

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]Ins-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.¹²⁵I-aggretin bound to platelets with a high affinity ($K_d = 4.0 \pm 1.1$ 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)P₃ 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 (trimucylin) from *Trimeresurus mucrosquamatus* (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 trimucylin [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, prostaglandin 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 Nordisc (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 aggrexin from *C. rhodostoma* venom

Aggrexin was purified from *C. rhodostoma* venom by a previously described method [10]. The partially purified aggrexin 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

Aggrexin was analysed on a 12% polyacrylamide gel as described by Laemmli [21]. A 5 µg portion of aggrexin 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 mM)/NaCl (136.9 mM)/KCl (2.7 mM)/NaHCO₃ (11.9 mM)/CaCl₂ (2 mM)/MgCl₂ (1 mM), 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 bioluminescence 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 by 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]InsP

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 µCi/ml of *myo*-[2-³H]Ins (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 InsP phosphatase. Because the levels of InsP₂ and InsP₃ were very low, we measured InsP as an index of the total inositol phosphates formed.

Radiolabelling of aggrexin

The purified aggrexin was labelled with Na¹²⁵I (Amersham International) using Enzymobeads (Bio-Rad) at room temperature for 25 min, and the labelled aggrexin was separated from free

Na¹²⁵I on a Sephadex G-25 column. The specific radioactivity of ¹²⁵I-aggrexin was about 20000 c.p.m./ μ g of protein.

Binding of ¹²⁵I-aggrexin 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 μ l; 3×10^8 platelets/ml) was composed of 400 μ l of platelet suspension (3.75×10^8 /ml), 10 μ l of ¹²⁵I-aggrexin and an appropriate volume of Tyrode solution. After the addition of ¹²⁵I-aggrexin, the platelet suspension was gently shaken briefly and incubated for another 10 min. Then 400 μ 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 γ -radiation counter. Non-specific binding was measured in the presence of a 100-fold excess of unlabelled aggrexin. Specific binding of ¹²⁵I-aggrexin was calculated as the difference between total binding and non-specific binding of ¹²⁵I-aggrexin 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 phosphatidylcholine 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 aggrexin and the induction of platelet aggregation by aggrexin

By means of various column-chromatographic techniques, aggrexin was isolated from *C. rhodostoma* venom. SDS/PAGE analysis of aggrexin 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 aggrexin was devoid of the phospholipase A₂, tosylarginine methyl esterase or thrombin-like activities detected in crude venom (results not shown). Aggrexin (> 5 nM, 0.15 μ g/ml) induced platelet aggregation and release reaction with a lag

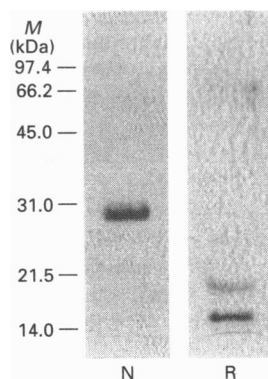


Figure 1 SDS/PAGE pattern of aggrexin

Aggrexin was pretreated with sample buffer in the presence (R) or absence (N) of reducing agent (5% β -mercaptoethanol) at 95 °C for 5 min. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. A 12% gel was used. Abbreviation: M, molecular mass.

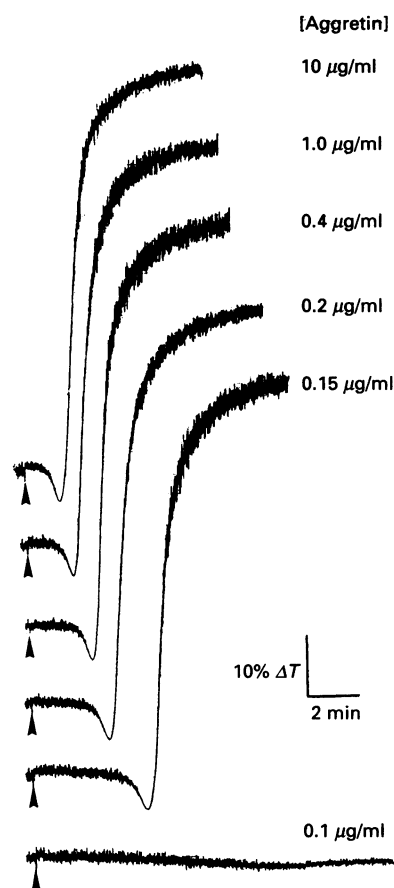


Figure 2 Platelet aggregation induced by various doses of aggrexin

Various concentrations of aggrexin (0.1–10 μ g/ml) were added to washed human platelet suspension (3×10^8 platelets/ml) to trigger platelet aggregation. The arrowhead marks the addition of aggrexin. A turbidimetric method was used to monitor the aggregation response. ΔT is change in light transmission.

