS Institute) was used for analysis, and *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. PDGF and β-TG release in the presence of platelet aggregation

Collagen (0.25 to 4 μg/mL), ADP (1.25 to 100 μM), and TRAP (1 to 100 μM) elicited platelet aggregation in a concentration-dependent manner (Fig. 1). In the presence of platelet aggregation, all agonists caused a concentration-dependent release of α-granule contents: PDGF and β-TG (Fig. 2). In subsequent studies to examine the effects of platelet aggregation inhibitors on α-granule secretion, the maximal concentrations of agonists (4 μg/mL collagen, 100 μM ADP, and 100 μM TRAP) that caused full responses were used.

3.2. Effects on PDGF and β-TG release in the presence of platelet aggregation

Treatment of platelets with SC-57101A (0.03 to 3 μM) and PGE_1 (0.03 to 1 μM) resulted in a concentration-dependent inhibition of collagen-, ADP-, and TRAP-induced platelet aggregation respectively (Figs. 3 and 4). The IC_{50} values of SC-57101A for collagen-, ADP-, and TRAP-induced platelet aggregation were similar (0.1 to

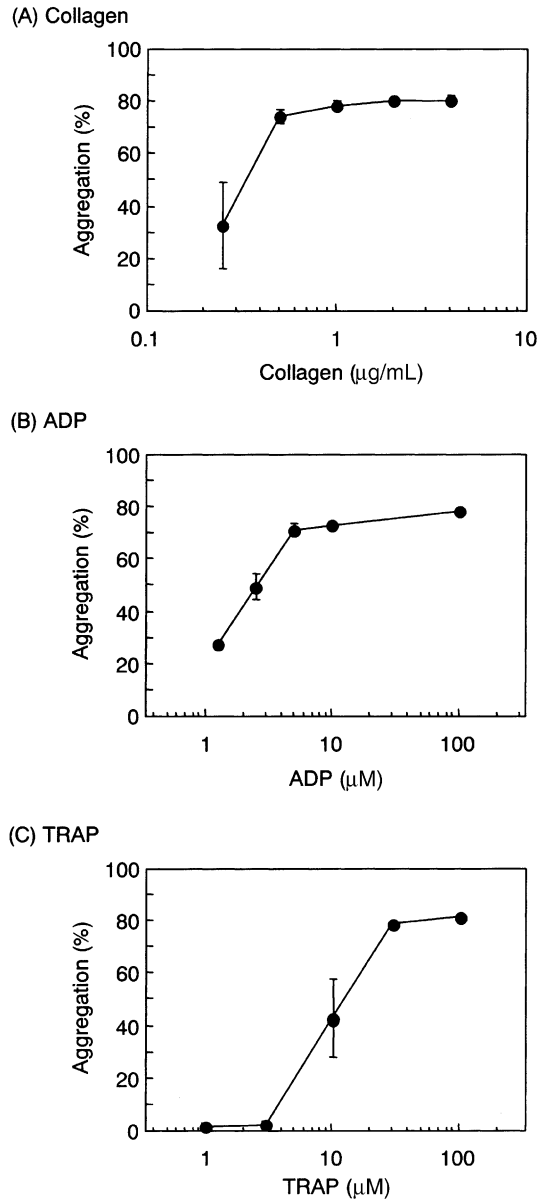

Fig. 1. Platelet aggregation induced by collagen (A), ADP (B), and TRAP (C). Data are presented as means ± S.E.M. (N = 6).

