Aggretin, a novel platelet-aggregation inducer from snake (*Calloselasma rhodostoma*) venom, activates phospholipase C by acting as a glycoprotein Ia/IIa agonist

Tur-Fu HUANG,* Chao-Zong LIU and Sheng-Hsin YANG
Pharmacological Institute, College of Medicine, National Taiwan University, 1 Jen-Ai Road, Sec. 1, Taipei, Taiwan

A potent platelet aggregation inducer, aggretin, was purified from Malayan-pit-viper (*Calloselasma rhodostoma*) venom by ionic-exchange chromatography, gel-filtration chromatography and HPLC. It is a heterodimeric protein (29 kDa) devoid of esterase, phospholipase A and thrombin-like activity. Aggretin (> 5 nM) elicited platelet aggregation with a lag period in both human platelet-rich plasma and washed platelet suspension. EDTA (5 mM), prostaglandin E1 (1 µM) and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (*TMB-8*: 100 µM) abolished its aggregating activity, indicating that exogenous bivalent cations and intracellular Ca²⁺ mobilization are essential for aggretin-induced platelet aggregation. Neomycin (4 mM) and mepacrine (50 µM) completely inhibited aggretin (33 nM)-induced aggregation; however, creatine phosphate/creatine phosphokinase (5 mM, 5 units/ml) and indomethacin (50 µM) did not significantly affect its aggregating activity. Aggretin caused a significant increase of [³H]InsP formation in [³H]InsP-loaded platelets, intracellular Ca²⁺ mobilization and thromboxane B₂ formation. Neomycin, a phospholipase C inhibitor, completely inhibited both the increase of [³H]InsP and intracellular Ca²⁺ mobilization of platelets stimulated by aggretin. A monoclonal antibody (6F1) directed against glycoprotein Ia/IIa inhibited platelet shape change and aggregation induced by aggretin.¹²¹-I-aggretin bound to platelets with a high affinity (Kᵰ = 4.0 ± 1.1 nM), and the number of binding sites was estimated to be 2119 ± 203 per platelet. It is concluded that aggretin may act as a glycoprotein Ia/IIa agonist to elicit platelet aggregation through the activation of endogenous phospholipase C, leading to hydrolysis of phosphoinositides and subsequent intracellular Ca²⁺ mobilization.

INTRODUCTION

Platelets are essential for the normal haemostasis. When blood vessels get injured, platelets adhere to exposed subendothelial matrix, collagen and von Willebrand factor. This adhesion results in platelet activation, release of biologically active substances (e.g. ADP and thromboxane A₂), followed by platelet aggregation, forming a haemostatic plug [1]. A variety of physiological inducers are able to activate platelets, including collagen, ADP, thromboxane A₂, adrenaline (epinephrine) and thrombin. There are two intracellular pathways in mediating platelet activation by most inducers [2]. Upon stimulation, PtdIns(4,5)P₂ is cleaved by phospholipase C to form Ins(1,4,5)P₃ (InsP₃) and diacylglycerol. InsP₃ releases Ca²⁺ from the intracellular stores and rises the cytosolic free Ca²⁺ level [3], while diacylglycerol activates protein kinase C, leading to protein phosphorylation, granule secretion and fibrinogen-receptor expression [4]. Thromboxane A₂ formation is the landmark of the second pathway. This pathway operates when arachidonate is released from membrane phospholipids either by the direct action of phospholipase A₂ or by the sequential action of phospholipase C and diacylglycerol lipase [5,6]. Through a cascade of enzyme-mediated transformation, the liberated arachidonate is then metabolized to prostaglandin endoperoxides and thromboxane A₂, which are potent inducers in activating platelets. It is also well recognized that the release of ADP from platelets accounts for, at least in part, the activation of platelets by collagen, especially at low concentrations [7].

