British Journal of Pharmacology (2001) 132, 843–850

www.nature.com/bjp

Pharmacological characterization and antithrombotic effect of agkistin, a platelet glycoprotein Ib antagonist

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1 Agkistin, purified from the snake venom of Formosan *Agkistrodon acutus*, belongs to the family of C-type lectin GPIb binding proteins. It is a heterodimeric molecule, consisting of α- (16.5 kDa) and β- (15.5 kDa) subunits with a molecular mass of 32,512 Daltons examined by SDS–PAGE and mass spectrometry.

2 *In vitro*, agkistin concentration-dependently inhibited ristocetin-induced human platelet agglutination and aggregation in the presence of vWF. It also inhibited TXA_2 formation and prolonged the latent period in triggering aggregation by a low concentration of thrombin (0.03 u ml^{-1}).

3 ^{125}I-agkistin specifically bound to unactivated human platelets in a saturable manner with a K_D value of 223 ± 10.6 nM. This binding reaction was rapid and reversible. Monoclonal antibodies, AP1 and 6D1 raised against platelet GPIb, almost completely blocked ^{125}I-agkistin binding to platelets. However, monoclonal antibody 7E3 raised against GPIIb/IIIa complex, trigramin, a GPIIb/IIIa antagonist, ADP and EDTA did not affect ^{125}I-agkistin binding reaction.

4 Agkistin (250 μg kg^{-1}) significantly prolonged the bleeding time and induced transient thrombocytopenia of mice when given intravenously. Furthermore, it markedly inhibited platelet plug formation in irradiated mesenteric venules of fluorescein-treated mice *in vivo*.

5 In conclusion, agkistin inhibits ristocetin induced platelet aggregation mainly through its specific binding to platelet GPIb, thereby blocking the interaction between GPIb and vWF. In addition, agkistin exhibits antithrombotic activity *in vivo*.

British Journal of Pharmacology (2001) **132,** 843–850

Keywords: Agkistin; platelet; glycoprotein Ib; anti-thrombotic; snake venom

Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediamine-tetraacetic acid; GP, glycoprotein; kDa, kilodalton; mAb, monoclonal antibody; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide electrophoresis; TX, thromboxane; vWF, von Willebrand factor

Introduction

Binding of platelet glycoprotein (GP) Ib by von Willebrand factor (vWF) can play a major role in platelet adhesion, allowing platelet aggregation and subsequent thrombosis formation either at sites of damaged endothelium or under high shear conditions associated with stenosed arteries (Kroll *et al*., 1996; Sakariassen *et al*., 1987). It is well established that inhibition of platelet adhesion is important for preventing the platelet-rich thrombus formation in many thrombogenic disorders (Roth, 1992). Additionally, thrombin is an important mediator of platelet aggregation under normal physiological and pathological conditions. The membrane GPIb receptor is implicated in enhancing the platelet response to low concentration of thrombin, and in regulating the interactions of platelets with their environment *via* linking with membrane-associated cytoskeleton (Greco *et al*., 1996; Clemetson & Clemetson, 1995). Therefore, platelet membrane GPIb, the receptor for both vWF and thrombin binding toward platelets may be a good target for preventing thrombosis.

Snake venoms affect blood coagulation and platelet aggregation in various ways (Ouyang *et al*., 1992). Recently many studies have focused on the characterization of antiplatelet components of snake venoms in the hope of finding some potential candidates for the development of antithrombotic agents, which may be useful for the treatment of arterial thrombosis in case of myocardial infarction, embolic stroke and atherosclerosis (Huang, 1998). Disintegrin (e.g. trigramin) represents a family of viper venom proteins that potently block platelet aggregation elicited by various agonists, acting as a platelet GPIIb/IIIa (i.e. fibrinogen receptor) antagonist by interfering with fibrinogen binding to its receptor of the activated platelets (Huang *et al*., 1987; Gould *et al*., 1990). The discovery of disintegrins has a great impact especially on the development of synthetic low molecular weight antithrombotic agents (e.g. Integrelin, a specific integrin $\alpha_{IIb}\beta_3$ antagonist) that are currently in a clinical trial (Roe *et al*., 2000; Scarborough, 1999). On the other hand, snake venoms also contain many components that modulate the GPIb-vWF axis (Fujimura *et al*., 1996). They belong to a member of the C-type lectin family and fall into three categoies: (1) botrocetin (Read *et al*., 1989) and bitiscetin (Hamako & Marsui, 1996) which bind to the A1

