Biochemical Pharmacology, Vol. 59, pp. 1345–1355, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00279-3

On the Mechanism of Plasmin-Induced Platelet Aggregation

IMPLICATIONS OF THE DUAL ROLE OF GRANULE ADP

Akiko Ishii-Watabe, Eriko Uchida, Hiroyuki Mizuguchi and Takao Hayakawa*

Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo, Japan

ABSTRACT. Plasmin-induced platelet aggregation has been considered to be a cause of reocclusion after thrombolytic treatment with plasminogen activators. However, little is known regarding the mechanism and regulation of plasmin-induced platelet aggregation. In this study, we demonstrated that plasmin causes the degranulation of platelets, and that ADP released from granules plays a crucial role in the induction of platelet aggregation. This conclusion is supported by results showing that both ADP antagonists and ADPase can inhibit the effect of plasmin on platelets. We also demonstrated that pretreatment of platelets with ADP makes the platelets more sensitive to plasmin, and plasmin-induced platelet aggregation is, therefore, observed at lower concentrations where no aggregation occurs in quiescent platelets. In other words, it is thought that ADP potentiates the plasmin-induced aggregation. The effect of ADP was inhibited by N^6-[2-(methylthio)-ethyl]-2-(3,3,3-trifluoropropyl)thio-5′-adenylic acid, monoanhydride with dichloromethylenebisphosphonic acid (AR-C69931), a selective antagonist for the $P2T_{AC}$ subtype of P2 receptor, but not by the P2Y1 receptor-selective antagonist adenosine 3′-phosphate 5′-phosphosulfate (A3P5PS). The P2X1 receptor agonist α,β-methylene adenosine 5′-triphosphate (α,β-MeATP) did not mimic the action of ADP. These data indicate that ADP potentiates plasmin-induced platelet aggregation via the $P2T_{AC}$ receptor. In addition, epinephrine, a typical G_i agonist against platelets, could potentiate the plasmin-induced platelet aggregation, suggesting that the signal via the G_i protein is involved in potentiating the plasmin-induced platelet aggregation, ADP is secreted from platelet granules, and concomitantly works in conjunction with plasmin in a $P2T_{AC}$ receptor-mediated manner. BIOCHEM PHARMACOL **59**;11:1345–1355, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. plasmin; ADP; platelet; aggregation; receptor; potentiation

Plasminogen activator is known to be effective in treating acute myocardial infarction, although reocclusion of the damaged vessel sometimes occurs following an initially successful reperfusion [1, 2]. Such reocclusion has been reported to occur in approximately 30% of cases in which treatment is initially successful [3]. Concerning the mechanism of reocclusion after thrombolytic treatment, platelets are thought to play a major role, and the participation of plasmin has been assumed because of its ability to activate platelets [2, 4].

Plasmin is a serine protease primarily responsible for the degradation of insoluble fibrin and the resorption of vascular thrombi. Plasmin is formed by the proteolytic action of plasminogen activators on the circulating zymogen plasminogen [5]. Under physiological conditions, highly efficient plasma proteinase inhibitors ensure that plasmin activity is restricted to the vicinity of the thrombus. However, when plasminogen activator is used for therapeutic thrombolysis, appreciable amounts of free active plasmin can be present in the circulation [6]. In regard to possible mechanisms by which plasmin causes platelet activation, Schafer *et al.* [7] have suggested the involvement of calcium mobilization, activation of protein kinase C, and phospholipase C. In addition, the involvement of fibrinogen-receptor activation by proteolytic cleavage, and von Willebrand factor secretion have also been considered [8, 9]. In our previous study [10], protease activity and the lysine-binding activity of plasmin were needed for platelet aggregation. GPIIb/IIIa was also involved in the aggregation. Moreover, the aggregation depended on extracellular calcium. Based on the results of the binding study between plasmin and platelets, we suggested that plasmin has its own receptor on the surface of platelets. However, the mechanism of plasmin-induced platelet aggregation will require further study.

To improve thrombolytic treatment with plasminogen activators, it seems very important to elucidate and determine a way of blocking the specific mechanisms of plasmin-induced platelet aggregation. In an attempt to identify inhibitors of plasmin-induced platelet aggregation, apyrase (ADPase) has shown the unique character of effectively

* Corresponding author: Dr. Akiko Ishii-Watabe, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. (81) 3-3700-9089; FAX (81) 3-3700-9084; E-mail: watabe@nihs.go.jp

Received 28 May 1999; accepted 9 November 1999.

inhibiting the aggregation induced by plasmin, although it does not inhibit the aggregation induced by thrombin or collagen at a concentration sufficient to inhibit the action of plasmin. Therefore, we have focused on the role of ADP in plasmin-induced platelet aggregation.

ADP is stored in the dense granules of human platelets and is released when platelets adhere to the sites of vascular injury or when platelets are stimulated by agonists [11]. Released ADP is primarily responsible for the activation, recruitment, and induction of aggregation of additional platelets in the microenvironment [12]. The molecular basis for ADP-induced platelet activation has been studied extensively [11, 13], and, recently, evidence of the presence of three distinct platelet receptors has been presented [14–18]. According to the nomenclature described by Kunapuli and coworkers [15–17], platelets have the following three subtypes of ADP receptors: the P2Y1 receptor, which is coupled to the activation of phospholipase C; the $P2T_{AC}$* receptor, which is coupled to the inhibition of adenylate cyclase via G_i; and P2X1, which is an intrinsic ion channel. The involvement of these receptor subtypes in cellular function can be investigated by using specific agonists or antagonists [15–17, 19]. In this report, we provide evidence that, in human platelets, ADP released from platelet granules plays an essential role in plasmin-induced platelet aggregation, and that ADP potentiates plasmin-induced platelet aggregation via the $P2T_{AC}$ subtype of the ADP receptor.

MATERIALS AND METHODS

Materials

The reagents used were obtained from commercial sources as follows: plasmin: Seikagaku Kogyo; ADP: Oriental Yeast; epinephrine: Wako Pure Chemical Industries, Ltd.; and apyrase, PPADS, suramin, ATPαS, αβ-MeATP, and A3P5PS: Sigma Chemical Co. The luciferase/luciferin reagent was from Dade. Fura-2-AM was from Dojindo. AR-C69931 [N^6-[2-(methylthio)-ethyl]-2-(3,3,3-trifluoropropyl)thio]-5′-adenylic acid, monoanhydride with dichloromethylene-bisphosphonic acid] was a gift of the Astra Research Laboratories. Heat-inactivated apyrase was obtained by incubating the aqueous solution of apyrase for 30 min at 95°.

Platelet Preparation

To prepare the washed human platelets, venous blood samples from informed human healthy volunteers were collected into plastic tubes containing 3.8% sodium citrate (1:10). Platelet-rich plasma (PRP) was obtained by centrifugation at 140 *g* for 10 min at room temperature. PRP was centrifuged at 1500 *g* for 10 min at room temperature in the presence of 20 μM adenosine, and the platelet pellet was washed with HEPES–Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.8 mM NaH_2PO_4, 5.5 mM glucose, 3.8 mM HEPES, 0.35% BSA, pH 6.5) in the presence of 20 μM adenosine. Then the washed platelets were resuspended in HEPES–Tyrode's buffer (pH 7.35).

Measurement of Platelet Aggregation and Shape Change

Platelet aggregation and shape change were measured in a Chronolog Lumi-aggregometer under constant stirring conditions (1000 rpm) at 37°. The total volume of the reaction mixture in the sample cuvette was 400 μL, and the platelet concentration was adjusted to 3×10^8/mL with HEPES–Tyrode's buffer (pH 7.35). A 1.7 mM concentration of $CaCl_2$ was added to each sample 1 min before the addition of test reagents. When ADP or other compounds were added to the platelet suspension, 8 μL of reagents of the appropriate concentrations was added 1 min before the plasmin. Changes in the light transmission were recorded in a chart recorder. The extent of the platelet aggregation was measured as an increase in the light transmission, and expressed as a percentage of the maximum variation in light transmission set up between the nonaggregated platelet suspension and the HEPES–Tyrode's buffer. The extent of the platelet shape change was measured as a decrease in the light transmission.

