

ORIGINAL**Inhibitory Effects of Aspirin and Cilostazol on Intracellular Ca²⁺ Mobilization and Aggregation in Thrombin-activated Human Platelets**Atsumi Sone¹, Kensaku Aki², Toshiyuki Yasui³, and Eiji Hosoi²¹Subdivision of Biomedical Laboratory Sciences, Graduate School of Health Sciences, Tokushima University, Tokushima, Japan, ²Department of Cells and Immunity Analytics, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan, ³Department of Reproductive and Menopausal Medicine, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Abstract : Platelets play an important role in physiological hemostatic mechanisms. In contrast, platelet activation has been implicated in pathological conditions, such as atherosclerosis, angiogenesis, and inflammation. Thrombin is considered to be of particular pathological importance as a platelet-activating substance, and thrombin-activated platelets are detected in the blood of patients with advanced occlusive arterial disease. Ca²⁺ acts as a second messenger in platelet activation, and the regulation of intracellular Ca²⁺ concentrations ([Ca²⁺]_i) is important for controlling platelet functions. However, changes in [Ca²⁺]_i by antiplatelet agents remain unclear. Therefore, we herein investigated the relationship between [Ca²⁺]_i and the intensity of platelet aggregation after a thrombin stimulation, the relationship between [Ca²⁺]_i and the intensity of platelet aggregation by antiplatelet agents, and the effects of antiplatelet agents on thrombin-activated platelets as a surrogate platelet model for arterial occlusive disease. Fura2-loaded platelets were treated with phosphate-buffered saline or a low concentration of thrombin (0.005 U/mL), followed by antiplatelet agents (aspirin or cilostazol), and changes in [Ca²⁺]_i and the intensity of platelet aggregation by the thrombin stimulation were measured using fluorescence spectrophotometry. Changes in [Ca²⁺]_i and the intensity of platelet aggregation after the thrombin stimulation as well as the relationship between [Ca²⁺]_i and the intensity of platelet aggregation by antiplatelet agents indicated that cilostazol exerted stronger antiplatelet effects than aspirin and also that antiplatelet effects may be attenuated in thrombin-activated platelets. The present results also suggest the utility of thrombin-activated platelets as a surrogate platelet model for arterial occlusive disease. These results may contribute to future drug development for antiplatelet therapy. *J. Med. Invest.* 70:94-100, February, 2023

Keywords : intracellular Ca²⁺ concentration, intensity of platelet aggregation, aspirin, cilostazol, thrombin

INTRODUCTION

Platelets are small cells (2-3 μm) that play an important role in hemostatic mechanisms. They form a hemostatic thrombus when vascular endothelial cells break down. When vascular endothelial cells are ruptured, platelets are activated by adhering to exposed collagen in endothelial tissue, and the release of intracellular granules further promotes platelet aggregation. The blood coagulation system is simultaneously activated, and the production of thrombin triggers the formation of fibrin, which strongly activates platelets (1). Ca²⁺ as a second messenger plays an important role in this chain of reactions (2, 3). However, platelet activation has been implicated in pathologies such as arteriosclerosis, angiogenesis, and inflammation (1, 4, 5).

Although many substances are involved in platelet activation, the representative activation mechanism may be summarized as the mobilization of intracellular Ca²⁺ concentrations ([Ca²⁺]_i) by the activation of phospholipase C (PLC) upon a stimulation and the production of thromboxane A₂ (TXA₂), a strong inducer of platelet aggregation (6-8). Thrombin is an aggregation attractant that activates PLC and increases [Ca²⁺]_i in platelets. Among platelet-activating substances, thrombin is considered to

be pathologically important, and thrombin-activated platelets have been detected in the blood of patients with advanced arterial occlusive diseases, particularly cerebral and myocardial infarction. Antiplatelet therapy with aspirin and cilostazol is used to treat and prevent the recurrence of these diseases (9-14). Aspirin is an antiplatelet agent that inhibits platelet aggregation by irreversibly suppressing cyclooxygenase-1 (COX-1), thereby reducing the production of TXA₂ (15-17). Cilostazol is another typical antiplatelet agent that selectively inhibits phosphodiesterase III and suppresses platelet aggregation by increasing the concentration of cAMP in platelets (18-21). Although aspirin and cilostazol have different mechanisms of action, they ultimately inhibit platelet activation by controlling [Ca²⁺]_i. Therefore, variations in [Ca²⁺]_i are important for the effects of antiplatelet agents in antiplatelet therapy. However, the mechanisms underlying [Ca²⁺]_i fluctuations and associated platelet aggregation by these antiplatelet agents have not yet been elucidated in detail. In the present study, we assessed [Ca²⁺]_i fluctuations and associated platelet aggregation during a thrombin stimulation, and examined the effects of [Ca²⁺]_i on platelet aggregation. We also investigated the relationship between [Ca²⁺]_i and the intensity of platelet aggregation in the platelet aggregation inhibition response to antiplatelet agents (aspirin and cilostazol) as well as the effects of these antiplatelet agents on thrombin-activated platelets as a surrogate platelet model for arterial occlusive disease.

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MATERIALS AND METHODS

Ethics Statement

The present study was approved by the Ethics Committee of the University of Tokushima Hospital (approval number 2072-6). Written informed consent was obtained from all volunteers, and all participants signed consent forms approved by the Ethics Committee.