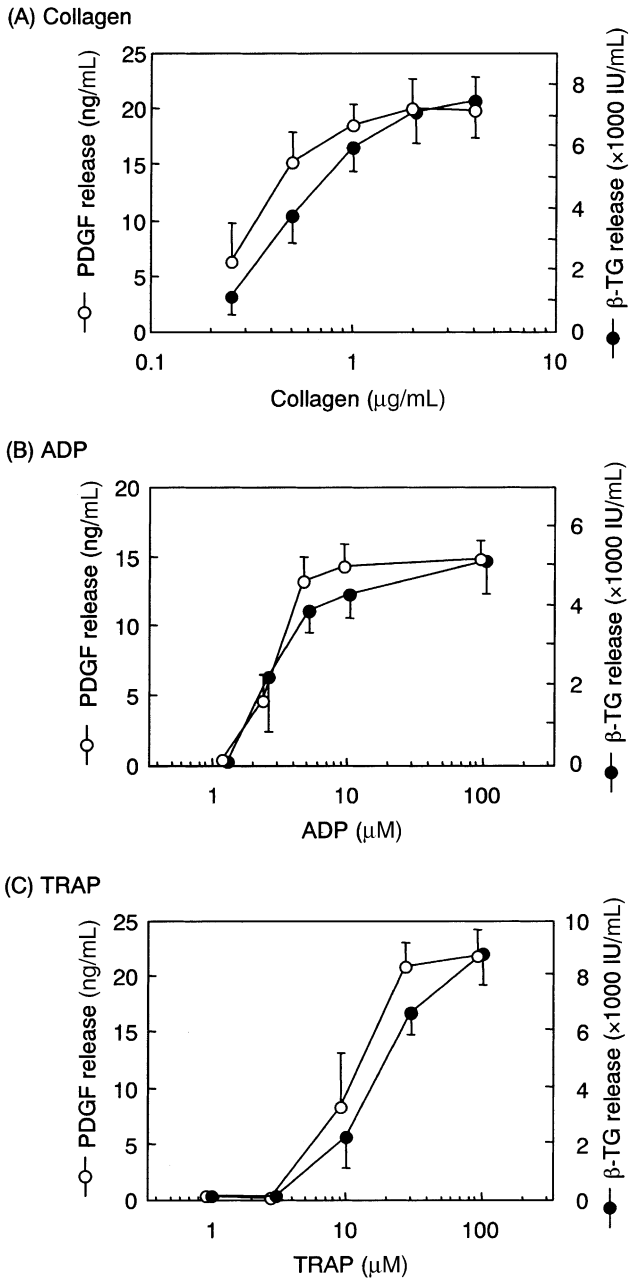

Fig. 2. PDGF and β-TG release from platelets stimulated with collagen (A), ADP (B), and TRAP (C) under stirring conditions. Data are presented as means ± S.E.M. (N = 6).

0.2 μM). PGE_1 also exhibited nearly the same inhibition of platelet aggregation induced by all the agonists used (IC_{50} values of 0.1 to 0.2 μM). PGE_1 inhibited the release of PDGF and β-TG from platelets stimulated with all the agonists used (Fig. 4). Likewise, SC-57101A (0.03 to 3 μM) inhibited collagen- and ADP-induced α-granule secretion in a concentration-dependent manner (Fig. 3A and B). However, SC-57101A did not inhibit the TRAP-induced release of PDGF and β-TG (Fig. 3C).

3.3. P-selectin expression and GPIIb/IIIa activation in the absence of platelet aggregation

P-selectin expression and GPIIb/IIIa activation in platelets were observed under non-stirring conditions to prevent platelet aggregation. Under these conditions, less than 4% of resting platelets were positive for P-selectin expression or GPIIb/IIIa activation (Fig. 5A). ADP (100 μM) and TRAP (100 μM) induced increases in P-selectin-positive cells to 31.2 ± 3.4% (N = 6) and 83.4 ± 2.0% (N = 6), respectively (Fig. 5B). In contrast, collagen failed to elicit P-selectin expression. GPIIb/IIIa activation was determined by the binding of PAC-1, an antibody against activated GPIIb/IIIa, to agonist-stimulated platelets. ADP (100 μM) and TRAP (100 μM) induced increases of PAC-1-positive cells to 89.5 ± 4.3% (N = 6) and 82.2 ± 7.6% (N = 6), respectively (Fig. 5C). Collagen