Measurement of Granule Release from Platelets

ATP secreted from the platelet dense granules following exposure to plasmin was estimated simultaneously with platelet aggregation by measuring the intensity of the chemiluminescence of luciferin luciferase reagent in a dual-channel Lumi-aggregometer. The assay was calibrated with a solution containing a known concentration of ATP.

Measurement of cAMP

Washed platelets were stirred at 37°. Forskolin (10 μM) was added 2 min prior to the addition of ADP (10 μM). AR-C69931 was added 1 min prior to ADP. Incubations were continued for 2 min after ADP addition, and the reactions then were stopped by the addition of 1 vol. of ice-cold 20% trichloroacetic acid (TCA). After the removal of TCA by washing with water-saturated diethyl ether, the cAMP content was determined using the cAMP EIA kit (Amersham Pharmacia Biotech, UK).

Measurement of $[Ca^{2+}]_i$

PRP was incubated with fura-2-AM (4 μM) at 37° for 45 min. Then the fura-2-loaded platelets were collected by centrifugation at 1500 *g* for 10 min. After washing once with HEPES–Tyrode's buffer (pH 6.5), the platelets were

* *Abbreviations:* $P2T_{AC}$, platelet ADP receptor coupled to inhibition of adenylate cyclase; A3P5PS, adenosine 3′-phosphate 5′-phosphosulfate; α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; ATPαS, adenosine-5′-*O*-(1-thiotriphosphate); PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium; CU, casein units; cAMP, cyclic AMP; and CP/CPK, creatine phosphate/creatine phosphokinase.

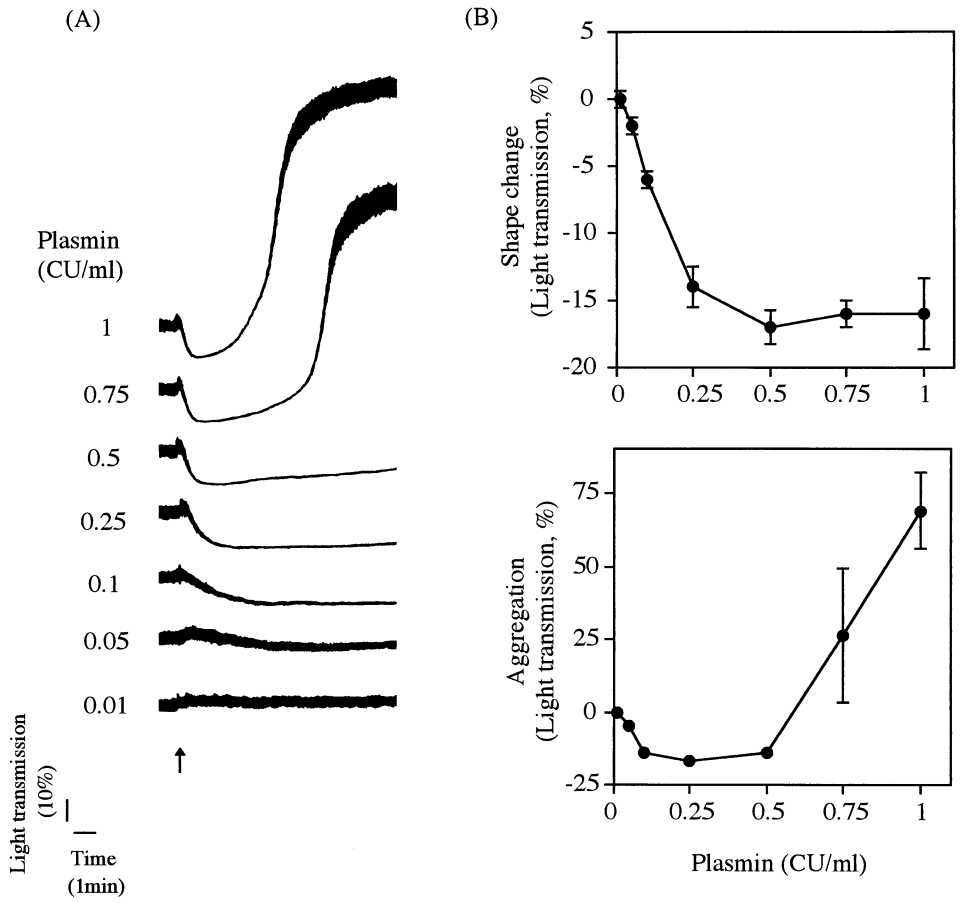

FIG. 1. Plasmin-induced platelet shape change and aggregation. Representative traces of platelet shape change and aggregation induced by plasmin (A) with its concentration dependency (B) are shown. Additions are indicated by an arrow. (A) Washed human platelets were stimulated by 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, or 1.0 CU/mL of plasmin. Each tracing is representative of at least three similar experiments. (B) The concentration dependencies of plasmin-induced platelet shape change and aggregation were evaluated by light transmission 1 and 5 min after the addition of plasmin, respectively. Values are means ± SD (N = 3).

resuspended in HEPES–Tyrode's buffer (pH 7.35). $CaCl_2$ (1.7 mM) was added to each sample just before setting it in the spectrofluorometer. The fura-2-labeled platelets were activated in a CAF-100 spectrofluorometer (JASCO, Japan) in a water-jacketed cuvette maintained at 37° with stirring. The stirred platelet suspension was excited alternately at 340 and 380 nm, with emitted light measured at 500 nm, and the intracellular Ca^{2+} concentrations were calculated from the ratio of intensities at 340 and 380 nm [20]. The maximum intensity ratio was determined following lysis of the platelets with 0.1% Triton X-100, and the minimum intensity ratio was determined by adding 20 mM Tris (hydroxymethyl) aminomethane followed by 10 mM [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid.

RESULTS

Plasmin-Induced Platelet Shape Change and Aggregation

A typical tracing of plasmin-induced washed human platelet aggregation is shown in Fig. 1A. Plasmin caused platelet aggregation at concentrations above 0.75 CU/mL. A plasmin concentration of 1 CU/mL was sufficient to observe the platelet aggregation. No aggregation occurred below 0.5 CU/mL of plasmin. Platelet shape change, monitored by decreases in light transmission, began to be observed at 0.05 CU/mL of plasmin. The level of shape change seemed to reach a maximum at 0.25 CU/mL of plasmin. The decrease in light transmission was maintained throughout the measurement if aggregation did not occur.