Study participants

Six healthy participants aged between 24 and 64 years old (4 males and 2 females; mean age \pm SD, 40.8 ± 17.2 years) with no history of drug use and no underlying health conditions affecting platelet function were included in the present study.

Chemicals

Thrombin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Aspirin (acetylsalicylic acid) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and cilostazol was provided from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Fura2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). CaCl_2 solution (0.025 M) was from Muto Pure Chemicals Co., Ltd. (Tokyo, Japan). Phosphate-buffered saline (PBS) was from Sigma-Aldrich Co. Dimethyl sulfoxide (DMSO) was purchased from Kanto Chemical Industry Co., Ltd. (Tokyo, Japan). Triton X-100 was from Wako Pure Chemical Industries, Ltd., acid citrate dextrose (ACD) was from Terumo (Tokyo, Japan), and ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) was from Sigma-Aldrich Co.

Preparation of Fura2-loaded platelets

Human platelets were collected from six healthy adult volunteers. Blood collected from humans was mixed with ACD (blood : ACD = 6 : 1) and centrifuged at $163 \times g$ at room temperature for 15 min. Supernatant platelet-rich plasma (PRP) was incubated with Fura2-AM (2 μL Fura2-AM in 1 mL PRP) at 37°C for 50 min in the dark. After being incubated, ACD at 15% of the total volume of PRP was added, and the mixture was centrifuged at $720 \times g$ for 10 min. After the removal of the supernatant, the platelet pellet was washed twice with 5 mL HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 10 mM HEPES, 5 mM glucose, and pH 7.4)/750 μL ACD ($720 \times g$ for 6 min). The platelet count was adjusted with HEPES buffer to $1 \times 10^8/\text{mL}$, and changes in $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation were measured.

Measurement of $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation by the thrombin stimulation in untreated and thrombin-activated platelets

First, 480 μL of the Fura2-AM-treated platelet suspension, resuspended in HEPES buffer at 1×10^8 cells/mL, was placed in a quartz cell, followed by preheating at 37°C for 20 seconds in a fluorescence spectrophotometer. Subsequently, 10 μL of 0.025 M CaCl_2 was added at a final concentration of 1 mM (set to the concentration of Ca^{2+} in blood), and 60 seconds later, 20 μL of 0.13 U/mL thrombin (or PBS as a control) was added at a final concentration of 0.005 U/mL for treatment for 60 seconds. Then, 10 μL of aspirin or cilostazol (or a DMSO-water mixture as a control) was added for 6 minutes, followed by stimulation with 20 μL of 1.1 U/mL thrombin at a final concentration of 0.04 U/mL. After 180 seconds, 100 μL of 1% Triton X-100 was added. Then, after 100 seconds, 100 mM EGTA was added. Fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, and the intensity of platelet aggregation was assessed by light scattering at an excitation wavelength of 380 nm and an emission wavelength of 400 nm using a fluorescence spectrophotometer (F-2500; Hitachi Ltd., Tokyo, Japan). $[\text{Ca}^{2+}]_i$ in human platelets and the intensity of platelet aggregation were denoised using the Savitzky Golay smoothing filter included in the $[\text{Ca}^{2+}]_i$ measurement software (Hitachi, Ltd.) and calculated using the analysis software with the formula of Grynkiewicz *et al.* (22).

Statistical analysis

Data are expressed as the mean \pm standard deviation. The significance of differences was analyzed using a one- or two-way ANOVA followed by a post hoc analysis using Dunnett's test or the Student's *t*-test (Excel Statistics : Bellcurve for Excel and Microsoft Excel) and set at $P < 0.05$.

RESULTS

Determination of thrombin concentrations for preparation of thrombin-activated platelets and the selection of a thrombin concentration for the stimulation

Figure 1 shows $[\text{Ca}^{2+}]_i$ in human platelets (Figure 1A) and the intensity of platelet aggregation (Figure 1B) after thrombin stimulations (0.005-0.1 U/mL). $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation at each concentration of the thrombin

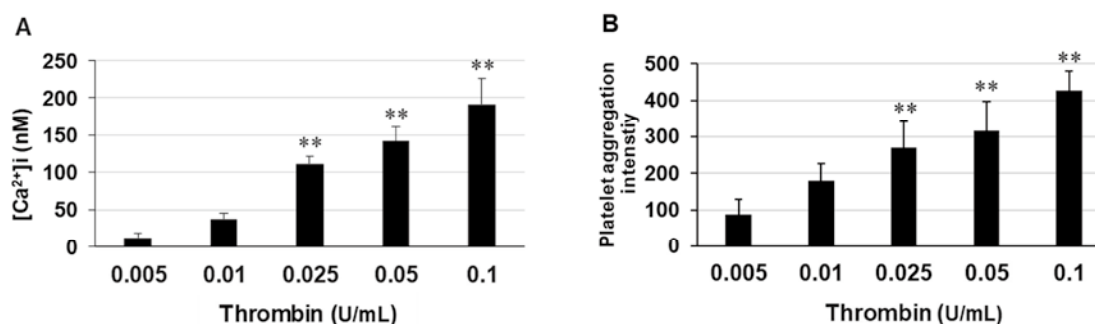


Figure 1. Determination of thrombin concentrations for preparation of thrombin-activated platelets and the selection of a thrombin concentration for the stimulation. Changes in $[\text{Ca}^{2+}]_i$ in human platelets (A) and the intensity of platelet aggregation (B) were assessed 15 seconds after a thrombin stimulation (0.005-0.1 U/mL). $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation at each concentration of the thrombin stimulation were compared with increases from pre-stimulation values of 0. Results are presented as the mean \pm SD, $n = 3$, a one-way ANOVA followed by Dunnett's test (** $P < 0.01$ vs. 0.005 U/ml thrombin).