Snake-venom components affect platelet function in various ways. Some venom proteins induce release reaction and platelet aggregation, while others inhibit these reactions [8]. The most pronounced anti-platelet constituents are Arg-Gly-Asp (RGD)-containing trigramin-like peptides, which have been identified as specific fibrinogen receptor [i.e. glycoprotein (GP) IIb/IIIa complex] antagonists [8] and have been termed ‘disintegrins’ [9]. Several non-coagulant, non-enzymic principles that cause platelet aggregation have been purified from different snake venoms [10–17]. However, the detailed action mechanisms of these platelet-aggregation inducers are still obscure. Recently a platelet-aggregation inducer (trimucytin) from *Trimeresurus microsquamatus* (Taiwan habu snake) venom was shown to be a collagen-like activator. It might activate platelets through the binding of a collagen-like receptor on the platelet membrane. A monoclonal antibody directed against GPIa inhibited platelet aggregation caused by collagen and trimucytin [18].

Thrombocytopenia is a common syndrome after *Calloselasma rhodostoma* (Malayan pit viper) has bitten [19], and the crude venom possessed a potent platelet-activating activity in washed rabbit platelet suspensions [10]. In the present study we purified the platelet-activating component (aggretin) from this snake venom and investigated its mechanism of action in activating human platelets.

Abbreviations used: InsP₃, Ins(1,4,5)P₃; GP, glycoprotein; ACD, acid citrate/dextrose; PGE₁, prostaglandin E₁; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(dimethylamino)octyl ester; Fura-2/AM, Fura-2 acetoxymethyl ester; CP/CPK, creatine phosphate/creatine phosphokinase; PRP, platelet-rich plasma.

* To whom correspondence should be addressed.
MATERIALS AND METHODS

Materials

The freeze-dried crude venom of C. rhodostoma was purchased from Latoxan (Rosans, France). Apyrase, Fura-2 acetoxy-methyl ester (Fura-2/AM), neomycin, indomethacin, protaglandin E1 (PGE1), creatine phosphate/creatine phosphokinase (CP/CPK), mepacrine, 3,4,5-dimethoxybenzoic acid 8- (dimethylamino)octyl ester (TMB-8), collagen (type I, bovine achilles tendon), thrombin (human), phosphatidylcholine, firefly-lantern extract (a mixture of luciferin and luciferase) and BSA were purchased from Sigma Chemical Co. Molecular-mass standards for electrophoresis were purchased from Bio-Rad. Rhodostomin is an Arg-Gly-Asp (RGD)-containing peptide purified from C. rhodostoma venom [20]. Heparin was purchased from NOVO Nordic (Bagsvaerd, Denmark). EDTA disodium salt was purchased from Kanto Chemical Co. (Osaka, Japan).

The synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) was purchased from The Peptide Institute Inc. (Tokyo, Japan). Monoclonal antibodies 6F1 (against glycoprotein Ia/IIa), 7E3 (against glycoprotein Iib/IIia) and 6D1 (against glycoprotein Ib) were kindly donated by Dr. Barry S. Coller (State University by New York, Stony Brook, N.Y.). Monoclonal antibody against glycoprotein Ib (AP-1) was generously given by Dr. Robert Montgomery (The Blood Center of Southeastern Wisconsin, Milwaukee, WI, U.S.A.).

Purification of aggretin from C. rhodostoma venom

Aggretin was purified from C. rhodostoma venom by a previously described method [10]. The partially purified aggretin was finally fractionated by HPLC (Waters; model 501 pump; 490E detector) controlled by a personal computer loaded with Baseline 810 software using a protein PAK 300 SW gel-filtration column (7.5 mm × 30 cm) equilibrated and eluted with 0.1 M phosphate buffer, pH 7.0.

SDS/PAGE

Aggretin was analysed on a 12% polyacrylamide gel as described by Laemmli [21]. A 5 μg portion of aggretin pretreated with sample buffer at 95 °C for 5 min in the presence or absence of β-mercaptoethanol (4%) was applied to each well in the gel slab and subjected to electrophoresis. After electrophoresis, gel was stained with Coomassie Blue (0.1%).