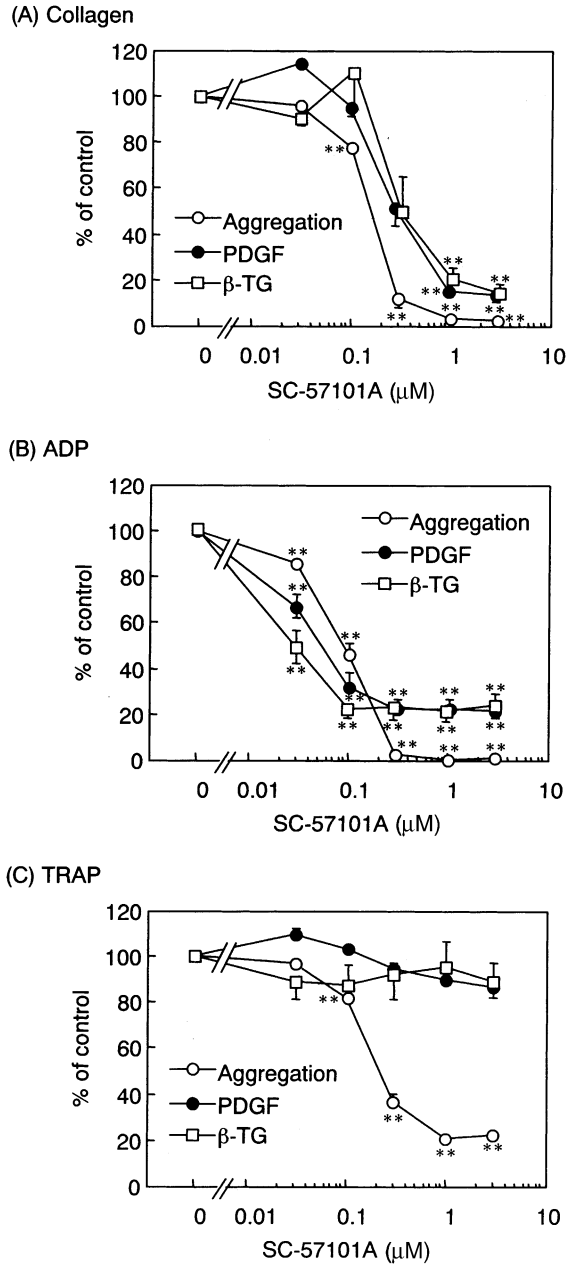

Fig. 3. Effects of SC-57101A on platelet aggregation, and release of PDGF and β-TG from platelets. Platelets were stimulated with 4 μg/mL collagen (A), 100 μM ADP (B), and 100 μM TRAP (C). Data are presented as means ± S.E.M. (N = 6). Key: (**) $P < 0.01$ vs. control. Platelet aggregation, PDGF, and β-TG levels without SC-57101A were as follows: (A) 81.2 ± 1.3 (%), 19.2 ± 3.0 (ng/mL), and 7.4 ± 0.8 ($\times 10^3$ IU/mL); (B) 77.8 ± 1.2 (%), 14.8 ± 1.3 (ng/mL), and 7.1 ± 2.4 ($\times 10^3$ IU/mL); (C) 81.2 ± 0.5 (%), 21.8 ± 2.3 (ng/mL), and 8.8 ± 1.2 ($\times 10^3$ IU/mL).