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domain of vWF, changing its conformation so that it is able to interact with GPIbα; (2) echicetin (Peng *et al*., 1993) and many similar proteins (Fujimura *et al*., 1995; Kawasaki *et al*., 1995; Taniuchi and Kawasaki, 1995) that bind to GPIbα and block its interaction with vWF and (3) alboaggregin A and B (Peng *et al*., 1991) which induce platelet activation by binding to and clustering GPIb. In this study, we characterize a member of C-type lectin venom protein isolated from Formosan *Agkistrodon acutus*, called agkistin (Huang & Yeh, 1994) in modulating the interactions of vWF and thrombin toward platelet GPIb by acting as a GPIb antagonist, and show its antithrombotic activity *in vivo* in a murine experimental model.

Methods

Materials

Lyophilized powder of Formosan *Agkistrodon acutus* venom was provided by Dr M.Y. Liau (National Institute of Preventive Medicine, Department of Health, Executive Yuan, Taipei, Taiwan) and its identity was verified by high-performance liquid chromatography (HPLC) and biological activity. Trigramin was purified from the snake venom of *T. gramineus* as previously described (Huang *et al*., 1987). Two monoclonal antibodies (mAbs), 7E3 and 6D1, raised against GPIIb/IIIa and GPIb, respectively, were kindly provided by Dr B. Coller (The Mount Sinai Medical Center, NY, U.S.A.), and mAb AP1 was kindly donated from Dr R. Montgomery (Southern Wisconsin Milwaukee Blood Center, Milwaukee, WI, U.S.A.). Collagen (type I, bovine tendon), ristocetin, apyrase, α-thrombin, acrylamide, bovine serum albumin (BSA), adenosine diphosphate (ADP), ethylenediamine-tetra-acetic acid (EDTA), fluorecein sodium, Sephadex G-10, Tris and thrombin receptor activating peptide were purchased from Sigma Chem. Co. U.S.A. $Na^{125}I$ was commercially obtained from Amersham, U.K. Enzymobead was purchased from Bio-Rad (Richmond, CA, U.S.A.). Thromboxane (TX) A_2 analogue U46619 was from Biomol. Res. Lab. Inc, U.S.A.

Characterization of agkistin

Purification of agkistin was performed as previously described (Huang & Yeh, 1994). Briefly, crude venom of *A. acutus* was dissolved in 0.02 M ammonia acetate buffer (pH 5.0). After centrifugation, the supernatant was applied to a Mono-S column equipped with fast protein liquid chromatography (FPLC) and the gradient elution was carried out with 0.02 M ammonia acetate buffer containing 0.75 M NaCl (pH 5.0). The active fraction that possessed antiplatelet activity was collected, dialyzed and further refractionated by using of Superose and Mono-Q columns. The homogeneity of agkistin was verified by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS–PAGE). The highly purified venom protein agkistin was dialyzed, lyophilized and then stored at −20°C.

For determination of N-terminal amino acid sequence of agkistin, 10 μg of the reductive alkylated agkistin was first separated into two subunits, designated as α and β subunits, by 12% SDS–PAGE, then electroblotted to a polyvinylidene fluoride (PVDF) membrane according to the method described previously (Matsudaira, 1987). After staining with Coomassie blue, each of the α- and β-subunits was subjected to determination of N-terminal amino acid sequencing by using an Applied Biosystems model 477A gas-pulsed liquid-phase sequencer equipped with an on-line 120 PTH amino acid analyzer. Amino acid composition was determined using a Beckman Model M121 analyzer after acid hydrolysis with 6 N HCl vapor and derivatization with phenylisothiocyanate according to the previously described method (Herinrikson & Meredith, 1984).