stimulation were compared as an increase from the pre-stimulation value of 0. With the 0.005 U/mL thrombin stimulation, $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation both slightly increased ($[\text{Ca}^{2+}]_i$: 12.5 ± 6.2 nM, intensity of platelet aggregation: 87.7 ± 41.6). However, at the thrombin stimulation at 0.025 U/mL, $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation both significantly increased ($[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation: 111.6 ± 10.1 nM and 269.5 ± 72.3 , respectively). Therefore, 0.005 U/mL thrombin was selected as the thrombin concentration to prepare thrombin-activated platelets in this study. We also used 0.04 U/mL as the thrombin concentration for the platelet stimulation.

Changes in $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation in untreated and thrombin-activated platelets

Figure 2 shows changes in $[\text{Ca}^{2+}]_i$ (Figure 2A) and the intensity of platelet aggregation (Figure 2B) in untreated and thrombin-activated platelets after the thrombin stimulation (0.04 U/mL). $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation were compared as an increase from the pre-stimulation value of 0. A significant difference was observed in changes in $[\text{Ca}^{2+}]_i$ between untreated and thrombin-activated platelets after the thrombin stimulation. $[\text{Ca}^{2+}]_i$ increased rapidly in untreated platelets (153.0 ± 29.8 nM) 15 seconds after the thrombin stimulation; however, no clear increase was subsequently observed until after 180 seconds. Although $[\text{Ca}^{2+}]_i$ also increased rapidly in thrombin-activated platelets after the stimulation, the increase (92.6 ± 29.1 nM) was significantly less than that in untreated platelets, was followed by a gradual increase after 60 seconds, and was similar to that in untreated platelets after 180 seconds. On the other hand, no significant difference was observed in changes in the intensity of platelet aggregation between untreated and thrombin-activated platelets after the thrombin stimulation, whereas the intensity of platelet aggregation also increased rapidly 15 seconds after the thrombin stimulation. The intensity of platelet aggregation by thrombin-activated platelets was 321.9 ± 182.6 15 seconds after the stimulation, which was lower than that of untreated platelets (413.0 ± 192.9), but there was no significantly different. Thereafter, untreated and thrombin-activated platelets both showed an increase until 180 seconds.

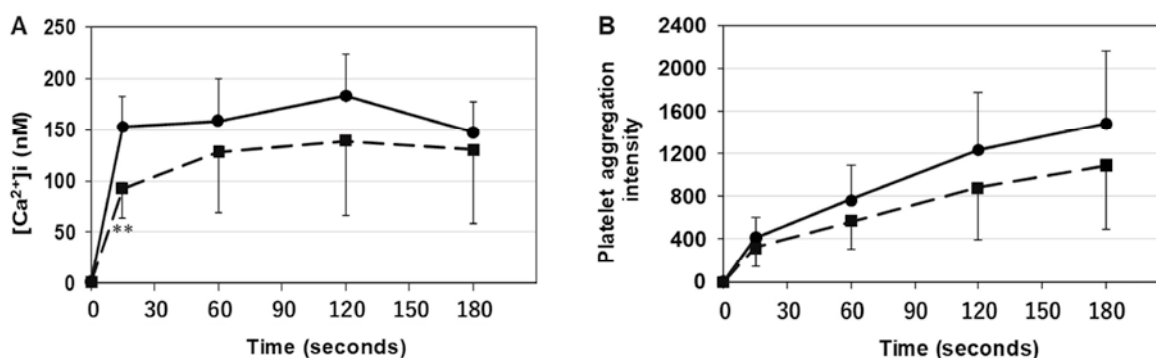


Figure 2. Changes in $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation in untreated and thrombin-activated platelets. Changes in $[\text{Ca}^{2+}]_i$ in human platelets (A) and the intensity of platelet aggregation (B) were measured after a thrombin stimulation (0.04 U/ml). ●, untreated platelets; ■, thrombin-activated platelets (thrombin: 0.005 U/mL). $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation were compared with increases from pre-stimulation values of 0. Results are presented as the mean \pm SD, $n = 5$, a two-way ANOVA followed by the Student's t -test (** $P < 0.01$, untreated platelets vs. thrombin-activated platelets, 15 seconds after the stimulation).