Preparation of human platelets

Blood was obtained from individuals who had not taken any medication in the preceding 2 weeks. Blood collected in acid citrate/dextrose (9:1, v/v) or in sodium citrate (3.8%, 9:1, v/v) was centrifuged at 100 g and at 25 °C for 10 min to obtain platelet-rich plasma (PRP). Human washed platelet suspension was prepared from acid citrate/dextrose (ACD)-anticoagulated PRP by the method of Mustard et al. [22] and suspended in Tyrode solution [NaH2PO4, 0.4 (M)/NaCl (136.9 (M))/KCl (2.7 (M))/NaHCO3 (11.9 (M))/CaCl2 (2 (M))/MgCl2 (1 (M)), pH 7.35] containing 3.5 mg/ml BSA.

Measurement of platelet aggregation and release reaction

Platelet aggregation was performed at 37 °C with stirring (900 rev./min) by a turbidimetric method [23], using an aggregometer (Payton) in the case of platelet suspension and PRP. Platelet counts for the aggregation test were adjusted to 3.0 × 10⁸ platelets/ml. The extent of aggregation was expressed in light-transmission units, and the percentage aggregation was calculated on the basis of the the final extent of aggregation of the test samples compared with that of controls. ATP released from activated platelets was simultaneously monitored by bio-luminescence changes in the presence of the mixture of luciferin and luciferase [24].

Thromboxane B2 assay

At 6 min after the addition of platelet-aggregation inducer, 2 mM EDTA and 50 μM indomethacin were added to halt thromboxane formation. After centrifugation in an Eppendorf microcentrifuge (model 5415C) for 2 min, thromboxane B2 in the supernatant was assayed by an EIA kit (Cayman Chemical Co.).

Measurement of the intracellular Ca2+ level

The concentration of cytosolic free Ca2+ was determined using a fluorescent probe, Fura-2, according to the method of Pollock and Rink [25]. In brief, Fura-2/AM (1 μM) was incubated with ACD-anticoagulated platelet-rich plasma at 37 °C for 50 min. Then Fura-2/AM-loaded platelet suspension was prepared similarly to the preparation of the platelet suspension mentioned above. The intracellular Ca2+ level was measured by the fluorescence change monitored by a Hitachi fluorescence spectrophotometer (excitation at 339 nm, emission at 500 nm). The intracellular Ca2+ concentration was calculated using the equation given by Grynkiewicz et al. [26]. To see whether the influx of extracellular Ca2+ contributes the cytosolic Ca2+ increase, isolated human platelets were suspended in Ca2+-free Tyrode solution after the last wash and these experiments were carried out simultaneously with those under normal conditions.

Measurement of the production of [3H]InsP3

After the centrifugation of ACD-anticoagulated PRP at 1500 g at 37 °C for 10 min, the platelet pellets were resuspended in 1 ml of Ca2+-free and BSA-free Tyrode solution containing 75 mM LiCl/ml of myo-[2-3H]Inos (Amersham International) and 1 mM EDTA. After incubation at 37 °C for 2 h, the platelets were collected by centrifugation and suspended in normal Tyrode solution mentioned above. Phosphoinositide breakdown was initiated by adding aggregation inducer to 1 ml of platelet suspension (3 × 10⁸ platelets/ml) with stirring at 900 rev./min and 37 °C for 6 min. Indomethacin (50 μM) was added in order to rule out the possible contribution of endogenous thromboxane-induced phosphoinositide hydrolysis. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 2000 g for 10 min, 1 ml of supernatant was pooled and trichloroacetic acid was removed by extraction with three 5 ml portions of diethyl ether. The aqueous phase containing the inositol phosphates was adjusted to pH 7–8 and diluted to 4 ml with distilled water before application to a Dowex-1 ion-exchange column for separation of the inositol phosphates as described by Neylon and Summers [27]. All the experiments were performed in the presence of 5 mM LiCl to inhibit InsP3 phosphatase. Because the levels of InsP4 and InsP2 were very low, we measured InsP3 as an index of the total inositol phosphates formed.

Radiolabelling of aggretin

The purified aggretin was labelled with Na125I (Amersham International) using Enzymobeads (Bio-Rad) at room temperature for 25 min, and the labelled aggretin was separated from free
Na\textsubscript{125}I on a Sephadex G-25 column. The specific radioactivity of \textsubscript{125}I-aggretin was about 20000 c.p.m./\mu g of protein.