Effect of Apyrase on Plasmin-Induced Platelet Aggregation

The effects of apyrase (ADPase) on plasmin-, thrombin-, and collagen-induced platelet aggregation are shown in Fig. 2. Pretreatment of platelets with apyrase effectively inhibited the effects of plasmin (Fig. 2A). The inhibition of plasmin-induced platelet aggregation was observed at apyrase concentrations of 0.01 U/mL or more. On the other hand, thrombin (0.1 U/mL)- or collagen (10 μg/mL)-induced platelet aggregation was not inhibited by apyrase even at a concentration of 0.1 U/mL, which is ten times

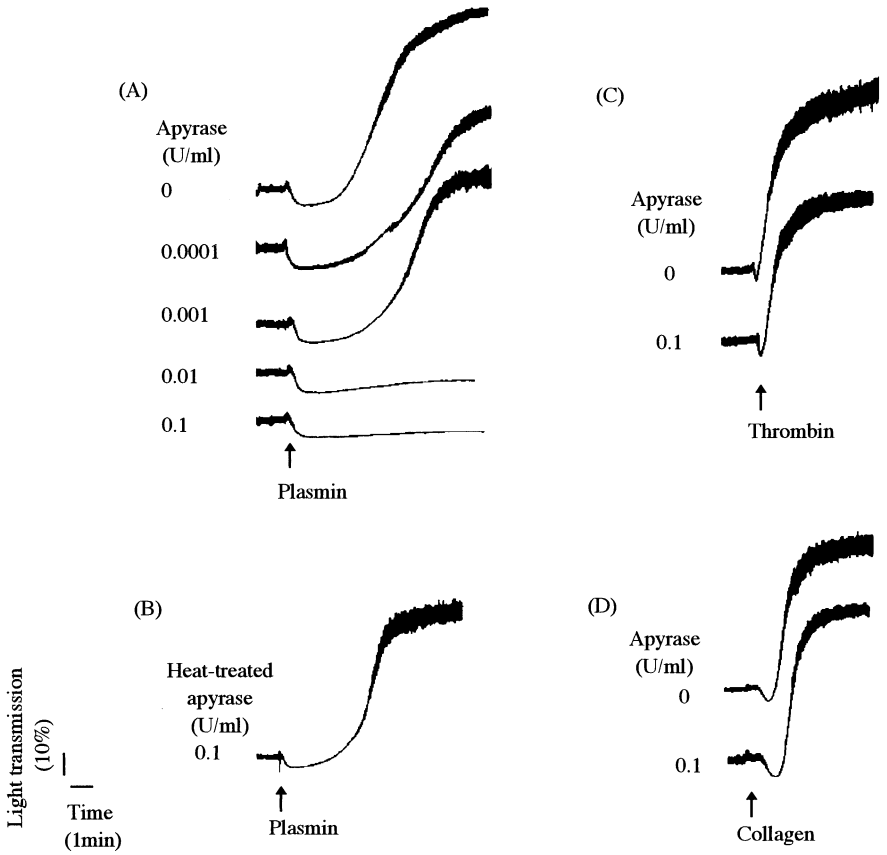

FIG. 2. Effect of apyrase on plasmin-induced platelet aggregation. Representative traces of platelet aggregation induced by plasmin (A and B), thrombin (C), or collagen (D) and the effects of apyrase are shown. Agonist-induced platelet aggregation was measured as described in Materials and Methods. Additions are indicated by arrows. (A) Washed human platelets were incubated with 0, 0.0001, 0.001, 0.01, or 0.1 U/mL of apyrase for 1 min at 37°, and stimulated by plasmin (1 CU/mL). (B) Platelets were incubated with 0.1 U/mL of heat-inactivated apyrase for 1 min at 37° and then were stimulated by plasmin (1 CU/mL). (C and D) Washed human platelets were incubated with 0 or 0.1 U/mL of apyrase for 1 min at 37° and then were stimulated by thrombin (0.1 U/mL) or collagen (10 μg/mL). Each tracing is representative of at least three similar experiments.

higher than that needed to inhibit the effects of plasmin (Fig. 2 C and D). Heat-inactivated apyrase did not inhibit plasmin-induced platelet aggregation, confirming that the inhibitory effect of apyrase is due to the enzymatic activity (Fig. 2B). These results suggest the importance of ADP in plasmin-induced platelet aggregation. Another ADP scavenger, CP/CPK, also inhibited the effects of plasmin (data not shown). Platelet shape change after the stimulation with plasmin, which was evaluated by the decrease in light transmission, was not affected by apyrase.

Effect of ADP Antagonists on Plasmin-Induced Platelet Aggregation

We next examined the effects of ADP antagonists on plasmin-induced platelet aggregation. Platelets were preincubated with suramin, ATPαS, or PPADS at the indicated concentrations, and then were stimulated by plasmin. Plasmin-induced platelet aggregation was inhibited completely by these ADP antagonists (Fig. 3).

Release of Granule Contents from Platelets Stimulated with Plasmin

Since ADP is a platelet agonist stored in platelet dense granules and is released following the activation of platelets, we supposed that degranulation from platelets is necessary to cause the plasmin-induced platelet aggregation. Therefore, we measured the granule content release from platelets after the stimulation of plasmin. The granule content release was monitored by the change in the concentrations of released ATP using luciferase/luciferin methods, as described in Materials and Methods. Because both ATP and ADP are stored in the same granules in platelets, ATP is used as a marker of dense granules. As shown in Fig. 4A, degranulation after the stimulation of plasmin was detected at the beginning of aggregation. The amount of released ATP was increased in a concentration-dependent manner (Fig. 4B).

Effect of ADP on Plasmin-Induced Platelet Aggregation

In considering the relationship between ADP and plasmin-induced platelet aggregation, we presumed that ADP could

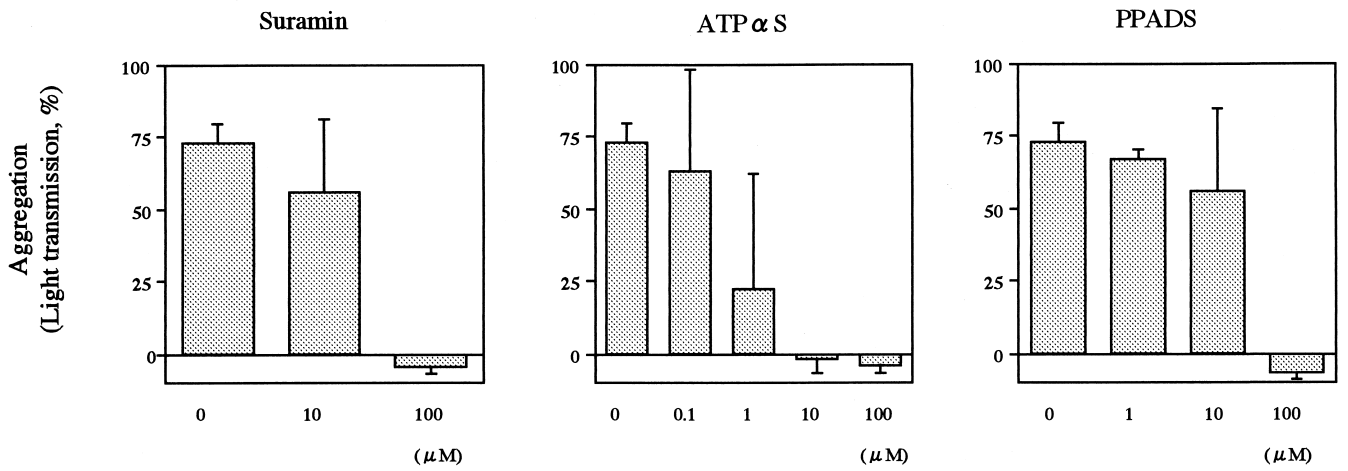

FIG. 3. Effects of ADP antagonists on plasmin-induced platelet aggregation. Platelets were incubated with suramin, ATPαS, or PPADS at the indicated concentrations for 1 min at 37° and then were stimulated by plasmin (1 CU/mL). Platelet aggregation was measured as described in Materials and Methods. Light transmission at 8 min after the stimulation with plasmin is indicated. Values are means ± SD (N = 3).

possibly potentiate the plasmin-induced platelet aggregation. We examined the effects of ADP on plasmin-induced platelet aggregation. To examine whether ADP works synergistically with plasmin, platelet aggregation was monitored during the incubation with ADP followed by plasmin. ADP (10 μM) alone did not cause an aggregation of washed platelets without the addition of fibrinogen. In this experiment, the concentration of plasmin used was 0.5 CU/mL. At this concentration, plasmin did not cause platelet aggregation. As shown in Fig. 5A, pretreatment with ADP enabled the platelets to aggregate in response to plasmin (0.5 CU/mL), indicating that ADP indeed potentiates plasmin-induced platelet aggregation. The effect of ADP was concentration-dependent from 0.1 to 10 μM (Fig. 5B).