Effects of aspirin on $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation by the thrombin stimulation in untreated and thrombin-activated platelets

Figure 3 shows the effects of aspirin on Ca^{2+} release 15 seconds after the thrombin stimulation (Figure 3A) and the intensity of platelet aggregation (Figure 3B and 3C) after 15 and 180 seconds in untreated and thrombin-activated platelets. Significant differences were noted in the inhibitory effects of aspirin on both $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation between untreated and thrombin-activated platelets 15 seconds after the thrombin stimulation. Aspirin at 5.6-560 μM inhibited increases in both $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation in a concentration-dependent manner in untreated platelets, with these increases being significantly inhibited at concentrations of 56 and 560 μM (Figure 3A and 3B). In thrombin-activated platelets, an aspirin concentration of 560 μM slightly inhibited $[\text{Ca}^{2+}]_i$, whereas the intensity of platelet aggregation was not affected at any of the concentrations tested (Figure 3A and 3B). In contrast, no significant differences were observed in the inhibitory effects of aspirin on the intensity of platelet aggregation of untreated and thrombin-activated platelets 180 seconds after thrombin stimulation, although the intensity platelet aggregation of untreated and thrombin-activated platelets was slightly inhibited by aspirin (Figure 3C). The inhibitory effects of aspirin were stronger in untreated platelets than in thrombin-activated platelets.

Effects of cilostazol on $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation by the thrombin stimulation in untreated and thrombin-activated platelets

Figure 4 shows the effects of cilostazol on $[\text{Ca}^{2+}]_i$ 15 seconds after the thrombin stimulation (Figure 4A) and the intensity of platelet aggregation (Figure 4B and 4C) after 15 and 180 seconds in untreated and thrombin-activated platelets. Significant differences were observed in changes in $[\text{Ca}^{2+}]_i$ between untreated and thrombin-activated platelets after the thrombin stimulation. Cilostazol at 0.5-5 μM inhibited $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner in both untreated and thrombin-activated platelets and significantly inhibited the increase in $[\text{Ca}^{2+}]_i$ at all concentrations tested (Figure 4A). On the other hand, no significant differences were noted in changes in the intensity of

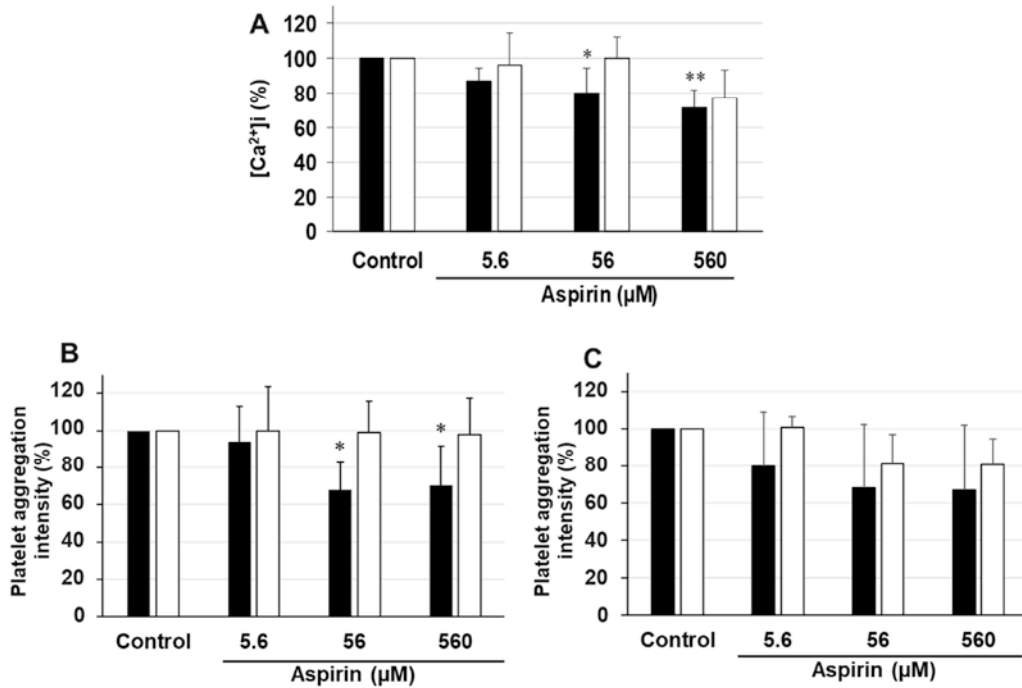


Figure 3. Effects of aspirin on [Ca²⁺]_i and the intensity of platelet aggregation by the thrombin stimulation in untreated and thrombin-activated platelets. Fura2-loaded untreated platelets or thrombin-activated platelets (thrombin : 0.005 U/mL) were treated with aspirin (5.6-560 µM) for 6 minutes and then stimulated with thrombin (0.04 U/mL). ■, untreated platelets ; □, thrombin-activated platelets. (A) ; [Ca²⁺]_i mobilization and (B), (C) ; the intensity of platelet aggregation (B ; 15 and C ; 180 seconds after the thrombin stimulation). Results are presented as the mean ± SD, n = 5, a two-way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01 vs. control).