period in both human PRP (results not shown) and washed platelet suspension (Figures 2 and 3). Aggrexin induced platelet aggregation in an all-or-none manner. A lower concentration of aggrexin (0.15 μ g/ml) also induced a maximal aggregation with a longer latent period. However, if the concentration of aggrexin was too low (≤ 0.1 μ g/ml), it did not trigger any aggregation (Figure 2).

Effects of some inhibitors on aggrexin-induced platelet aggregation

In order to determine the contribution of endogenous thromboxane in aggrexin-induced platelet activation, we examined the effect of indomethacin, an inhibitor of cyclo-oxygenase. Indomethacin (50 μ M), which inhibited collagen (10 μ g/ml)-induced human platelet aggregation completely (results not shown), only partially attenuated the aggregation induced by aggrexin (1.0 μ g/ml) (Table 1). The ADP scavengers, apyrase (1 unit/ml) and CP/CPK (5 mM, 5 units/ml), had no effect on aggrexin-induced aggregation (Table 1). Tosylarginine methyl ester (100 μ M) blocked the aggregation of washed platelets induced by thrombin (0.2 units/ml), while it did not affect aggrexin (1 μ g/ml)-induced aggregation (Table 1). On the other hand, PGE₁ (1 μ M), neomycin (4 mM), mepacrine (50 μ M), TMB-8

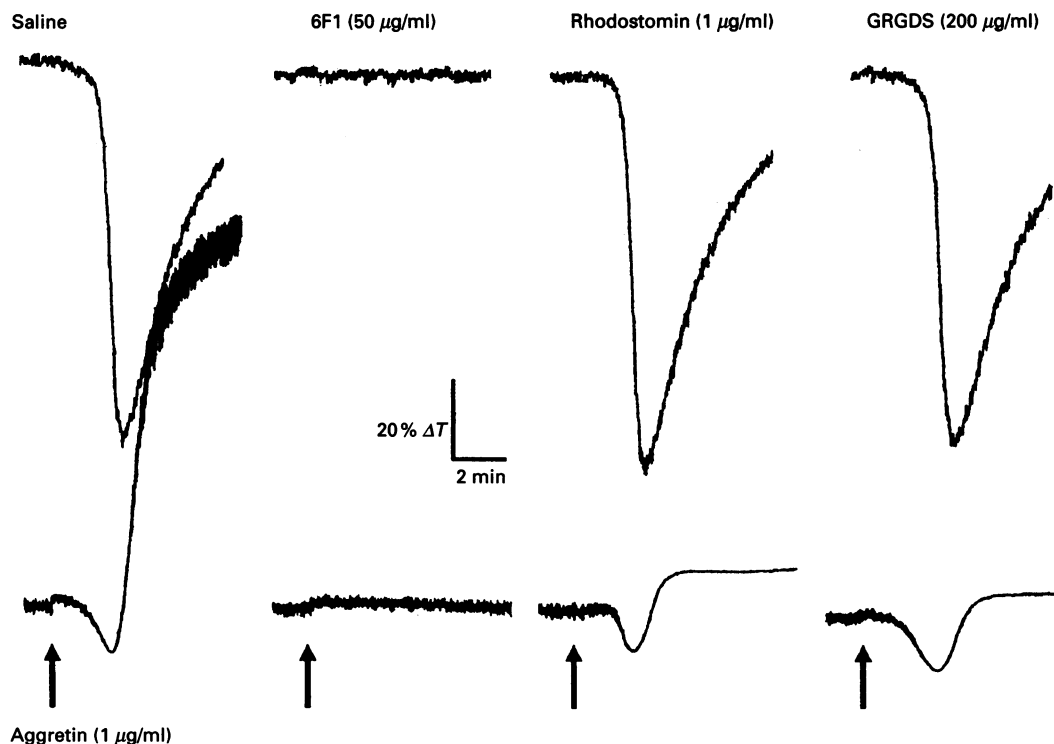


Figure 3 Effects of 6F1 (monoclonal antibody against GPIa/IIa) and RGD-containing peptides (rhodostomin and GRGDS) on aggregin-induced platelet aggregation and ATP release