**Binding of \textsubscript{125}I-aggretin to platelets**

Ligand–platelet binding studies were performed at room temperature (25 °C) by a previously described method [28]. In brief, the incubation mixture (total volume 500 \mu l; 3 \times 10^9 platelets/ml) was composed of 400 \mu l of platelet suspension (3.75 \times 10^8/ml), 10 \mu l of \textsubscript{125}I-aggretin and an appropriate volume of Tyrode solution. After the addition of \textsubscript{125}I-aggretin, the platelet suspension was gently shaken briefly and incubated for another 10 min. Then 400 \mu l of platelet suspension was centrifuged through the sucrose solution (20 \%, w/v) at 14000 rev./min (8000 \(g\)) for 5 min using an Eppendorf microcentrifuge (model 5415C). The radioactivities of the supernatants and the scissored tips containing pellets were counted in an LKB \(\gamma\)-radiation counter. Non-specific binding was measured in the presence of a 100-fold excess of unlabelled aggretin. Specific binding of \textsubscript{125}I-aggretin was calculated as the difference between total binding and non-specific binding of \textsubscript{125}I-aggretin and analysed by means of a Scatchard plot.

**Enzyme activity**

Phospholipase A activity was estimated by the indirect haemolysis method described by Brown and Bowles [29] using phosphatidylycholine as substrate. Esterase activity was measured by the method of Hestrin [30]. Thrombin-like activity was measured by the method of Ouyang et al. [10].

**RESULTS**

**Characterization of aggretin and the induction of platelet aggregation by aggretin**

By means of various column-chromatographic techniques, aggretin was isolated from *C. rhodostoma* venom. SDS/PAGE analysis of aggretin showed that it is a double-chain protein with a molecular mass of 29 kDa consisting of two subunits with molecular masses of 18 kDa and 15 kDa (Figure 1). The purified aggretin was devoid of the phospholipase A\textsubscript{2}, tosylarginine methyl esterase or thrombin-like activities detected in crude venom (results not shown). Aggretin (> 5 nM, 0.15 \mu g/ml) induced platelet aggregation and release reaction with a lag period in both human PRP (results not shown) and washed platelet suspension (Figures 2 and 3). Aggretin induced platelet aggregation in an all-or-none manner. A lower concentration of aggretin (0.15 \mu g/ml) also induced a maximal aggregation with a longer latent period. However, if the concentration of aggretin was too low (\(< 0.1 \mu g/ml\)), it did not trigger any aggregation (Figure 2).

**Effects of some inhibitors on aggretin-induced platelet aggregation**

In order to determine the contribution of endogenous thromboxane in aggretin-induced platelet activation, we examined the effect of indomethacin, an inhibitor of cyclo-oxygenase. Indomethacin (50 \mu M), which inhibited collagen (10 \mu g/ml)-induced human platelet aggregation completely (results not shown), only partially attenuated the aggregation induced by aggretin (1.0 \mu g/ml) (Table 1). The ADP scavengers, apyrase (1 unit/ml) and CP/CPK (5 mM, 5 units/ml), had no effect on aggretin-induced aggregation (Table 1). Tosylarginine methyl ester (100 \mu M) blocked the aggregation of washed platelets induced by thrombin (0.2 units/ml), while it did not affect aggretin (1 \mu g/ml)-induced aggregation (Table 1). On the other hand, PGE\textsubscript{1} (1 \mu M), neomycin (4 mM), mepacrine (50 \mu M), TMB-8
Figure 3 Effects of 6F1 (monoclonal antibody against GPIIb/IIIa) and RGD-containing peptides (rhodostomin and GRGDS) on aggretin-induced platelet aggregation and ATP release

6F1 (50 μg/ml) and RGD-containing peptides, rhodostomin (1 μg/ml) and GRGDS (200 μg/ml), were incubated with human platelets for 3 min prior to the addition of aggretin (1 μg/ml). After the addition of aggretin, platelet aggregation (upward tracing; ΔT = change in light transmission) and ATP release (downward tracing) were continuously recorded for 6 min. Platelet aggregation and ATP release reaction were monitored by the turbidimetric method and by bioluminescence changes respectively.