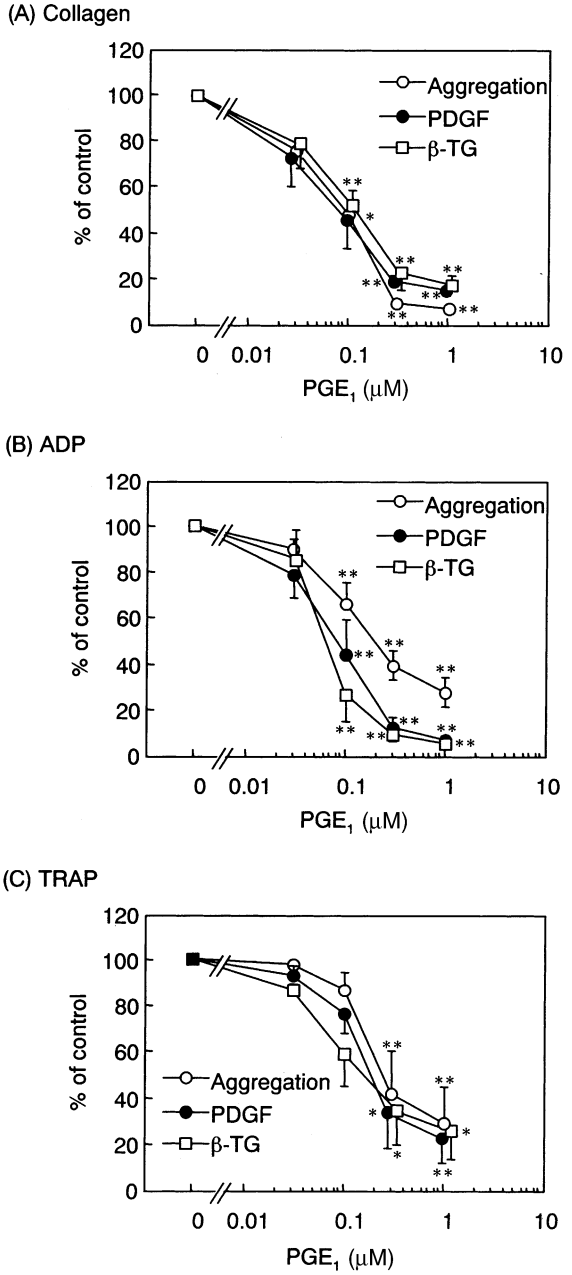

Fig. 4. Effects of PGE_1 on platelet aggregation, and release of PDGF and β-TG from platelets. Platelets were stimulated with 4 μg/mL collagen (A), 100 μM ADP (B), and 100 μM TRAP (C). Data are presented as means ± S.E.M. (N = 6). Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs. control. Platelet aggregation, PDGF and β-TG levels without PGE_1 were as follows: (A) 80.2 ± 1.4 (%), 18.1 ± 2.4 (ng/mL), and 6.1 ± 0.6 ($\times 10^3$ IU/mL); (B) 77.2 ± 1.4 (%), 13.0 ± 1.9 (ng/mL), and 6.1 ± 2.7 ($\times 10^3$ IU/mL); (C) 80.2 ± 1.1 (%), 18.3 ± 2.0 (ng/mL), and 6.6 ± 0.6 ($\times 10^3$ IU/mL).

did not induce any apparent PAC-1 binding under non-stirring conditions.

3.4. Effects on P-selectin expression and PAC-1 binding in the absence of platelet aggregation

SC-57101A (0.1 to 1 μM) caused a partial but statistically significant ($P < 0.05$) inhibition of ADP-induced P-selectin expression (Fig. 6A), whereas this agent had no effect on the P-selectin expression of TRAP-activated platelets (Fig. 6B). As shown in Fig. 6C, RGDS peptide (1 mM) inhibited ADP-induced P-selectin expression to a similar extent. PAC-1 binding was inhibited significantly by SC-57101A in a concentration-dependent manner (Fig. 7). PGE_1 (0.01 to 1 μM) inhibited ADP- and TRAP-induced P-selectin expression and PAC-1

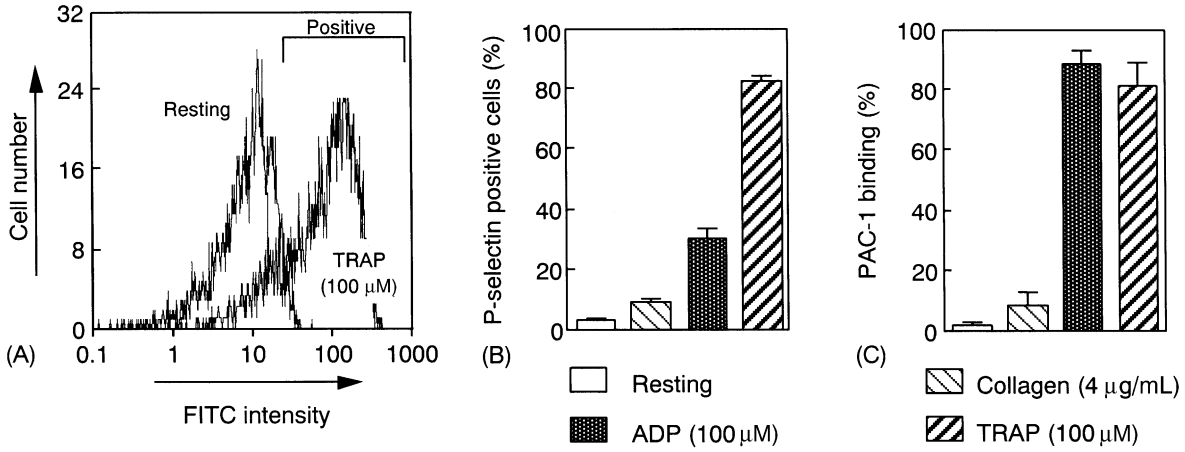

Fig. 5. (A) Flow cytometric histogram of FITC-conjugated anti-P-selectin MoAb binding to platelets stimulated with TRAP (100 μM). (B) P-selectin expression in platelets stimulated with collagen (4 μg/mL), ADP (100 μM), and TRAP (100 μM). (C) Binding of PAC-1 to platelets stimulated with collagen (4 μg/mL), ADP (100 μM), and TRAP (100 μM). Data are presented as means ± S.E.M. (N = 6).