Assays of human platelet aggregation and TXB_2 formation

Human peripheral blood collected from healthy adults, who had not taken any medicine for 2 weeks, was anti-coagulated with acid citrate dextrose (9 : 1, v v^{-1}; citric acid, 65 mM; sodium citrate, 56 mM and glucose, 14 mM; ACD). Washed platelets were prepared mainly according to the previously described method (Mustard *et al*., 1972). Isolated platelets were suspended in Tyrode's solution (in mM): NaH_2PO_4 0.4, NaCl 136.9, KCl 217, $NaHCO_3$ 11.9, CaC_2, 2, $MgCl_2$ 1, BSA 3.5 mg ml^{-1}; pH 7.35 and the platelet count was adjusted to 3.75×10^8 ml^{-1}. For preparing human platelet-rich plasma, whole blood was anticoagulated with 1/10 volume of sodium citrate (3.8%, w v^{-1}) and centrifuged at $150 \times g$, 25°C for 9 min.

Platelet aggregation was measured with an aggregometer (Payton Scientific) at 37°C under stirring (900 r.p.m.). The extent of platelet aggregation was continuously monitored for 6 min by turbidimetry and expressed as increase of light transmission. For assay of GPIb-dependent platelet aggregation, ristocetin (0.6 mg ml^{-1}) was added to induce agglutination of platelet suspension in the presence of small amount of plasma (10 μl) as the source of vWF.

For platelet TXB_2 formation assay, EDTA (2 mM) and indomethacin (50 μM) were added to platelet suspension at 6 min after the addition of agonist. After centrifugation, the TXB_2 level of supernatant was determined by TXB_2 enzyme immunoassay (EIA) kit (Amersham, U.K.).

Radiolabelling of agkistin

Agkistin was labelled with $Na^{125}I$ by Enzymobead method as previously described (Huang *et al*., 1987). Briefly, the vial of enzymebead reagent was hydrated with 50 μl distilled water at 4°C for 1 h, followed by adding 50 μl phosphate buffer, 50 μg agkistin, 0.5 mCi $Na^{125}I$ and 25 μl β-D-glucose (1%). The mixture was incubated at room temperature for 25 min. I^{125}-labelled agkistin was separated from free $Na^{125}I$ on a Sephadex G-10 column (bed volume: 10 ml) at 25°C.

Binding studies of ^{125}I-agkistin to human platelets

Ligand-platelet binding studies were performed as previously described (Niewiarowski *et al*., 1981). Briefly, platelet suspension and ^{125}I-agkistin were incubated for 5 min in an appropriate volume of Tyrode's solution. Then 400 μl of mixture was centrifuged through the sucrose solution (20%, w v^{-1}) at $800 \times g$ for 5 min. The total binding of radio-activities of supernatant and the cut-off tips containing platelet pellet were separately counted in an LKB-γ-radiation counter. Non-specific binding was measured in the presence of excess unlabeled agkistin (200 μg ml^{-1}). Specific binding of

^{125}I-agkistin was calculated as the difference between total binding and non-specific binding of ^{125}I-agkistin. The number of agkistin binding site per platelet and its dissciation constant (K_D) were analysed by the method of Scatchard (Scatchard, 1949).

In vivo *and* ex vivo *experimental studies*

Mice were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p) to perform both *in vivo* and *ex vivo* experimental studies. Bleeding time of mice was measured by the method described previously with minor modifications (Dejana & de Gaetano, 1982). Saline or various doses of agkistin was injected intravenously through a tail vein of the mouse (ICR, male, with an average body weight of 20.2±1.4 g). A sharp cut of 3 mm from tail tip of mouse was made 10 min (except as noted) after injection. The tail was then immediately immersed in a saline-filled beaker, kept at 37°C and the bleeding time was measured.