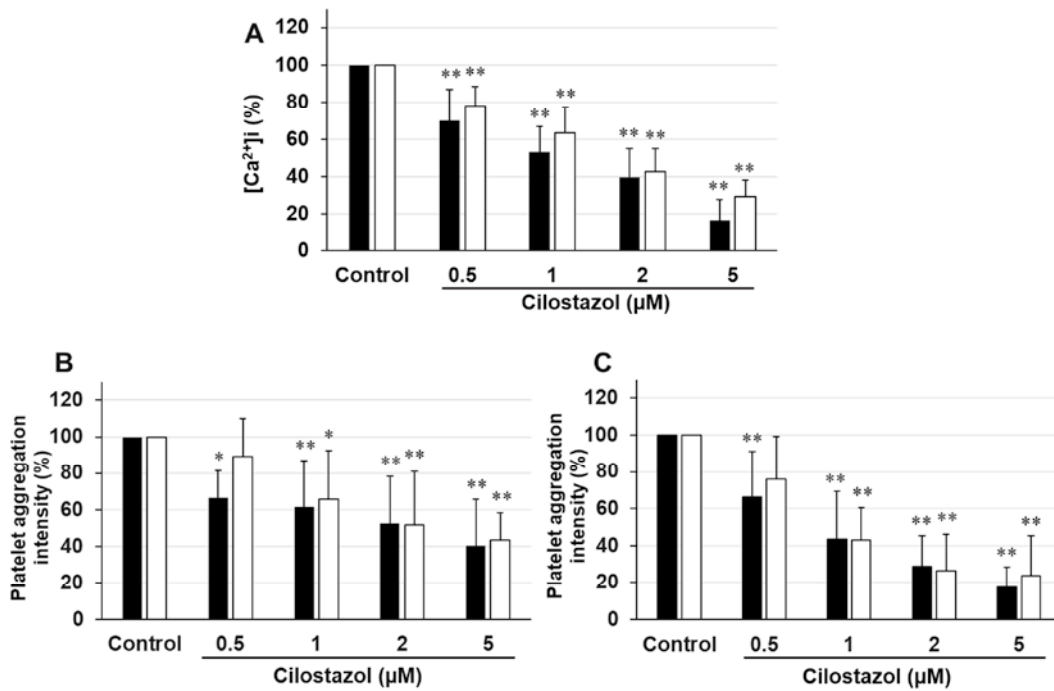


Figure 4. Effects of cilostazol on [Ca²⁺]_i and the intensity of platelet aggregation by the thrombin stimulation in untreated and thrombin-activated platelets. Fura2-loaded untreated platelets or thrombin-activated platelets (thrombin : 0.005 U/mL) were treated with cilostazol (0.5-5 µM) for 6 minutes and stimulated with thrombin (0.04 U/mL). ■, untreated platelets ; □, thrombin-activated platelets. (A) ; [Ca²⁺]_i mobilization and (B), (C) ; the intensity of platelet aggregation (B ; 15 and C ; 180 seconds after the thrombin stimulation). Results are presented as the mean ± SD, n = 6, a two-way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01 vs. control).

platelet aggregation between untreated and thrombin-activated platelets 15 and 180 seconds after the thrombin stimulation. The intensity of platelet aggregation was significantly inhibited in untreated platelets at all concentrations, but was not suppressed in thrombin-activated platelets at a concentration of 0.5 μM (Figure 4B and 4C). The inhibitory effects of cilostazol at concentrations of 0.5-5 μM 180 seconds after the stimulation were slightly stronger in both untreated and thrombin-activated platelets (Figure 4C). The inhibitory effects of cilostazol were very strong and similar between untreated and thrombin-activated platelets.

Relationship between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation in platelets treated with antiplatelet agents (aspirin and cilostazol)

Figure 5 shows the relationship between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation 15 seconds after the thrombin stimulation (0.04 U/mL) in platelets (untreated and thrombin-activated platelets) treated with aspirin (Figure 5A and 5B) or cilostazol (Figure 5C and 5D) at various concentrations. A correlation was observed between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation in untreated and thrombin-activated platelets following the addition of aspirin or cilostazol ($r=0.616-0.802$, $P<0.001$).

DISCUSSION

Platelets play an important role in physiological hemostatic mechanisms. In contrast, activated platelets have been implicated in pathological conditions, such as atherosclerosis, angiogenesis, and inflammation (1-5). Antiplatelet therapy with aspirin and cilostazol is used to treat arterial hemoperfusion, such as cerebral and myocardial infarctions, and also prevent recurrence (12-14). Therefore, the evaluation of platelet functions is of importance, particularly the effects of these antiplatelet agents. In the present study, we investigated the relationship between $[\text{Ca}^{2+}]_i$ fluctuations and platelet aggregation after a thrombin stimulation as well as that between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation in the platelet aggregation inhibitory reaction by antiplatelet agents (aspirin and cilostazol). In addition, platelets are activated by many substances, among which thrombin is pathologically important, and thrombin-activated platelets have been detected in the blood of patients with advanced arterial occlusive disease (9-11). Therefore, we also examined the effects of these antiplatelet agents on thrombin-activated platelets prepared as a surrogate platelet model for arterial occlusive disease.

Thrombin-activated platelets as a surrogate platelet model for arterial occlusive disease were pretreated with 0.005 U/mL thrombin, which exerted the weakest effects on $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation at each thrombin concentration. To maintain the linear response with 0.04 U/mL thrombin, this concentration was selected for stimulation.