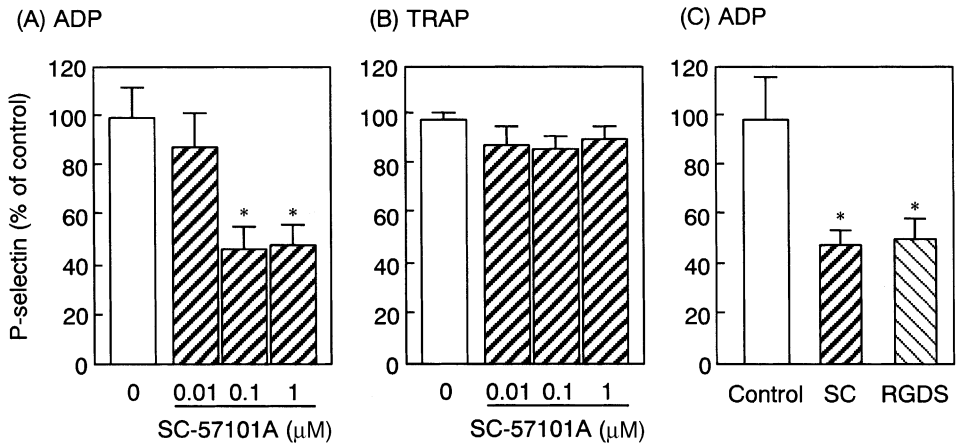

Fig. 6. Effects of SC-57101A on P-selectin expression in platelets stimulated with (A) 100 μM ADP and (B) 100 μM TRAP. (C) Effects of SC-57101A (1 μM) and RGDS peptide (1 mM) on P-selectin expression in platelets stimulated with 100 μM ADP. Data are presented as means ± S.E.M. (N = 5–6). Key: (*) $P < 0.05$ vs. control. The amount of P-selectin-positive cells (%) without inhibitors was as follows: (A) 31.2 ± 3.4 (N = 6), (B) 83.4 ± 2.0 (N = 6), and (C) 35.8 ± 5.8 (N = 5).

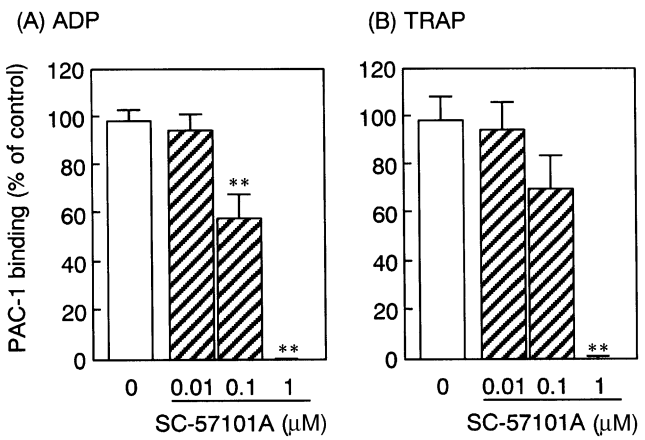

Fig. 7. Effects of SC-57101A on PAC-1 binding to platelets stimulated with (A) 100 μM ADP and (B) 100 μM TRAP. Data are presented as means ± S.E.M. (N = 6). Key: (**) $P < 0.01$ vs. control. The amount of PAC-1-positive cells (%) without SC-57101A was as follows: (A) 89.5 ± 4.3 and (B) 82.2 ± 7.6.

binding in a concentration-dependent manner (data not shown).

3.5. Influence of pretreatment with aspirin on the antisecretory effects of SC-57101A

To prevent *de novo* TXA_2 formation in ADP-activated platelets, platelets were pretreated with aspirin (1 mM), an irreversible cyclooxygenase inhibitor. The levels of PDGF and β-TG released from ADP-activated platelets were reduced to 30% of the levels released from control platelets (data not shown). While SC-57101A showed no apparent inhibition of the ADP-induced release of PDGF and β-TG, the agent abolished the platelet aggregation induced by ADP (Fig. 8A). In addition, neither SC-57101A (1 μM) nor RGDS (1 mM) elicited any apparent inhibition of the ADP-induced P-selectin expression in the absence of platelet aggregation in aspirin-pretreated platelets (Fig. 8B).