Table 1 Effects of some antiplatelet agents on the aggregation of washed human platelets induced by aggretin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>100</td>
</tr>
<tr>
<td>Indomethacin (50 μM)</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>TAME (100 μM)</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>Apyrase (1 unit/ml)</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>CP/CPK (5 mM/5 units/ml)</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>Neomycin (4 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Mepacrine (50 μM)</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>PGE (1 μM)</td>
<td>0</td>
</tr>
<tr>
<td>EDTA (5 mM)</td>
<td>0</td>
</tr>
<tr>
<td>TMB-8 (100 μM)</td>
<td>0</td>
</tr>
</tbody>
</table>

(100 μM) and EDTA (5 mM) completely blocked the aggregation elicited by aggretin (1 μg/ml) (Table 1).

Effect of indomethacin on aggretin-induced thromboxane B₂ formation

Thromboxane B₂ is the stable metabolite of thromboxane A₂. Therefore we measured the amount of thromboxane B₂ instead of thromboxane A₂. Thromboxane B₂ formation of washed human platelets in response to aggretin (1 μg/ml) was 88 ± 6.8 ng/ml (resting level, 2.3 ± 0.2 ng/ml, n = 4). Indomethacin (50 μM) profoundly attenuated the thromboxane B₂ formation caused by aggretin (1 μg/ml) (82 % inhibition), whereas it only slightly blocked aggregation induced by aggretin (23 % inhibition) (Table 1).

Ca²⁺ mobilization

By using the specific Ca²⁺ probe Fura-2, we further investigated the mechanisms involved in aggretin-induced platelet activation and aggregation. Aggretin (1 μg/ml) caused an increase (from 75 ± 2 to 298 ± 20 nM, n = 3) in intracellular Ca²⁺ in platelets with a lag period (Figure 4). Even in Ca²⁺-free medium, aggretin showed an equal ability to elevate the level of intracellular Ca²⁺ (results not shown). This enhanced Ca²⁺ flux was completely inhibited by neomycin (4 mM). However, indomethacin (50 μM) did not affect the increase in the level of intracellular Ca²⁺ caused by aggretin (Figure 4). Pretreatment of the RGD-containing peptides rhodostomin and GRGDS partially inhibited (by 50 %) the aggretin-induced cytosolic Ca²⁺ increase (results not shown).

Phosphoinositide breakdown

Many aggregation inducers trigger phosphoinositide breakdown, which subsequently leads to platelet activation and aggregation [31–33]. Aggretin (1 μg/ml) and thrombin (0.4 unit/ml) caused 4.0 ± 0.7- and 4.7 ± 1.5-fold increase of the InsP₃ formation in the presence of indomethacin (50 μM) respectively as compared with
that of resting platelets. Pretreatment with neomycin (4 mM) completely blocked aggretin- and thrombin-induced InsP₃ formation (Table 2).

### Effects of monoclonal antibodies and RGD-containing peptides on platelet aggregation and the release reaction caused by aggretin

To analyse the possible receptor of aggretin on the platelet membrane, the effect of some monoclonal antibodies (6F1, AP-1, 7E3 and 6D1) were tested. At 10 μg/ml, the monoclonal antibody (6F1) raised against GPIa/IIa, which has been proposed to be a collagen receptor [34,35], dramatically inhibited collagen-induced platelet shape-change (reflected by the initial decrease in transmission) and aggregation [36]. Similarly, 6F1 also blocked the platelet shape-change, aggregation and release reaction induced by Aggretin (Figure 3) without exerting any inhibitory effect on those elicited by other inducers (e.g. thrombin) (T. F. Huang and C. Z. Liu, unpublished work). However, a higher concentration (50 μg/ml) of 6F1 was required in order to block the platelet aggregation and ATP release completely (Figure 3). By contrast, other monoclonal antibodies (AP-1 and 6D1, raised against platelet GPIb) prolonged human thrombin (0.2 unit/ml)-induced platelet aggregation without exerting any appreciable effect on platelet aggregation induced by aggretin (T. F. Huang and C. Z. Liu, unpublished work). Monoclonal antibody (7E3) against GPIIb/IIIa (results not shown), rhodostomin and the synthetic peptide GRGDS, which are GPIIb/IIIa antagonists, inhibited platelet aggregation without interfering with the shape-change and release reaction elicited by several aggregation agonists [20], including aggretin (Figure 3).