Regarding changes induced by the stimulation with 0.04

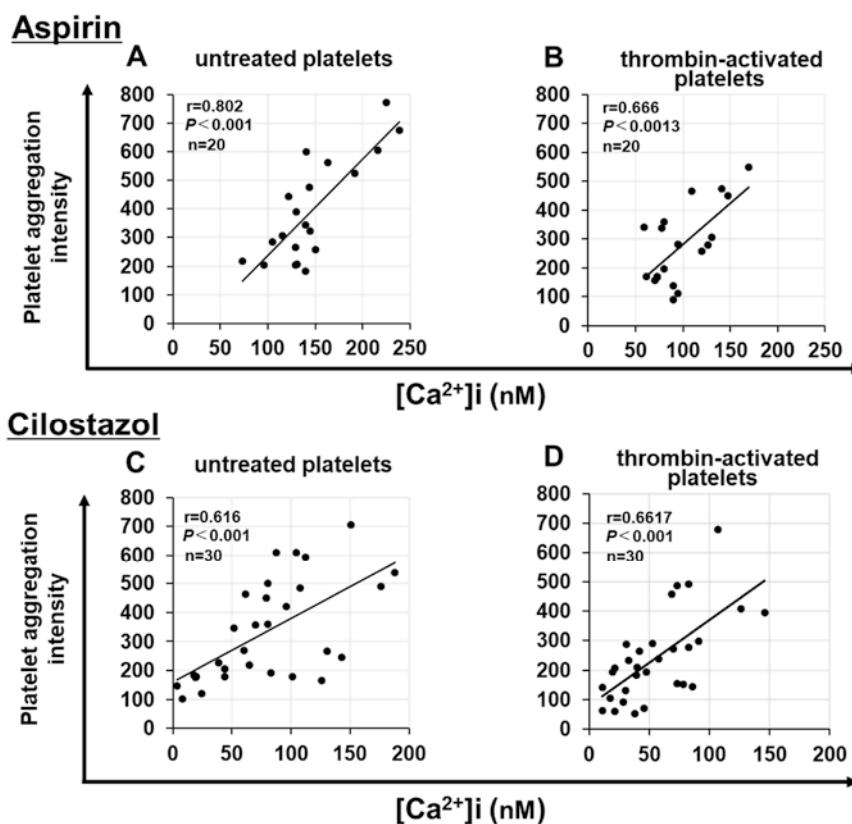


Figure 5. Relationship between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation in platelets treated with antiplatelet agents (aspirin and cilostazol). Scatter diagrams show the relationship between $[\text{Ca}^{2+}]_i$ and the intensity of platelet aggregation 15 seconds after the thrombin stimulation (0.04 U/mL) [(A) and (C), untreated platelets; (B) and (D), thrombin-activated platelets (thrombin : 0.005 U/mL)] in platelets treated with various concentrations of aspirin [(A) and (B)] or cilostazol [(C) and (D)]. (A) and (B) : $n=20$, (C) and (D) : $n=30$.

U/mL thrombin, $[Ca^{2+}]_i$ increased rapidly in untreated platelets up to 15 seconds after the stimulation and did not significantly change until after 180 seconds. In thrombin-activated platelets, $[Ca^{2+}]_i$ also increased rapidly up to 15 seconds, but was lower than that in untreated platelets, and reached a similar value to that in untreated platelets after 180 seconds. On the other hand, the intensity of platelet aggregation also increased rapidly up to 15 seconds in untreated and thrombin-activated platelets, and then increased slowly up to 180 seconds. Based on these results, we investigated the relationship between $[Ca^{2+}]_i$ and the intensity of platelet aggregation 15 seconds after the thrombin stimulation. Regarding the intensity of platelet aggregation, we examined changes at two time points, 15 and 180 seconds after the thrombin stimulation; however, it became clear that the intensity of platelet aggregation needed to be evaluated 180 seconds after the thrombin stimulation. Furthermore, the sensitivity of platelets to the thrombin stimulation appeared to vary among different individuals.

In the typical thrombin platelet-activating pathway, thrombin activates thrombin receptors (PAR-1 and PAR-4) on the platelet membrane, which subsequently evoke PLC to produce IP3. IP3 then binds to IP3 receptors in the endoplasmic reticulum and promotes the aggregation of platelets by mobilizing $[Ca^{2+}]_i$. In addition, increases in $[Ca^{2+}]_i$ produce TXA2, a potent inducer of platelet aggregation, which promotes platelet aggregation (6). The main thrombin receptor on platelets is PAR-1, which has a higher affinity for thrombin than PAR-4 and is activated by low concentrations of thrombin. In contrast, PAR-4 was shown to be activated by high concentrations of thrombin (6, 23). Therefore, in thrombin-activated platelets, PAR-1 may have been activated and processed by the pretreatment with a low concentration of thrombin (0.005 U/mL), resulting in low $[Ca^{2+}]_i$ 15 seconds after the subsequent thrombin stimulation (0.04 U/mL).