Effects of P2 Antagonists on ADP-Induced Platelet Aggregation

Having established that ADP potentiates plasmin-induced platelet aggregation, we next attempted to determine which ADP receptor subtype, P2Y1, $P2T_{AC}$, or P2X1 [15, 16], is involved in this process. Several compounds have been shown to be effective for use in specifying among these receptor subtypes. A3P5PS works as an antagonist of P2Y1, the subtype that is involved in ADP-induced intracellular

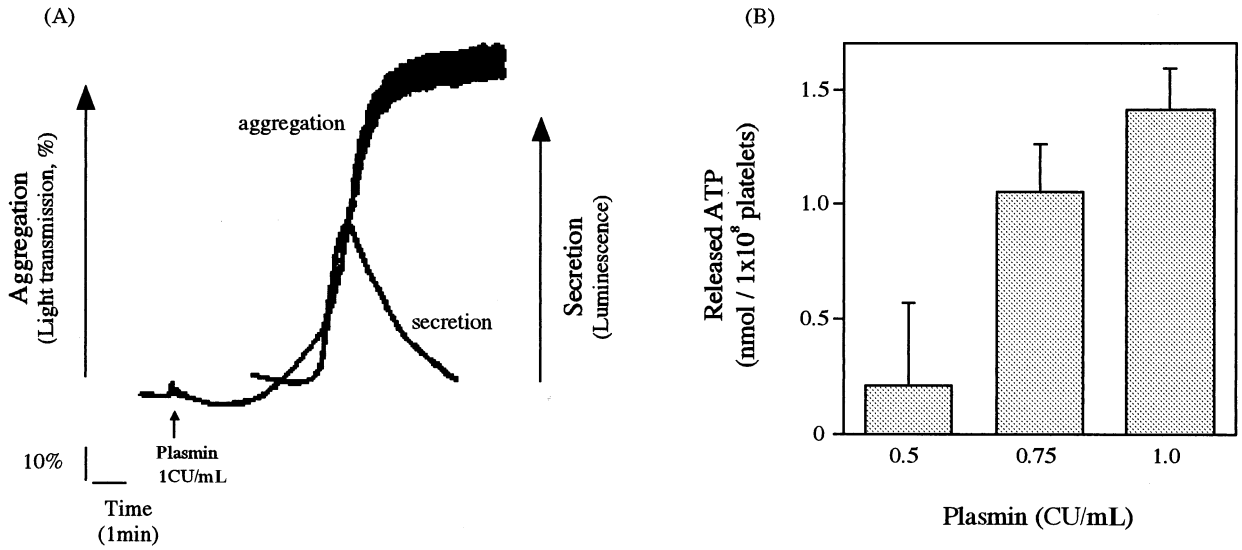

FIG. 4. Granule content release from platelets after the stimulation of plasmin. Representative traces of platelet aggregation and ATP release induced by plasmin are shown (A). Washed platelets were subjected to plasmin (1 CU/mL) in the presence of luciferase/luciferin reagent. The platelet aggregation and ATP released were monitored simultaneously with aggregation with a dual-cannel Lumi-aggregometer. Addition is indicated by an arrow. Each tracing is representative of at least three similar experiments. The amounts of ATP released from platelets induced by increasing concentrations of plasmin are also shown (B). Values are means ± SD (N = 3).

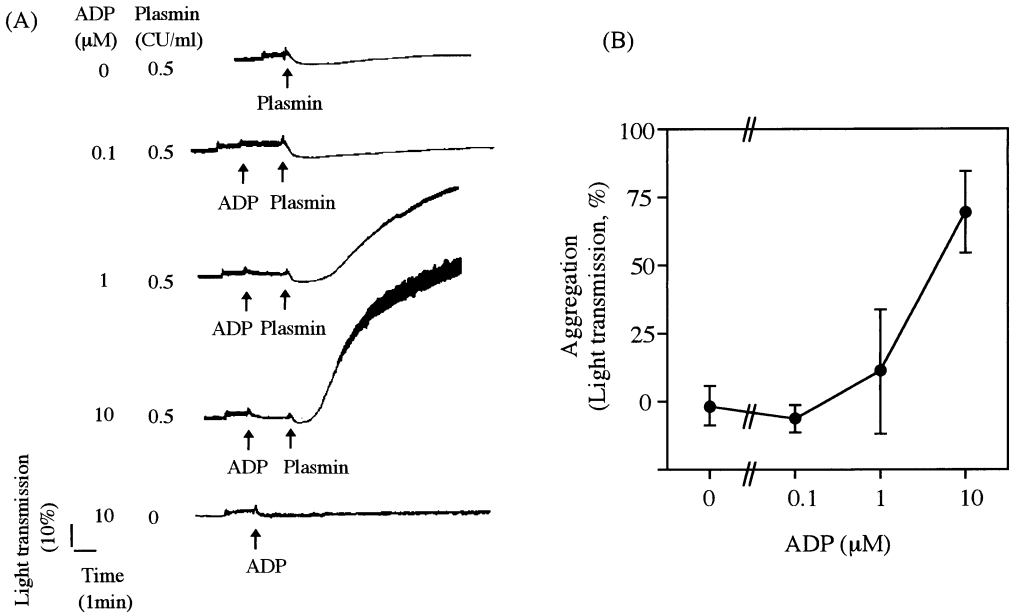

FIG. 5. Effect of ADP on plasmin-induced platelet aggregation. Representative traces of platelet aggregation induced by plasmin and/or ADP are shown (A). Agonist-induced platelet aggregation was measured as described in Materials and Methods. Additions are indicated by arrows. Platelets were treated with 0, 0.1, 1, or 10 μM ADP for 1 min at 37° and then were stimulated by plasmin (0.5 CU/mL). As a control, platelets were stimulated by 10 μM ADP alone. Each tracing is representative of at least three similar experiments. The light transmission at 8 min after the stimulation with increasing concentrations of ADP with 0.5 CU/mL of plasmin is indicated (B). Values are means ± SD (N = 3).

calcium increase and shape change. ARL66096 (2-propylthio-D-β, γ-difluoromethylene adenosine 5′-triphosphate) and AR-C69931 are antagonists for $P2T_{AC}$. ARL66096, which has been shown recently to be a specific antagonist for $P2T_{AC}$, blocks ADP-induced inhibition of adenylate cyclase and aggregation, but does not affect ADP-mediated intracellular calcium increases or shape change [15, 16]. AR-C69931, a more potent analogue of ARL66096, is currently being developed as an antithrombotic agent for clinical use [19]. Because there are no antagonists specific for P2X1, the P2X1-specific agonist α,β-MeATP was used here to examine the contribution of P2X1. We first examined the specificity of these two classes of antagonists because the subtype selectivity of ARL66096, a leading compound of AR-C69931, as a $P2T_{AC}$ antagonist has been examined in detail [15, 16], but that of AR-C69931 has not been reported. As shown in Fig. 6A, both the $P2T_{AC}$ antagonist AR-C69931 and the P2Y1 antagonist A3P5PS inhibited ADP-induced platelet aggregation in the presence of fibrinogen. Furthermore, while the P2Y1 antagonist A3P5PS inhibited ADP-induced platelet shape change, the $P2T_{AC}$ antagonist AR-C69931 did not affect this change (Fig. 6B). Therefore, AR-C69931 was shown to inhibit the $P2T_{AC}$-mediated action of ADP without inhibiting the P2Y1-mediated action of ADP without inhibiting the P2Y1-mediated action of ADP.

Effects of Subtype-Specific Ligands for ADP Receptor on the Effect of ADP

The effects of these antagonists and this agonist on the effect of ADP were examined. As shown in Fig. 7, the effect of ADP was inhibited by the $P2T_{AC}$-selective antagonist AR-C69931, but not by the P2Y1-selective antagonist A3P5PS. The P2X1-selective agonist α,β-MeATP did not mimic the effect of ADP (Fig. 7). AR-C69931 had an IC_{50} of 3×10^{-9} M (Fig. 8), which is similar to the value reported for ARL66096 to inhibit $P2T_{AC}$-mediated signals [19]. These data indicate that ADP potentiates plasmin-induced platelet aggregation in a $P2T_{AC}$ receptor-mediated manner.