Platelet plug formation in mesenteric microvessels was performed according to a well-known thrombogenic animal model (Sato & Ohshima, 1984; Chang & Huang, 1994). Briefly, an external jugular vein of the anesthetized mice was cannulated for the administration of dye and drugs. After exteriorizing the small intestine, a mesenteric membrane with a microvascular bed was placed on a plastic plate for microscopic observation. Venules with a diameter of 30–40 nm were selected to be irradiated to produce a microthrombus. Ten minutes after injection of fluorescein sodium (150 ng mouse^{-1}), the irradiation by filtered light was started and the aggregating platelets were simultaneously monitored on a TV-monitor. The time to occlusive thrombus formation (cessation of blood flow) was measured. Agkistin or saline was injected in a volume of less than 50 μl by a continuous infusion throughout the experiment.

Recalcification time of the whole blood was measured for determination of the whole blood clotting time. In brief, the whole blood of mice was collected in a glass tube (0.3 ml per tube) with sodium citrate (3.8 %, w v^{-1}) as anticoagulant. After incubation for 1 min at 37°C, saline or agkistin was added and incubated for 1 min, followed by adding 0.05 ml of 1.29% $CaCl_2$ (w v^{-1}), the clotting time was measured immediately.

For platelet counts, blood either from saline or agkistin treated mice was collected and anticoagulated with sodium citrate at the indicated time intervals. The platelet number of the whole blood sample was immediately counted by a hemacytomer (Hemalaster 2, Sebia; Paris, France).

Statistical analysis

All data are presented as mean±s.e.mean for the indicated number of separate experiments. Student's *t*-test was used to assess the statistical differences.

Results

Physicochemical characterization of agkistin

Agkistin was characterised by both SDS–PAGE (12%) stained with Coomassie blue and N-terminal amino acid sequencing. As shown in Figure 1A, agkistin migrated as a single band with an apparent molecular mass of 28 kDa under nonreducing conditions and it was shown to be a heterodimer containing two subunits with molecular weight of 16.5 and 15.5 kDa, respectively. The molecular mass of agkistin was determined to be 32,512 daltons by mean of matrix assisted laser desorption-time of flight (MALD-TOF) mass spectrometry (Bruker, U.S.A., data not shown). The N-terminal sequences of each subunit of agkistin are highly homologous with those of the recently reported GPIb binding proteins (Polgar *et al*., 1997 and Figure 1B). The results of amino acid analysis demonstrated the following composition: Lys (25), His (5), Arg (6), Asp/Asn (21), Thr (17), Ser (46), Glu/Gln (36) Pro (7), Gly (23), Ala (16), Cys (5), Ile (10), Val (11), Met (3), Leu (14), Tyr (8), Phe (14). Moreover, agkistin, even at 40 μg ml^{-1}, exhibited little enzymatic activities such as fibrinogenolysis, phospholipase A_2 and esterase (data not shown).

Inhibition of agkistin on ristocetin-induced platelet aggregation

Ristocetin, a macrolide antibiotic known to cause GPIb-dependent binding of vWF to unstimulated human platelets,