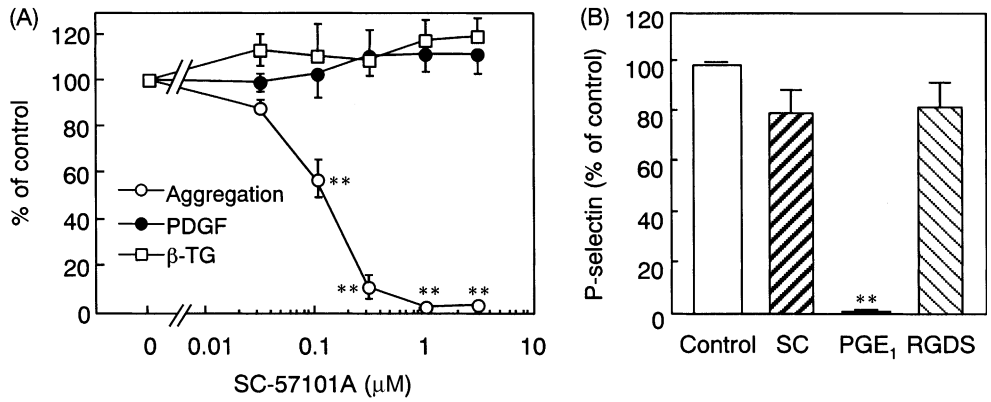

Fig. 8. (A) Effects of SC-57101A on platelet aggregation, and release of PDGF and β-TG from platelets pretreated with aspirin. (B) Effects of SC-57101A (1 μM), PGE_1 (1 μM), and RGDS peptide (1 mM) on ADP (100 μM)-induced P-selectin expression in platelets pretreated with 1 mM aspirin. Data are presented as means ± S.E.M. (N = 3–4). Key: (**) $P < 0.01$ vs. control. Platelet aggregation, PDGF, and β-TG without SC-57101A were as follows: 67.5 ± 2.7 (%), 5.3 ± 1.1 (ng/mL), and 1.7 ± 0.3 ($\times 10^3$ IU/mL) (N = 4). The amount of P-selectin-positive cells (%) without SC-57101A was 32.4 ± 0.2 (N = 3).

4. Discussion

The fibrinogen receptor GPIIb/IIIa plays a pivotal role in platelet adhesion and aggregation through both inside-out signaling and outside-in signaling [13–15]. However, conflicting results have clouded our understanding of the role of GPIIb/IIIa activation in platelet granule secretion [16–21]. In the present study, we examined the effects of SC-57101A, a non-peptide GPIIb/IIIa antagonist, on α-granule secretion in collagen-, ADP-, and TRAP-activated human platelets in comparison with the effects of PGE_1, a stimulator of adenylyl cyclase.

One of the common characteristics of SC-57101A and PGE_1 is that their antiaggregatory effects are independent of the agonists used. This was confirmed in the present study. In addition to inhibiting platelet aggregation, PGE_1 inhibited α-granule secretion induced by the three agonists used in our study. In contrast, the antisecretory effects of SC-57101A were agonist-dependent. While SC-57101A inhibited collagen- and ADP-induced PDGF and β-TG release in a concentration-dependent manner, it failed to inhibit TRAP-induced PDGF and β-TG release. This is consistent with previous studies reporting a dissociation between antiaggregatory and antisecretory effects of GPIIb/IIIa antagonists in TRAP- [22] and thrombin-activated platelets [18]. The differential effects of SC-57101A on PDGF and β-TG release among the three agonists are probably not due to the aggregatory potencies of the agonists, since the antiaggregatory effects (IC_{50} values) of SC-57101A were shown to be comparable among the three agonists. The present results suggest that platelet aggregation contributes in different ways to the secretion induced by collagen, ADP, and TRAP.

To further examine the effect of SC-57101A on α-granule secretion, we also measured the expression of α-granule membrane-associated P-selectin with flow cytometry under non-stirring conditions in the absence of platelet aggregation. Under these conditions, both ADP and TRAP-induced P-selectin expression and PAC-1 binding, reflecting α-granule secretion and GPIIb/IIIa activation, respectively, while collagen induced neither of these effects to an apparent degree (Fig. 5). A previous study has reported that while high concentrations of collagen (>10 μg/mL) are required to cause P-selectin expression, lower concentrations suffice to induce platelet aggregation [23]. The concentration of collagen used in our study (4 μg/mL) might have been too low to elicit P-selectin expression under non-stirring conditions. TXA_2 formation is believed to be essential for the onset of secretory and aggregatory responses in collagen-activated platelets. Indeed, the addition of U-46619 (10 μM), a TXA_2 analogue, to non-stirring platelets resulted in an increase of P-selectin expression (data not shown). These results suggest that α-granule secretion is closely linked to aggregation-dependent TXA_2 formation in collagen-activated platelets.