Regarding the inhibition of platelet aggregation by antiplatelet agents, we examined aspirin at a low dose of 5.6 μ M, which is used as an antiplatelet agent, and at higher doses (56 and 560 μ M). Aspirin significantly inhibited $[Ca^{2+}]_i$ and the intensity of platelet aggregation in untreated platelets at concentrations of 56 and 560 μ M 15 seconds after the thrombin stimulation, but had no significant effects on thrombin-activated platelets, with only the slight inhibition of $[Ca^{2+}]_i$ being observed at 560 μ M aspirin. However, 180 seconds after the thrombin stimulation, the intensity of platelet aggregation slightly decreased in untreated platelets or thrombin-activated platelets, whereas no significant changes were detected. Aspirin irreversibly inactivates enzyme activity by acetylating the 529th serine residue of COX-1, thereby inhibiting TXA2 production and platelet function by suppressing increases in $[Ca^{2+}]_i$ (16, 17). The present results strongly suggest that the antiplatelet effects of aspirin were attenuated in thrombin-activated platelets. A previous study reported that a significant proportion of young stroke patients have platelets that are hyperactive to thrombin and are not normalized by aspirin, which is consistent with the present results (24). We attributed the effects of aspirin to the partial inhibition of thrombin-stimulated activation and not to the strong suppression of aggregation. We intend to investigate the combined effects of aspirin and antiplatelet agents with different mechanisms of action in the future. On the other hand, cilostazol has a different antiplatelet mechanism of action than aspirin, selectively inhibiting a cAMP-inactivating metabolic enzyme (phosphodiesterase III) and exerting its antiplatelet effects by lowering $[Ca^{2+}]_i$ through increases in the concentration of cAMP in platelets (18-21). These antiplatelet drugs control platelet functions by suppressing signaling through several pathways after a thrombin stimulation and ultimately inhibit $[Ca^{2+}]_i$. Cilostazol significantly suppressed thrombin-stimulated increases in $[Ca^{2+}]_i$ and the intensity of

platelet aggregation in both untreated and thrombin-activated platelets; however, the inhibitory effects of 0.5 μ M cilostazol on thrombin-activated platelets were weaker. This result suggests that the effects of cilostazol were attenuated in thrombin-activated platelets. The effects of the drugs on the intensity of platelet aggregation did not significantly differ between untreated and thrombin-activated platelets, which may have been due to the potent effects of cilostazol. Ikeda *et al.* previously reported that cilostazol inhibited the aggregation of platelets induced by ADP, collagen, and arachidonic acid in patients with cerebral infarction and arteriosclerosis obliterans, and also that its effects were stronger than those of aspirin (25). The effects of aspirin and cilostazol were attenuated in thrombin-activated platelets. In the present study, thrombin-activated platelets were prepared by a treatment with a low concentration of thrombin (0.005 U/mL). Low concentrations of thrombin have been shown to activate PAR-1, which produces phospholipase C β (PLC β)-mediated IP3 and diacylglycerol and/or decreases cAMP concentrations due to a reduction in adenylate cyclase activity (23, 26). Although alterations in these signaling processes within platelets have been suggested, to the best of our knowledge, the mechanisms of action of these antiplatelet agents have not yet been fully clarified; therefore, further studies are warranted.

$[Ca^{2+}]_i$ and the intensity of platelet aggregation after the thrombin stimulation positively correlated ($r=0.616-0.802$) in both untreated and activated platelets following the addition of aspirin or cilostazol. We confirmed that the inhibition of $[Ca^{2+}]_i$ by aspirin and cilostazol regulated the respective intensities of platelet aggregation.

The present results suggest that thrombin-activated platelets as a surrogate platelet model for arterial occlusive disease attenuate the effects of antiplatelet agents. Therefore, prior to the initiation of antiplatelet therapy by patients with arterial occlusive disease, the assessment of platelet $[Ca^{2+}]_i$ may be a useful indicator of not only the platelet status, but also the appropriate dose of antiplatelet agents to be used.

There are some limitations that need to be addressed. The number of participants was small and their ages widely varied. In addition, the ratio of males to females was skewed. Therefore, further studies on a larger number of participants analyzed by age and sex are needed. We intend to increase the number of participants and conduct more detailed examinations in the future.

In conclusion, the present study revealed a relationship between changes in $[Ca^{2+}]_i$ and the intensity of platelet aggregation after a thrombin stimulation. The relationship between $[Ca^{2+}]_i$ and the intensity of platelet aggregation by antiplatelet agents revealed that cilostazol exerted stronger antiplatelet effects than aspirin. We also found that the antiplatelet effects of aspirin and cilostazol were attenuated in platelets activated with a low concentration of thrombin (0.005/mL). Thrombin-activated platelets in the present study may be useful as a surrogate platelet model in future studies on platelet function in arterial occlusive disease. The present results may contribute to future drug development for antiplatelet therapy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