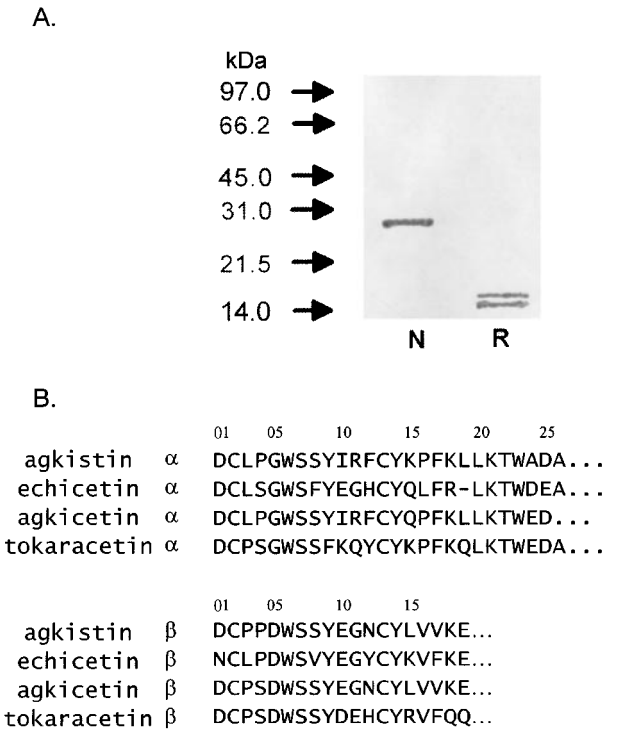

Figure 1 Physicochemical characterization of agkistin. (A) SDS–PAGE patterns of non-reduced (N) and reduced (R) agkistin. Agkistin was analysed on a 12% polyacrylamide gel and stained with Coomassie Brilliant blue. The protein standards used in order of decreasing molecular mass were phosphorylase b (97 kDa), bovine serum albumin (66.2 kDa) ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14 kDa). (B) N-terminal amino acid sequences of the α- and β-subunits of agkistin compared to those of other reported GPIb-binding proteins including echicetin (Peng *et al*., 1993), agkicetin (Chen & Tsai, 1995) and tokaracetin (Kawasaki *et al*., 1995). Single-letter amino acid code was used.

provides a tool for the study of vWF-GPIb interaction *in vitro* (Coller & Gralnick, 1977). Agkistin exhibited a concentration-dependent inhibition on human platelet aggregation triggered by ristocetin in the presence of vWF (Figure 2A). IC_{50} values of agkistin in platelet suspension and platelet-rich plasma were estimated to be 3.1 ± 0.1 and 4.9 ± 0.2 μg ml^{-1} (i.e. 96.6 ± 3.1 and 149.8 ± 6.1 nM, $n=4$), respectively. The inhibitory effects were independent of the incubation time of agkistin with platelets and not affected by EDTA treatment (5 mM, less than 10% inhibition, data not shown). In addition, agkistin was specific in suppressing ristocetin-induced platelet aggregation because it had little effect on platelet aggregation caused by collagen (10 μg ml^{-1}), U46619 (1 μM) and ADP (20 μM) plus fibrinogen (200 μg ml^{-1}) even at a concentration of 40 μg/ml (Figure 2B).

Inhibition of agkistin on platelet aggregation and TXB_2 formation caused by low concentration of thrombin

Agkistin did not affect platelet aggregation caused by a high concentration of thrombin (>0.05 u ml^{-1}, data not shown). However, agkistin (3–20 μg ml^{-1}) concentration-dependently prolonged the latent period (1.5–3.5 fold) in triggering aggregation by a low concentration of thrombin (0.03 u ml^{-1}) with a slight inhibition on the maximal aggregation (less than 20% inhibition at 20 μg ml^{-1}, Figure 3). In addition, TXB_2 formation was measured in a human platelet suspension challenged with various agonists for 6 min by an EIA kit. Agkistin exhibited a concentration-dependent inhibition of TXB_2 formation caused by either ristocetin in the presence of vWF (Figure 4A) or low concentration of thrombin (0.03 u ml^{-1}, Figure 4B), but not that challenged by high concentration of thrombin (>0.05 u ml^{-1}) or collagen (10 μg ml^{-1}, data not shown).

Characterization of the binding of ^{125}I-agkistin to human platelets

^{125}I-agkistin bound to unstimulated platelets in a concentration-dependent manner, reaching a saturated binding at 15 μg ml^{-1} (i.e. 484 nM, Figure 5A). According to Scatchard analysis, the number of binding sites was estimated to be $61{,}183 \pm 3{,}643$ per platelet and the K_D value was estimated to be 223 ± 10.6 nM (Figure 5A insert). The binding reaction reached a maximum within 1 min and was reversible as evidenced by the displacement of ^{125}I-agkistin by the subsequent addition of unlabelled agkistin (Figure 5B). As shown in Figure 6, the binding reaction of ^{125}I-agkistin to unactivated human platelets was almost completely inhibited by either mAb AP1 or 6D1 (both in 8 μg ml^{-1}) that specifically inhibit vWF binding to GPIb (93.4 and 95.8% inhibition, respectively). However, mAb 7E3 (40 μg ml^{-1}) and trigramin (20 μg ml^{-1}) that specifically bind to platelet GPIIb/IIIa complex, ADP (20 μM) and EDTA (5 mM) apparently exhibited little effect on this binding reaction (less than 10% inhibition).