### Binding of aggretin to human platelets

As shown in Figure 5, ¹²⁵I-aggretin bound to human platelets in a saturable manner. Pretreatment of platelet suspension with EDTA (5 mM) or PGE₁ (1 μM) for 3 min did not significantly affect the binding of aggretin to platelets (T. F. Huang and C. Z. Liu, unpublished work). Its binding is bivalent-cation-independent. Scatchard analysis of ¹²⁵I-aggretin binding data reveals that ¹²⁵I-aggretin bound to human platelets with a high affinity (Kₛ = 4.0 ± 1.1 nM) and the number of binding sites was estimated to be 2119 ± 203/platelet (n = 4).

### DISCUSSION

Physiological agonists, such as thrombin, induce platelet activation through a transmembrane signalling system involving the hydrolysis of inositol phospholipids, generation of second messenger, diacylglycerol and InsP₃, thereby enhancing the intracellular Ca²⁺ level, protein phosphorylation and release.

![Figure 4](image)

**Figure 4** Effects of indomethacin (50 μM) and neomycin (4 mM) on the increase of intracellular Ca²⁺ concentration of human platelets triggered by aggretin.

Fura-2/AM-loaded platelets were incubated with control buffer, indomethacin (50 μM) or neomycin (4 mM) for 3 min; then aggretin (1 μg/ml, 33 nM) was added to induce an increase in the level of cytosolic Ca²⁺.

![Table 2](image)

**Table 2** Effects of neomycin on the formation of InsP₃ formation in washed human platelets caused by aggretin and thrombin

<table>
<thead>
<tr>
<th>[³H]InsP increase in platelets (fold)</th>
<th>Unstimulated</th>
<th>Aggretin</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4 mM)</td>
<td>1</td>
<td>4.0 ± 0.7</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>Neomycin (4 mM)</td>
<td>ND</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

![Figure 5](image)

**Figure 5** Isotherm of ¹²⁵I-aggretin binding to washed human platelets

Human platelet suspension (3 x 10⁹ platelets/ml) was treated under non-stirring conditions with various concentrations of ¹²⁵I-aggretin for 10 min at 25 °C. Open triangles represent the specific binding of ¹²⁵I-aggretin after the subtraction of non-specific binding (open circle) from total binding (closed circle). The inset shows the Scatchard plot. Results are from a representative of four similar experiments.
reaction [32]. Aggretin induced platelet aggregation, ATP release (Figures 2 and 3) and thromboxane formation. It also enhanced the total InsP formation (Table 2) and cytosolic Ca**+ level (Figure 4). Platelet aggregation and the increase in cytosolic Ca**+ caused by aggretin were not significantly blocked by indomethacin (Table 1 and Figure 4), even though thromboxane formation was almost abolished. Furthermore, aggretin-induced aggregation was resistant to ADP-scavenging systems, CP/CPK and apyrase (Table 1). These results indicate that the positive feedback mechanisms involving ADP release and thromboxane A**+ formation do not contribute to platelet aggregation caused by aggretin. Neomycin, a cationic aminoglycoside antibiotic that interacts with polyphosphoinositides resulting in blockade of phosphoinositide breakdown [37], completely inhibited platelet aggregation, Ca**+ mobilization and InsP formation caused by aggretin (Tables 1 and 2, and Figure 4). This provides evidence supporting the involvement of phosphoinositide breakdown in the platelet activating process of aggretin. Other pieces of evidence also support this inference, because aggretin-induced aggregation was inhibited by the following compounds affecting phosphoinositide turnover or Ca**+ mobilization (Table 1): mepacrine, an inhibitor of both phospholipase A**+ and phospholipase C [38]; PGE**+, an activator of adenylate cyclase; and TMB-8, an inhibitor of intracellular calcium mobilization (39).