Effects of AR-C69931 on ADP-Induced Adenylate Cyclase Inhibition and Increase in Intracellular Ca^{2+}

Based on the data indicating that the effects of ADP are inhibited by the $P2T_{AC}$ antagonist AR-C69931, ADP was thought to work via activation of the G_i protein in potentiating the plasmin-induced platelet aggregation. To further confirm that the effects of ADP are mediated by the G_i protein, we examined the ability of AR-C69931 to block ADP-induced adenylate cyclase inhibition. As shown in Fig. 9, AR-C69931 blocked the ADP-induced inhibition of forskolin-stimulated adenylate cyclase activity. This response was observed in the same concentration range as it inhibited the potentiating effect of ADP on plasmin-induced platelet aggregation. In contrast, AR-C69931, in the concentration range that inhibited the potentiating effect of ADP, did not inhibit the ADP-induced increase in $[Ca^{2+}]_i$, although there was a slight decrease in $[Ca^{2+}]_i$ at an AR-C69931 concentration of 1×10^{-6} M (Fig. 10).

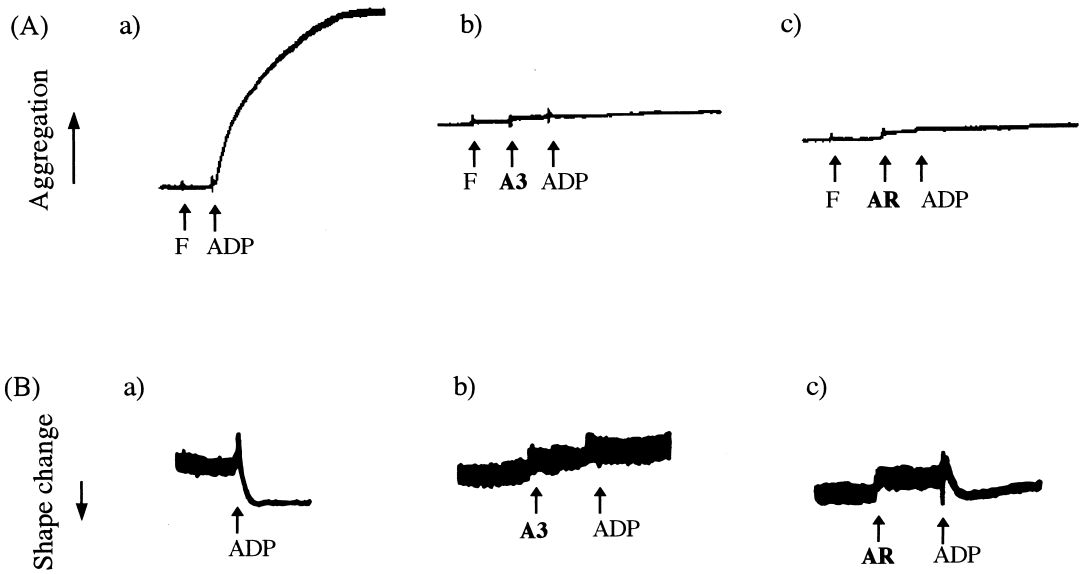

FIG. 6. Effects of A3P5PS and AR-C69931 on ADP-induced platelet aggregation and shape change. Representative traces of platelet aggregation (A) and shape change (B) induced by ADP and the effects of subtype-specific P2 antagonists are shown. Platelet aggregation was measured as described in Materials and Methods. Additions are indicated by arrows. A3: A3P5PS (100 μM) (b) or AR: AR-C69931 (1 μM) (c) was added prior to ADP, and the ADP-induced platelet aggregation was monitored (A). When ADP-induced platelet aggregation was monitored, 1 mg/mL of fibrinogen was added to the platelet suspension. Each tracing is representative of at least three similar experiments.

Effect of Epinephrine on Plasmin-Induced Platelet Aggregation

Since ADP was shown to potentiate the plasmin-induced platelet aggregation via the $P2T_{AC}$ receptor, which couples to the G_i protein, we next examined the involvement of other G_i-mediated signals in potentiating plasmin-induced platelet aggregation. Epinephrine is known to be a typical G_i-coupled agonist against platelet via the α_2 receptor [21, 22], and therefore its effect on plasmin-induced platelet aggregation was examined. Epinephrine (1 μM) cannot by itself cause the aggregation of washed platelets. When platelets were preincubated with epinephrine, however, aggregation occurred at a plasmin concentration of 0.5 μM, indicating that epinephrine potentiates the plasmin-induced platelet aggregation (Fig. 11). The effect of epinephrine was concentration dependent from 0.1 to 1 μM.

DISCUSSION

Platelets contain four distinct intracellular storage granules: dense granules, alpha granules, lysosomes, and peroxisomes [23]. The dense granules contain ADP, ATP, serotonin, calcium, and pyrophosphate. The alpha granules contain adhesive molecules, including fibrinogen, growth factors, coagulation factors, and other proteins. The contents of granules, such as ADP, are released when platelets are activated and work to potentiate the aggregation of platelets. Although the involvement of ADP receptor-mediated signals in plasmin-induced platelet aggregation has been a subject of controversy [24–26], in this study, plasmin-induced platelet aggregation was shown to be highly sensitive to apyrase (ADPase) (Fig. 2) and to be inhibited by ADP antagonists (Fig. 3), suggesting the importance of ADP in plasmin-induced platelet aggregation. After the stimulation with plasmin, granular contents were released at the beginning of aggregation (Fig. 4). These data suggest that in plasmin-induced platelet aggregation, ADP released from granules plays a critical role in mediating the aggregation. Since plasmin can cause the aggregation of washed platelets without the addition of fibrinogen, it may cause a release of fibrinogen from alpha granules in addition to the release of ADP from dense granules.

Although platelet shape change occurred just after the stimulation by plasmin, a period of several minutes was required before the start of granule release and aggregation (Fig. 1A). This time lag between the stimulation of plasmin and the start of aggregation is one of the features of plasmin-induced platelet aggregation. Plasmin-induced platelet shape change was observed at a much lower concentration of plasmin (0.05 CU/mL) compared with that needed to cause aggregation (>0.75 CU/mL) (Fig. 1B). While platelet aggregation was inhibited effectively by apyrase in this study, platelet shape change was not inhibited (Fig. 2A). These data suggest that the initial effects of plasmin are independent of ADP. To investigate the mechanism of plasmin-induced platelet shape change, we examined the effects of phenylarsine oxide (PAO), a protein tyrosine phosphatase inhibitor. PAO is reported to inhibit collagen-induced platelet shape change but not that induced by thrombin [27]. In our experiments, PAO did not inhibit plasmin-induced platelet shape change (data not shown), although we were able to confirm its effect against