Evaluation of biological activity of agkistin in vivo *and* ex vivo

As shown in Table 1, when a dose of agkistin (5 μg mouse^{-1}, i.e. 250 μg kg^{-1}) was intravenously administrated to mice, it

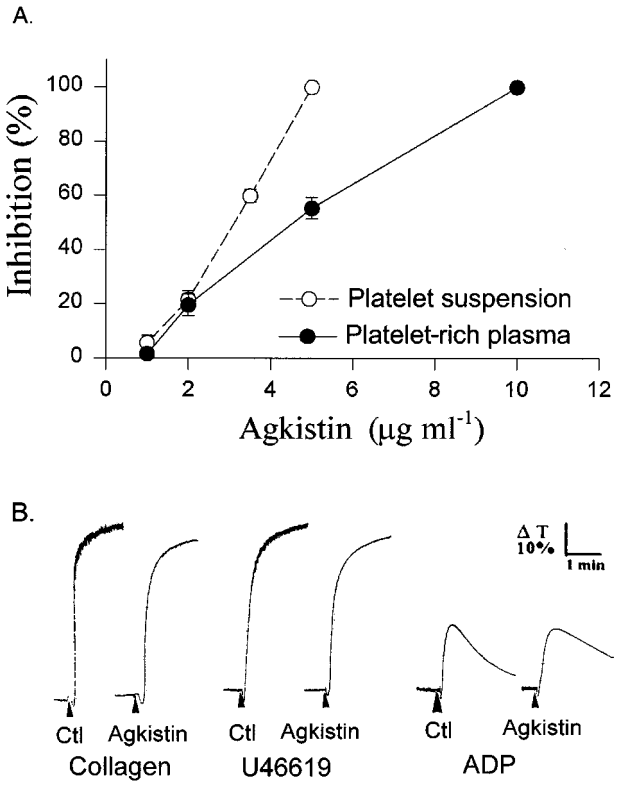

Figure 2 Effect of agkistin on platelet aggregation caused by ristocetin and other inducers in human platelet suspension (PS) and platelet-rich plasma (PRP). (A) Concentration-inhibition curve of agkistin on ristocetin induced platelet aggregation in platelet suspension (○) in the presence of vWF, and in platelet-rich plasma (●). Data are presented as mean±s.e.mean ($n=4-6$). (B) Effect of agkistin (40 μg ml^{-1}) or saline (Ctl) on aggregation of washed platelets caused by collagen (10 μg ml^{-1}), U46619 (1 μM) or ADP (20 μM) plus human fibrinogen (200 μg ml^{-1}). Platelets were incubated with various concentrations of agkistin for 3 min prior to addition of inducer.

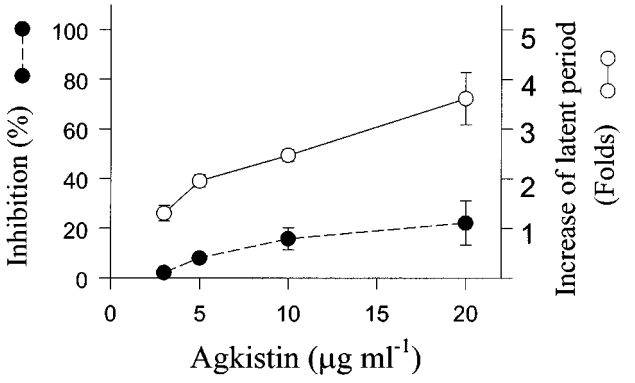

Figure 3 Effects of agkistin on human platelet aggregation and the latent period in triggering platelet aggregation by low concentration of thrombin. Platelets were preincubated with various concentrations of agkistin for 3 min prior to addition of thrombin (0.03 u ml^{-1}). The per cent inhibiton of maximal aggregation response (●) and folds in increase of latent period (○) were determined. Data are presented as means±s.e.mean ($n=4$).