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 aggregin (1 µg/ml). After the addition of aggregin, 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 aggregin

Platelets were pretreated with indomethacin (50 µM), neomycin (4 mM), mepacrine (50 µM), TMB-8 (100 µM), PGE₁ (1 µM), EDTA (5 mM) for 3 min or tosylarginine methyl ester (TAME) (100 µM), apyrase (1 unit/ml), CP/CPK (5 mM/5 units/ml) for 1 min, then aggregin (1 µg/ml) was added to trigger platelet aggregation. Values are presented as mean \pm S.E.M. ($n = 5$).

Treatment	Aggregation (%)
Saline	100
Indomethacin (50 µM)	77 \pm 2
TAME (100 µM)	91 \pm 3
Apyrase (1 unit/ml)	96 \pm 4
CP/CPK (5 mM/5 units/ml)	93 \pm 4
Neomycin (4 mM)	0
Mepacrine (50 µM)	8 \pm 3
PGE ₁ (1 µM)	0
EDTA (5 mM)	0
TMB-8 (100 µM)	0

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

Effect of indomethacin on aggregin-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 aggregin (1 µg/ml) was 88 \pm 6.8 ng/ml (resting level, 2.3 \pm 0.2 ng/ml, $n = 4$). Indomethacin (50 µM) profoundly attenuated the thromboxane B₂ formation caused by aggregin (1 µg/ml) (82% inhibition), whereas it only slightly blocked aggregation induced by aggregin (23% inhibition) (Table 1).

Ca²⁺ mobilization

By using the specific Ca²⁺ probe Fura-2, we further investigated the mechanisms involved in aggregin-induced platelet activation and aggregation. Aggregin (1 µg/ml) caused an increase (from 75 \pm 2 to 298 \pm 20 nM, $n = 3$) in intracellular Ca²⁺ in platelets with a lag period (Figure 4). Even in Ca²⁺-free medium, aggregin 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 aggregin (Figure 4). Pretreatment of the RGD-containing peptides rhodostomin and GRGDS partially inhibited (by 50%) the aggregin-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]. Aggregin (1 µg/ml) and thrombin (0.4 unit/ml) caused 4.0 \pm 0.7- and 4.7 \pm 1.5-fold increase of the InsP formation in the presence of indomethacin (50 µM) respectively as compared with

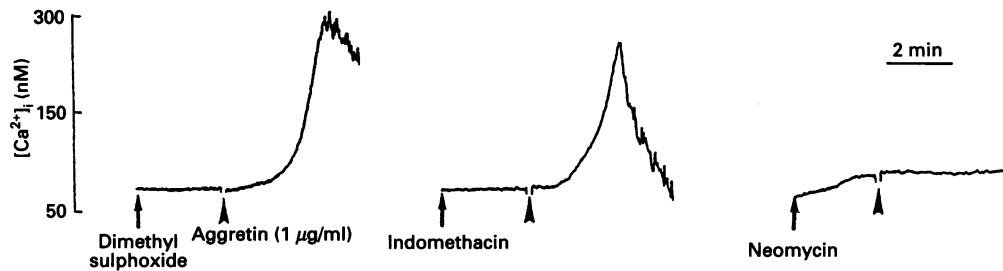


Figure 4 Effects of indomethacin (50 μM) and neomycin (4 mM) on the increase of intracellular Ca^{2+} concentration of human platelets triggered by aggretin

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

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

$[\text{H}^3]\text{InsP}$ -labelled platelets were preincubated with or without neomycin (4 mM) for 3 min in the presence of indomethacin (50 μM), then aggretin (1 $\mu\text{g}/\text{ml}$) or thrombin (0.4 unit/ml) was added and the mixture stirred for a further 6 min. The measurement of $[\text{H}^3]\text{InsP}$ was carried out as described in the Materials and methods section. The resting level of $[\text{H}^3]\text{InsP}$ in unstimulated platelet was 687 ± 142 c.p.m. The fold increase of $[\text{H}^3]\text{InsP}$ (relative to unstimulated platelets) caused by aggretin and thrombin are presented as mean \pm S.E.M. ($n = 3$). Abbreviation: ND, not determined.