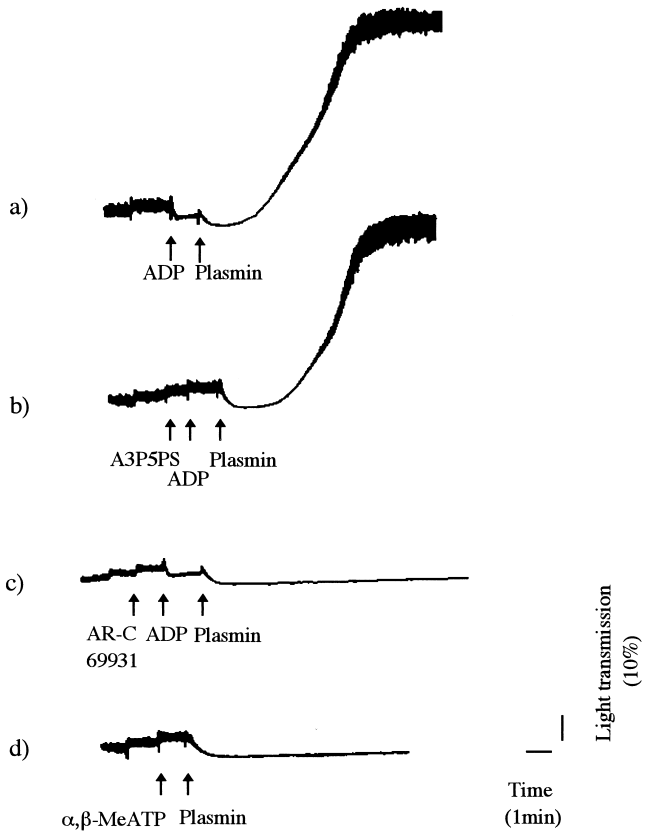

FIG. 7. Effects of subtype selective P2 antagonists or agonist on the effect of ADP. Representative traces of ADP and plasmin-induced platelet aggregation in the presence or absence of P2 antagonists or agonist are shown. (a) Platelets were treated with ADP (10 μM) for 1 min at 37°, and then were stimulated by plasmin. (b and c) Platelets were treated with (b) the P2Y1 antagonist A3P5PS (100 μM) or (c) the $P2T_{AC}$ antagonist AR-C69931 (1 μM) for 1 min at 37° and then with ADP (10 μM) for 1 min at 37°. Then the platelets were stimulated by plasmin (1 CU/mL). (d) Platelets were treated with the P2X1 agonist α,β-MeATP (100 μM) for 1 min at 37°, and then were stimulated by plasmin. Additions are indicated by arrows. Platelet aggregation was monitored by the change in light transmission. Each tracing is representative of at least three similar experiments.

thrombin- and collagen-induced platelet shape change. Therefore, judging by its sensitivity to PAO, plasmin was thought to activate a different signaling pathway than those activated by collagen. In future studies, a confirmation of the intracellular event that occurs within this lag time followed by the granule release will lead to further elucidation of the mechanisms of plasmin-induced platelet aggregation and the target of plasmin on the platelet surface.

In addition to its role as a second mediator of platelet aggregation after the stimulation of plasmin, ADP was shown to have a potentiating effect on plasmin-induced platelet aggregation via the $P2T_{AC}$ receptor (Figs. 5 and 7). That is, in quiescent platelets, ADP is considered to make the platelets more sensitive to plasmin-induced platelet aggregation. The specificity of the ADP antagonists used in this study was confirmed as follows. In the presence of

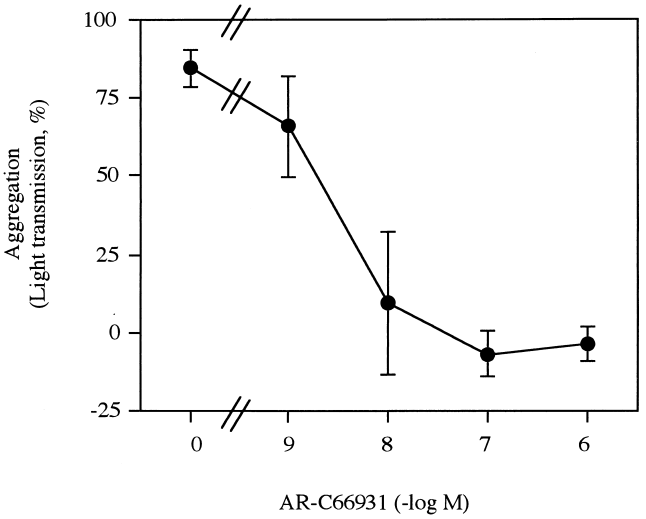

FIG. 8. Effect of the $P2T_{AC}$ antagonist AR-C69931 on the activity of ADP. Platelets were treated with various concentrations of AR-C69931 for 1 min at 37° and with ADP for 1 min at 37°. Then plasmin (1 CU/mL) was added to the platelet suspension, and aggregation was monitored by the change in light transmission. Values are means ± SD (N = 3).

fibrinogen, AR-C69931 inhibited ADP-induced platelet aggregation without inhibiting the platelet shape change (Fig. 6). On the other hand, A3P5PS inhibited both ADP-induced platelet shape change and aggregation (Fig. 6). Based on these results, AR-C69931 appears to inhibit $P2T_{AC}$ without inhibiting the P2Y1 subtype. Although both AR-C69931 and A3P5PS inhibited ADP-induced

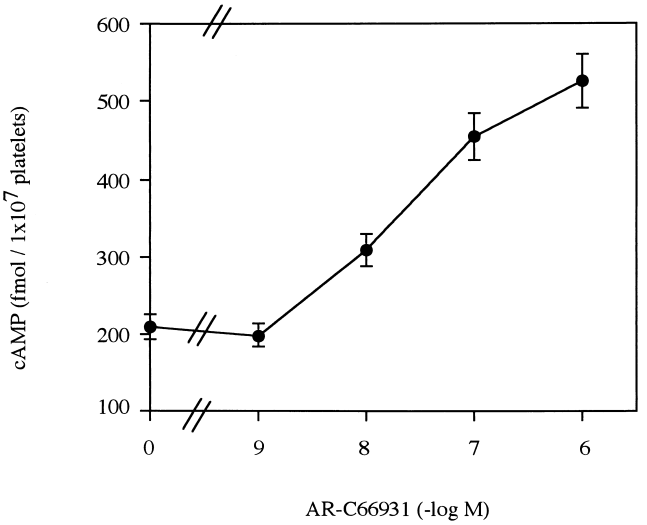

FIG. 9. Effect of AR-C69931 on ADP-induced adenylate cyclase inhibition. The effect of the $P2T_{AC}$ antagonist AR-C69931 on ADP-induced inhibition of adenylate cyclase was determined as described in Materials and Methods. Washed platelets were treated with forskolin (10 μM) and the indicated concentrations of AR-C69931, followed by ADP (10 μM), and the reaction was stopped with the addition of ice-cold 20% TCA. Basal and forskolin-stimulated cyclic AMP levels in the absence of ADP were 146 and 854 fmol/1 × 10^7 platelets, respectively. Values are means ± SD (N = 3).

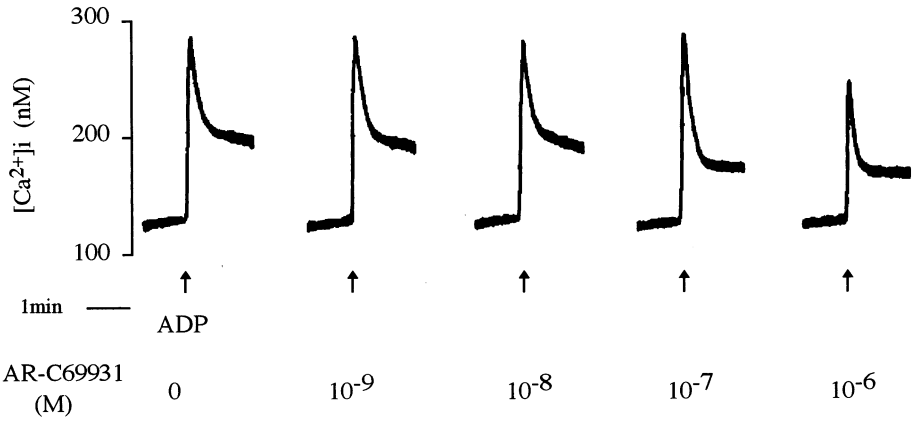

FIG. 10. Effect of AR-C69931 on ADP-induced increase in $[Ca^{2+}]_i$. Platelets labeled with fura-2 were stimulated with ADP in the presence of the indicated concentrations of AR-C69931 in a cuvette that was stirred and maintained at 37°. The fluorescence intensity ratio was measured, and the Ca^{2+} concentration was calculated as described in Materials and Methods. Each tracing is representative of at least three similar experiments.