RGD-containing peptides (rhodostomin and GRGDs) are glycoprotein IIb-IIIa (GPIIb/IIIa) antagonists which inhibit the binding of fibrinogen to activated GPIIb/IIIa and platelet aggregation without affecting the shape-change and release reaction of platelets [20]. They also inhibited human platelet aggregation, but not the shape-change and release reaction elicited by aggretin (Figure 3), even though they attenuated (50% inhibition) the cytosolic Ca**+ increase in platelets (results not shown). This indicates that the binding of fibrinogen to GPIIb-IIIa and the subsequent platelet aggregation is not essential for the initial activation of platelets triggered by aggretin, but platelet aggregation may potentiate the increase in cytosolic Ca**+

GPIIa/IIa has been demonstrated to be a candidate for the collagen receptor [34,35], because monoclonal antibody (6F1), raised against GPIIa/IIa, specifically inhibited platelet shape-change and aggregation induced by collagen [36]. Since 6F1 blocked the platelet shape-change and aggregation elicited by aggretin, and the number of GPIIa/IIa molecules (2000/platelet) expressed on platelets [40] is similar to the number of binding sites (2191/platelet) of aggretin, GPIIa/IIa is essential, and might be the functional receptor for aggretin to activate platelets. Although GPIIIa/IIa expressed on platelet membrane is important for both collagen and aggretin to induce platelet aggregation, there is still some diversity. As shown in Figure 2, aggretin induced human platelet aggregation in an all-or-none manner, which was characterized by a longer latent period required to initiate shape-change and the subsequent aggregation when a lower dose of aggretin was added. Unlike aggretin, collagen dose-dependently induced platelet aggregation with an identical latent period in causing shape-change (T. F. Huang and C. Z. Liu, unpublished work). Indomethacin (50 µM), which completely inhibited collagen (10 µg/ml)-induced platelet aggregation, only partially attenuated the platelet aggregation induced by aggretin (Table 1), even though thromboxane formation was almost abolished. In addition, the ADP scavenger CP/CPK, which inhibited collagen (5 µg/ml)-induced platelet aggregation remarkably, had no significant effect on the aggregation triggered by aggretin (0.2–1.0 µg/ml) (results not shown).

Tricumycin, a collagen-like inducer purified from T. macro-

miquamatus venom, activates rabbit platelets through phospho-

inositide breakdown and Ca**+ mobilization independent of ADP release and thromboxane formation [14]. Although signal transduction in platelet activation and aggregation evoked by trimucycin is similar to that of aggretin, pretreatment of trimucycin did not displace the binding of aggretin to human platelets (T. F. Huang and C. Z. Liu, unpublished work). It suggests that trimucycin and aggretin may bind to different epitopes of GPIIa or GPIIa.

All these data obtained in the present study suggest that aggretin might bind to a collagen-like receptor (either Ia or IIa) with a high affinity (K**+ = 4.0±1.1 nM) in a bivalent-cation-independent manner and then activate human platelets through the activation of endogenous phospholipase C, leading to InsP**+ formation and intracellular Ca**+ mobilization, and, subsequently, platelet aggregation. Aggretin may become an useful probe for the detection of membrane GPIIa or GPIIIa and for the elucidation of signal-transduction pathways involved with platelet GPIIa or GPIIa.

This program was financially supported by a grant from the National Science Council of Taiwan (NSC 79-0418-B002-01 and NSC 82-0412-B002-086). We thank Dr. Barry S. Collier for kindly providing monoclonal antibodies 6D1, 6F1 and 7E3, and Dr. Robert Montgomery for providing monoclonal antibody 4-1.

REFERENCES

A potent platelet aggregation inducer from snake (Calloselasma rhodostoma) venom

Received 31 October 1994/23 March 1995; accepted 3 April 1995