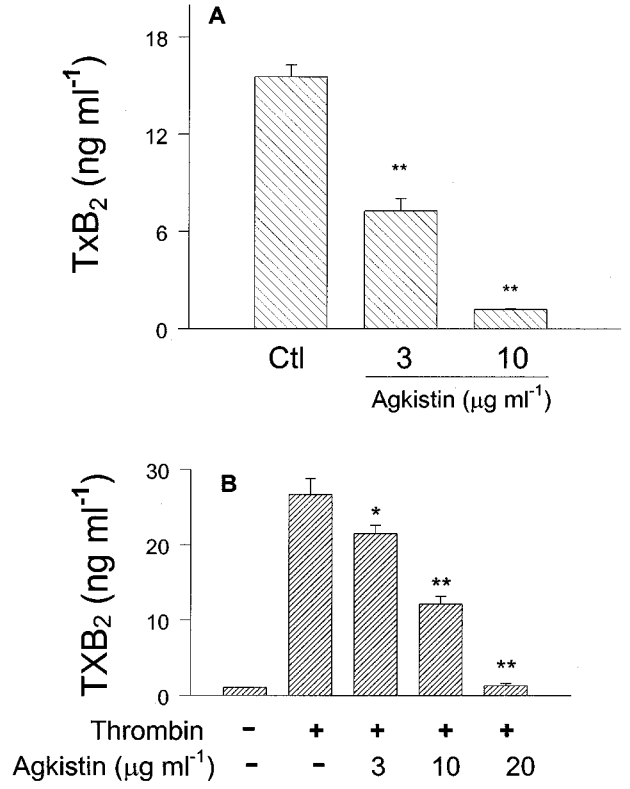

Figure 4 Effect of agkistin on TXB_2 formation triggered by ristocetin in the presence of vWF by low concentration of thrombin. Human washed platelets were incubated with saline (Ctl) or various concentration of agkistin prior to addition of ristocetin (0.6 mg ml^{-1}) in the presence of vWF (A) or low concentration of thrombin (0.03 u ml^{-1}, B). After 6 min, the TXB_2 formation was terminated by EDTA and indomethacin, and then the level of TXB_2 was measured by EIA kit. Values are presented as means ± s.e.mean ($n=5$). *$P<0.05$, **$P<0.01$, as compared with respective control (ristocetin or thrombin alone).