SC-57101A did not inhibit TRAP-induced P-selectin expression significantly in the absence of stirring. However, PAC-1 binding in platelets stimulated with TRAP was inhibited by SC-57101A in a concentration-dependent manner. These findings suggest that the blockade of fibrinogen binding to activated GPIIb/IIIa has no effect on TRAP-induced α-granule secretion, and that GPIIb/IIIa plays a minor role in α-granule secretion of human platelets. Previous studies have demonstrated that TRAP-activated TXA_2 formation in platelets is resistant to GPIIb/IIIa antagonists [22,24]. On this basis, we can postulate that *de novo* TXA_2 formation probably contributes to TRAP-induced α-granule secretion.

ADP-induced P-selectin expression was partially but significantly ($P < 0.05$) inhibited by SC-57101A. This is not likely to be a non-specific effect of SC-57101A, since RGDS peptide (1 mM) also inhibited ADP-induced P-selectin expression. It is well known that ADP stimulation produces TXA_2 in platelets with stirring under conditions

with low extracellular calcium concentrations [25]. In the present study, we examined the release of PDGF and β-TG from platelets anticoagulated with citrate, in which the extracellular Ca^{2+} concentrations were low. This raised the possibility that aggregation-dependent TXA_2 formation contributes substantially to α-granule secretion in ADP-activated platelets under stirring but not under non-stirring conditions. To test this hypothesis, we pretreated platelets with aspirin to prevent *de novo* TXA_2 formation during the ADP stimulation. Treatment with aspirin resulted in a reduction of the ADP-induced α-granule secretion. In addition, the inhibition of the ADP-induced PDGF and β-TG release by SC-57101A was not observed in aspirin-treated platelets. While GPIIb/IIIa antagonists have been reported to inhibit TXA_2 formation in ADP-activated platelets, the mechanism of this inhibition has not been elucidated [26,27]. Thus, the difference in the amounts of ADP-induced α-granule secretion in the presence and absence of stirring could be explained by the different amounts of TXA_2 formed after the ADP stimulation.

It was reported recently that platelet secretion and aggregation induced by U-46619, a TXA_2 mimetic, require signaling through the $P2Y_{12}$ receptor (G_i-linked P2T receptor) by secreted ADP [28,29]. This suggests that secreted ADP is an important factor in aggregation-dependent α-granule secretion, in addition to generated TXA_2. We did not measure the secretion of dense granules such as ADP in the present study. However, it is generally known that α-granule secretion is closely linked with dense-granule secretion. Therefore, involvement of secreted ADP in aggregation-dependent α-granule secretion could not be excluded. Further studies will be necessary to clarify this point.

Klinkhardt *et al.* [21] reported that there was a discoupling between the antiaggregatory effects and the antisecretory effects of GPIIb/IIIa antagonists. However, they have not determined any difference in the effects of GPIIb/IIIa antagonists among the agonists. They measured the release induced by collagen only under stirring conditions and that by TRAP only under non-stirring conditions using whole blood. In contrast, we measured granule secretion by three major agonists, ADP, collagen, and TRAP, under both stirring and non-stirring conditions using PRP. We clearly showed that there is a dissociation between the antiaggregatory and the antisecretory effects of the GPIIb/IIIa antagonist with TRAP but not with collagen or ADP. Further, we showed that the antisecretory effect of GPIIb/IIIa antagonists on ADP-induced secretion might be related to the block of the outside-in signaling through the GPIIb/IIIa, leading to TXA_2 production.

In summary, SC-57101A inhibited the α-granule secretion of collagen- and ADP-activated platelets, but not TRAP-activated platelets, despite the uniform inhibition of platelet aggregation. The inhibition of ADP-induced α-granule secretion by SC-57101A was probably due to the blocking of aggregation-dependent TXA_2 formation. These results provide additional evidence that GPIIb/IIIa activation plays a minor role, if any, in human platelet α-granule secretion.

Acknowledgments

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