platelet aggregation, the potentiating activity of ADP was inhibited by AR-C69931 and not by A3P5PS (Fig. 7). Therefore, ADP was thought to potentiate the plasmin-induced platelet aggregation in a $P2T_{AC}$-mediated manner. Since no specific antagonist for P2X1 is known, an agonist was used to examine the contribution of P2X1 to the priming effect of ADP. The P2X1 agonist α,β-MeATP is reported to work also as a P2X3 agonist; however, this subtype does not exist in platelets [11]. The role of P2X1 in the platelet response against ADP has not been clarified yet, and this subtype was also not involved in the potentiation of plasmin-induced platelet aggregation. In regard to the signal by which the plasmin-induced platelet aggregation is potentiated, the G_i-coupled signal would seem to be involved, since the $P2T_{AC}$ receptor couples to the G_i protein [15, 16]. Actually, AR-C69931, which inhibited the potentiating effect of ADP, inhibited the ADP-mediated inhibition of adenylate cyclase but did not affect Ca^{2+} mobilization (Figs. 8–10). Our finding that the typical G_i agonist epinephrine shows potentiating activity supports this possibility (Fig. 11). Moreover, mastoparan, a peptide that can directly activate the G_i protein [28, 29], showed a slight but marked potentiating effect on plasmin-induced platelet aggregation (data not shown). Further information on the molecular structure of the $P2T_{AC}$ receptor will be needed to help clarify the relationship between plasmin and $P2T_{AC}$-mediated signals.

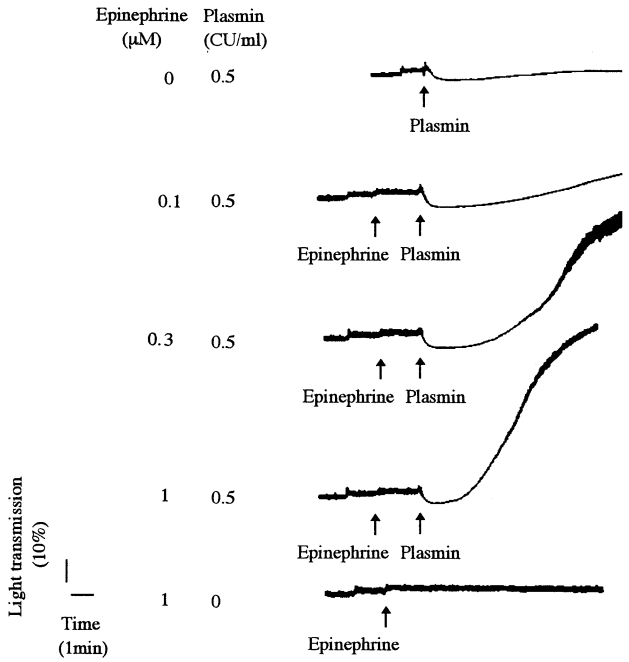

FIG. 11. Effect of epinephrine on plasmin-induced platelet aggregation. Representative traces of platelet aggregation induced by plasmin and/or epinephrine are shown. Platelets were treated with 0, 0.1, 0.3, or 1 μM epinephrine for 1 min at 37° and then were stimulated by plasmin (0.5 CU/mL). As a control, platelets were stimulated by 1 μM epinephrine. Platelet aggregation was monitored by the change in light transmission. Each tracing is representative of at least three similar experiments.

Our present hypothesis on this matter, together with the participation of a G_q-mediated signal in plasmin-induced platelet aggregation suggested by Schafer *et al.* [7], is presented in Fig. 12. Schafer *et al.* have shown that plasmin receptor activation leads to intracellular Ca^{2+} mobilization, and phospholipase C and protein kinase C activation, suggesting the involvement of G_q protein. Protein kinase C activation has been thought to be an essential step in secretion. Other investigators have also reported a plasmin-induced increase in $[Ca^{2+}]_i$ [25, 26], and we were able to confirm that plasmin causes an increase in $[Ca^{2+}]_i$, which reaches a maximum level about 1 min after stimulation by plasmin (data not shown). As it has now been established that both the G_q and G_i signaling pathways are required for fibrinogen receptor activation [17, 18], our data indicating that ADP works via G_i protein activation concomitantly with plasmin, which may activate the G_q signaling pathway, does not conflict with the current model of platelet aggregation. The inability of serotonin, which is known to activate the G_q-mediated signaling pathway in platelets, to potentiate plasmin-induced platelet aggregation (data not shown) may also support our model.

Both ADP and epinephrine are physiological ligands for platelets in blood vessels, and both may also be effective in pathological situations [11, 21]. If the concentrations of ADP or epinephrine are increased in a limited area, there is a greater chance that plasmin-mediated reocclusion will occur. Therefore, the concentrations of these ligands must

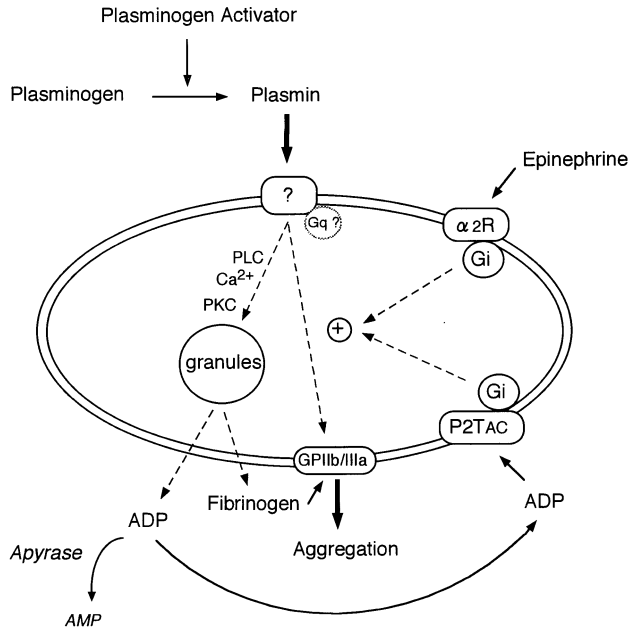

FIG. 12. Proposed scheme of plasmin-induced platelet aggregation. In this scheme, a proposed mechanism of plasmin-induced platelet aggregation through GPIIb/IIIa activation is presented. Plasmin causes degranulation from platelets, and ADP plays the essential role of mediating the aggregation. ADP potentiates the plasmin-induced platelet aggregation via the $P2T_{AC}$ subtype of the P2 receptor. The proposed involvement of G_q, Ca^{2+}, phospholipase C (PLC), and protein kinase C (PKC) in the plasmin signaling pathway is based on the report by Schafer *et al.* [7].

be regulated adequately in order to avoid excessive platelet aggregation. To date, ecto-apyrase (CD39) has been reported to exist on the apical surface of endothelial cells [30, 31]. This enzyme breaks down ADP and ATP to AMP, and prevents ADP-induced platelet activation [31]. This system is important in the regulation of the blood coagulation system. Our finding that plasmin-induced platelet aggregation is highly sensitive to apyrase suggests that the interaction of platelets with endothelial cells may be important in preventing the plasmin-mediated platelet activation under normal circumstances. However, under pathological conditions, such as a fissured atherosclerotic plaque, the normal hemostatic process can evade the control of normal regulation systems, resulting in irreversible vessel occlusion [12]. Indeed, the loss of ADPase activity following endothelial cell injury, as would occur in vascular inflammatory states or xenograft rejection, has been reported [32, 33]. Taking these findings into consideration, we hypothesize that, at the site of an endothelial cell defect accompanied by thrombus, plasmin might easily stimulate the aggregation of platelets.