	InsP increase in platelets (fold)		
	Unstimulated	Aggretin	Thrombin
Control	1	4.0 ± 0.7	4.7 ± 1.5
Neomycin (4 mM)	ND	1.2 ± 0.2	1.1 ± 0.1

that of resting platelets. Pretreatment with neomycin (4 mM) completely blocked aggretin- and thrombin-induced InsP 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 $\mu\text{g}/\text{ml}$, 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 $\mu\text{g}/\text{ml}$) 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

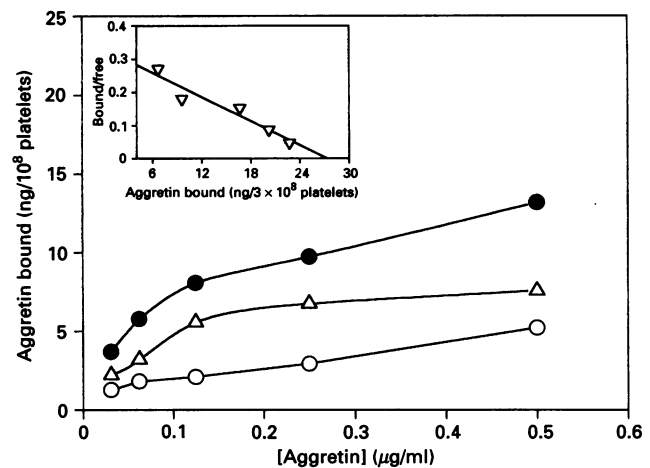


Figure 5 Isotherm of ^{125}I -aggretin binding to washed human platelets

Human platelet suspension (3×10^8 platelets/ml) was treated under non-stirring conditions with various concentrations of ^{125}I -aggretin for 10 min at 25 $^{\circ}\text{C}$. Open triangles represent the specific binding of ^{125}I -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.

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, ^{125}I -aggretin bound to human platelets in a saturable manner. Pretreatment of platelet suspension with EDTA (5 mM) or PGE_1 (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 ^{125}I -aggretin binding data reveals that ^{125}I -aggretin bound to human platelets with a high affinity ($K_d = 4.0 \pm 1.1$ 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 InsP_3 , thereby enhancing the intracellular Ca^{2+} level, protein phosphorylation and release

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^{2+} level (Figure 4). Platelet aggregation and the increase in cytosolic Ca^{2+} caused by aggretin were not significantly blocked by indomethacin (Table 1 and Fig. 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_2 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^{2+} 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^{2+} mobilization (Table 1): mepacrine, an inhibitor of both phospholipase A_2 and phospholipase C [38]; PGE_1 , 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^{2+} 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^{2+} .

GPIa/IIa has been demonstrated to be a candidate for the collagen receptor [34,35], because monoclonal antibody (6F1), raised against GPIa/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 GPIa/IIa molecules (2000/platelet) expressed on platelets [40] is similar to the number of binding sites (2119/platelet) of aggretin, GPIa/IIa is essential, and might be the functional receptor for aggretin to activate platelets. Although GPIa/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 $\mu\text{g}/\text{ml}$)-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 $\mu\text{g}/\text{ml}$)-induced platelet aggregation remarkably, had no significant effect on the aggregation triggered by aggretin (0.2–1.0 $\mu\text{g}/\text{ml}$) (results not shown).

Trimucylin, a collagen-like inducer purified from *T. mucronatum* venom, activates rabbit platelets through phospho-

inositide breakdown and Ca^{2+} mobilization independent of ADP release and thromboxane formation [14]. Although signal transduction in platelet activation and aggregation evoked by trimucylin is similar to that of aggretin, pretreatment of trimucylin did not displace the binding of aggretin to human platelets (T. F. Huang and C. Z. Liu, unpublished work). It suggests that trimucylin and aggretin may bind to different epitopes of GPIa 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 ($K_d = 4.0 \pm 1.1 \text{ nM}$) in a bivalent-cation-independent manner and then activate human platelets through the activation of endogenous phospholipase C, leading to InsP_3 formation and intracellular Ca^{2+} mobilization, and, subsequently, platelet aggregation. Aggretin may become an useful probe for the detection of membrane GPIa or GPIIa and for the elucidation of signal-transduction pathways involved with platelet GPIa or GPIIa.

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