REFERENCES

1. Wagner DD, Burger PC: Platelets in inflammation and thrombosis. *Arterioscler Thromb Vasc Biol* 23: 2131-2137,

- 2003
2. Rink TJ, Hallam TJ : What turns platelets on?. *Trends Biochem Sci* 9(5) : 215-219, 1984
 3. Johnson PC, Ware JA, Cliveden PB, Smith M, Dvorak AM, Salzman EW : Measurement of ionized calcium in blood platelets with the photoprotein aequorin. Comparison with Quin 2. *J Biol Chem* 260(4) : 2069-2076, 1985
 4. Massberg S, Vogt F, Dickfeld T, Brand K, Page S, Gawaz M : Activated platelets trigger an inflammatory response and enhance migration of aortic smooth muscle cells. *Thromb Res* 110(4) : 187-194, 2003
 5. Wu KK : Platelet activation mechanisms and markers in arterial thrombosis. *J Intern Med* 239 : 17-34, 1996
 6. Jurk K, Kehrel BE : Platelets : Physiology and Biochemistry. *Semin Thromb Hemost* 31(4) : 381-92, 2005
 7. Blockmans D, Deckmyn H, Vermynen J : Platelet Activation. *Blood Reviews* 9 (3) : 143-156, 1995
 8. Varga-szabo D, Braun A, Nieswandt B : Calcium signaling in platelets. *J Thromb Haemost* 7(7) : 1057-1066, 2009
 9. Jang IK, Gold HK, Ziskind AA, Leinbach RC, Fallon JT, Collen D : Prevention of platelet-rich arterial thrombosis by selective thrombin inhibition. *Circulation* 81(1) : 219-225, 1990
 10. Winters JK, Santro SA, Miletich JP, Eisenberg PR : Relative importance of thrombin compared with plasmin-mediated platelet activation in response to plasminogen activation with streptokinase. *Circulation* 84(4) : 1552-1560, 1991
 11. De Candia E : Mechanisms of platelet activation by thrombin : A short history. *Thromb Res* 129(3) : 250-256, 2012
 12. Laine M, Paganelli F, Bonello L : P2Y₁₂-ADP receptor antagonists : Days of future and past. *World J Cardiol* 8(5) : 327-332, 2016
 13. Yasue H, Ogawa H, Tanaka H, Miyazaki S, Hattori R, Saito M, Ishikawa K, Masuda Y, Yamaguchi T, Motomiya T, Tamura Y : Effects of aspirin and trapidil on cardiovascular events after acute myocardial infarction. Japanese Antiplatelets Myocardial Infarction Study (JAMIS) Investigators. *Am J Cardiol* 83(9) : 1308-1313, 1999
 14. Uchiyama S, Toyoda K, Omae K, Saita R, Kimura K, Hoshino H, Sakai N, Okada Y, Tanaka K, Origasa H, Naritomi H, Houkin K, Yamaguchi K, Isobe M, Minematsu K, Matsumoto M, Tominaga T, Tomimoto H, Terayama Y, Yasuda S, Yamaguchi T : Dual antiplatelet therapy using cilostazol in patients with stroke and intracranial arterial stenosis. *J Am Heart Assoc* 10(20) : e022575, 2021
 15. Lecomte M, Laneville O, Ji C, DeWitt DL, Smith WL : Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J Biol Chem* 269(18) : 13207-13215, 1994
 16. Catella-Lawson F, Reilly MP, Kapoor SC, Cucchiara AJ, DeMarco S, Tournier B, Vyas SN, FitzGerald GA : Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *N Engl J Med* 345(25) : 1809-1817, 2001
 17. Patrono C : Aspirin as an antiplatelet drug. *N Engl J Med* 330(18) : 1287-1294, 1994
 18. Umekawa H, Tanaka T, Kimura Y, Hidaka H : Purification of cyclic adenosine monophosphate phosphodiesterase from human platelets using new-inhibitor sepharose chromatography. *Biochem Pharmacol* 33(21) : 3339-3344, 1984
 19. Sudo T, Tachibana K, Toga K, Tochizawa S, Inoue Y, Kimura Y, Hidaka H : Potent effects of novel anti-platelet aggregatory clostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 59(4) : 347-356, 2000
 20. Igawa T, Tani T, Chijiwa T, Shiragiku T, Shimidzu S, Kawamura K, Kato S, Unemi F, Kimura Y : Potentiation of anti-platelet aggregation activity of cilostazol with vascular endothelial cells. *Thromb Res* 57(4) : 617-623, 1990
 21. Minami N, Suzuki Y, Yamamoto M, Kihira H, Imai E, Wada H, Kimura Y, Ikeda Y, Shiku H, Nisikawa M : Inhibition of shear stress-induced platelet aggregation by cilostazol, a specific inhibitor of cGMP-inhibited phosphodiesterase, in vitro and ex vivo. *Life Sci* 61(25) : 383-389, 1997
 22. Gryniewicz G, Poenie M, Tsien RY : A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 60(6) : 3440-3450, 1985
 23. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR : Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* 103(6) : 879-887, 1999
 24. Vanschoonbeek K, Feijge MAH, Keuren JFW, Coenraad Hemker H, Lodder JJ, Hamulyák K, van Pampus ECM, Heemskerk JWM : Thrombin-induced hyperactivity of platelets of young stroke patients : involvement of thrombin receptors in the subject-dependent variability in Ca²⁺ signal generation. *Thromb Hemost* 88(6) : 931-937, 2002
 25. Ikeda Y, Kikuchi M, Murakami H, Satoh K, Murata M, Watanabe K, Ando Y : Comparison of the inhibitory effects of cilostazol, acetylsalicylic acid and ticlopidine on platelet functions ex vivo. Randomized, double-blind cross-over study. *Arzneimittelforschung* 37(5) : 563-566, 1987
 26. Coughlin SR : Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 3(8) : 1800-1814, 2005