Our results show that the priming and development of plasmin-induced platelet aggregation are inhibited effectively by blocking the action of ADP. From the viewpoint of therapeutic applications, apyrase or ADP antagonists may be useful in preventing reocclusion after thrombolytic therapy with plasminogen activators. However, when preventing platelet function by medication, excess bleeding must be avoided. And because the platelet aggregation induced by thrombin or collagen was not inhibited by apyrase at a concentration sufficient to prevent plasmin-induced platelet aggregation, apyrase may possibly inhibit reocclusion without excess bleeding. Recombinant soluble apyrase, a mutant lacking in the N- and C- terminals of CD39, has been used successfully to inhibit platelet function *in vitro* [12]. As for AR-C69931, at maximum effective antithrombotic doses there is little prolongation of bleeding time (1.4-fold), which is in marked contrast to the action of GPIIb/IIIa antagonists [19]. Therefore, this ADP antagonist may also be useful for preventing reocclusion without prolonging bleeding time. Since both the apyrase and ADP antagonists lack any inhibitory activity on the proteolytic activity of plasmin, they can work without preventing the degradation of fibrin clots by plasmin. Thus, the information obtained here may eventually help in improving treatment by plasminogen activators.

We are grateful to Dr. Teruhide Yamaguchi and Dr. Toru Kawanishi for their kind and helpful advice. We also thank Astra Research Laboratories for providing AR-C69931. This work was supported by a grant-in-aid from the Japan Health Sciences Foundation.

References

1. Collen D and Lijnen HR, Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* **78:** 3114–3124, 1991.
2. Coller BS, Platelets and thrombolytic therapy. *N Engl J Med* **322:** 33–42, 1990.
3. Gram J, Jespersen J, Kluft C and Rijken DC, On the usefulness of fibrinolysis variables in the characterization of a risk group for myocardial reinfarction. *Acta Med Scand* **221:** 149–153, 1987.
4. Puri RN, Hu CJ, Matsueda R, Umeyama H and Colman RW, Aggregation of washed platelets by plasminogen and plasminogen activators is mediated by plasmin and is inhibited by a synthetic peptide disulfide. *Thromb Res* **65:** 533–547, 1992.
5. Collen D, On the regulation and control of fibrinolysis. *Thromb Haemost* **43:** 77–89, 1980.
6. Califf RM, Fortin DF, Tenaglia AN and Sane DC, Clinical risks of thrombolytic therapy. *Am J Cardiol* **69:** 12a–20a, 1992.
7. Schafer AI, Maas AK, Ware JA, Johnson PC, Rittenhouse SE and Salzman EW, Platelet protein phosphorylation, elevation of cytosolic calcium, and inositol phospholipid breakdown in platelet activation induced by plasmin. *J Clin Invest* **78:** 73–79, 1986.
8. Rabhi Sabile S and Pidard D, Exposure of human platelets to plasmin results in the expression of irreversibly active fibrinogen receptors. *Thromb Haemost* **73:** 693–701, 1995.
9. Rabhi Sabile S, de Romeuf C and Pidard D, On the mechanism of plasmin-induced aggregation of human platelets: Implication of secreted von Willebrand factor. *Thromb Haemost* **79:** 1191–1198, 1998.
10. Watabe A, Ohta M, Matsuyama N, Mizuno K, el Borai N, Tanimoto T, Kawanishi T and Hayakawa T, Characterization of plasmin-induced platelet aggregation. *Res Commun Mol Pathol Pharmacol* **96:** 341–352, 1997.
11. Boarder MR and Hourani SMD, The regulation of vascular function by P2 receptors: Multiple sites and multiple receptors. *Trends Pharmacol Sci* **19:** 99–107, 1998.
12. Gayle RB III, Maliszewski CR, Gimpel SD, Schoenborn MA,

Caspary RG, Richards C, Brasel K, Price V, Drosopoulos JHF, Islam N, Alyonycheva TN, Broekman MJ and Marcus AJ, Inhibition of platelet function by recombinant soluble ecto-ADPase/CD39. *J Clin Invest* **101:** 1851–1859, 1998.
13. Puri R, ADP-induced platelet aggregation and inhibition of adenylyl cyclase activity stimulated by prostaglandins. *Biochem Pharmacol* **57:** 851–859, 1999.
14. Geiger J, Honig Liedl P, Schanzenbacher P and Walter U, Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* **351:** 235–246, 1998.
15. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB and Kunapuli SP, Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273:** 2024–2029, 1998.
16. Jin J, Daniel JL and Kunapuli SP, Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* **273:** 2030–2034, 1998.
17. Jin J and Kunapuli SP, Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* **95:** 8070–8074, 1998.
18. Savi P, Beauverger P, Labouret C, Delfaud M, Salel V, Kaghad M and Herbert JM, Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett* **422:** 291–295, 1998.
19. Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, Humphries RG, Leff P, Clegg JA, Smith JA and Tomlinson W, Antagonists of the platelet P2T receptor: A novel approach to antithrombotic therapy. *J Med Chem* **42:** 213–220, 1999.
20. Grynkiewicz G, Poenie M and Tsien RY, A new generation of $Ca^{2+}{}_i$ indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
21. Motulsky HJ and Insel PA, Adrenergic receptors in man: Direct identification, physiologic regulation, and clinical alterations. *N Engl J Med* **307:** 18–29, 1982.
22. Cerione RA, Regan JW, Nakata H, Codina J, Benovic JL, Gierschik P, Somers RL, Spiegel AM, Birnbaumer L and Lefkowitz RJ, Functional reconstitution of the α_2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J Biol Chem* **261:** 3901–3909, 1986.
23. McNicol A, Functional responses: Secretion. In: *Platelets* (Eds. Watson SP and Authi KS), pp. 13–19, IRL Press, New York, 1995.
24. Puri RN, Zhou FX, Colman RF and Colman RW, Plasmin-induced platelet aggregation is accompanied by cleavage of aggregin and indirectly mediated by calpain. *Am J Physiol* **259:** C862–C868, 1990.
25. Penny WF and Ware JA, Platelet activation and subsequent inhibition by plasmin and recombinant tissue-type plasminogen activator. *Blood* **79:** 91–98, 1992.
26. Nakamura K, Kimura M, Fenton JW II, Andersen TT and Aviv A, Duality of plasmin effect on cytosolic free calcium in human platelets. *Am J Physiol* **268:** C958–C967, 1995.
27. Greenwalt DE and Tandon NN, Platelet shape change and Ca^{2+} mobilization induced by collagen, but not thrombin or ADP, are inhibited by phenylarsine oxide. *Br J Haematol* **88:** 830–838, 1994.
28. Weingarten R, Ransnäs L, Mueller H, Sklar LA and Bokoch GM, Mastoparan interacts with the carboxyl terminus of the α subunit of G_i. *J Biol Chem* **265:** 11044–11049, 1990.
29. Higashijima T, Burnier J and Ross EM, Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. *J Biol Chem* **265:** 14176–14186, 1990.
30. Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH and Robson SC, Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem* **271:** 33116–33122, 1996.
31. Marcus AJ, Broekman MJ, Drosopoulos JH, Islam N, Alyonycheva TN, Safier LB, Hajjar KA, Posnett DN, Schoenborn MA, Schooley KA, Gayle RB and Maliszewski CR, The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest* **99:** 1351–1360, 1997.
32. Bach FH, Robson SC, Winkler H, Ferran C, Stuhlmeier KM, Wrighton CJ and Hancock WW, Barriers to xenotransplantation. *Nat Med* **1:** 869–873, 1995.
33. Robson SC, Candinas D, Siegel JB, Kopp C, Millan M, Hancock WW and Bach FH, Potential mechanism of abnormal thromboregulation in xenograft rejection: Loss of ecto-ATPases upon endothelial cell activation. *Transplant Proc* **28:** 536, 1996.