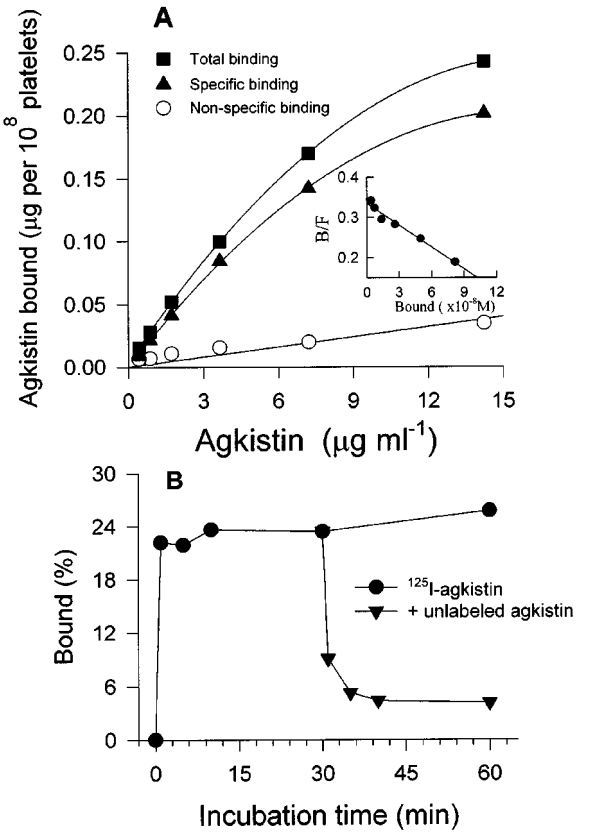

Figure 5 Characterization of ^{125}I -agkistin binding to human platelets. (A) Binding isotherm of various concentrations of ^{125}I-agkistin to washed platelets. Specific binding (▲) is calculated by subtracting the nonspecific binding (○, in the presence of unlabelled 200 μg ml^{-1} agkistin) from total binding (■). Insert: Scatchard plot of the ^{125}I-agkistin binding reaction. (B) Time course of ^{125}I-agkistin (4 μg ml^{-1}) binding to human platelets. Unlabelled agkistin (▼, 200 μg ml^{-1}) was added to suspension 30 min after incubation of platelets with ^{125}I-agkistin. The total binding (●) was determined in the absence of unlabeled agkistin. This is a representative one of three similar experiments.

exerted a pronounced effect in prolonging the elapsed time (379.2 ± 30.9 vs 93.0 ± 22.0 s for control, $P<0.01$, $n=5$) for induction of platelet plug formation in venules upon light irradiation in the fluorescein dye (150 μg $mouse^{-1}$)-pretreated mice *in vivo*, as compared with that of receiving PBS (control). However, it did not affect the clotting time of whole blood *ex vivo* (188.2 ± 37.5 s for control vs 176.3 ± 29.4 s for agkistin, $P=0.65$, $n=4$). In the mean time, the prothrombin time (PTT) and the partial thromboplastin time (PT) of mice after the administration of agkistin were also not affected (data not shown). On the other hand, intravenous administration of agkistin to mice significantly prolonged the bleeding time in a dose-dependent manner (1–5 μg $mouse^{-1}$, Figure 7A). When the dose of agkstin was increased to 250 μg kg^{-1} (5 μg $mouse^{-1}$), a significant prolongation of tail bleeding time (>10 min vs 1.0 ± 0.2 min for control, $P<0.01$, $n=3$) was observed (Table 1). Additionally, the pronounced prolongation of bleeding time (>10 min) of mice after agkistin administration (5 μg $mouse^{-1}$) was detected within 10 min, and this effect lasted for at least 2 h (data not shown). Moreover, a significant reduction in platelet count was observed within 5 min after the administration of agkistin (5 μg $mouse^{-1}$, 30.2 ± 7.8 vs 112.3 ± 5.5 × 10^4 μl^{-1} for control, $P<0.01$, $n=4$) and the

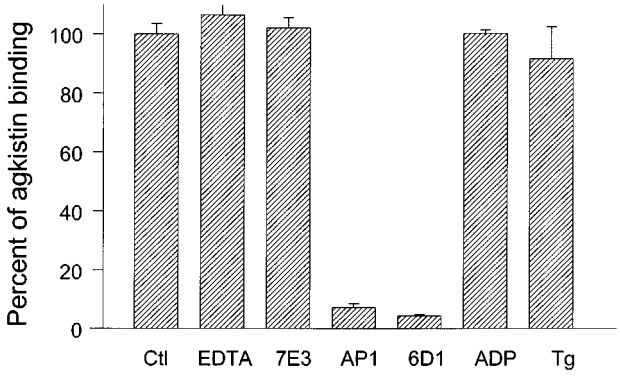

Figure 6 Characterization of the binding of ^{125}I-agkistin to human platelets. Platelet suspension was preincubated with saline (Ctl), EDTA (5 mM), 7E3 (40 μg ml^{-1}), AP1 (8 μg ml^{-1}), 6D1 (8 μg ml^{-1}), ADP (20 μM) and trigramin (Tg, 10 μg ml^{-1}) prior to the addition of ^{125}I-agkistin (4 μg ml^{-1}), and the total binding of ^{125}I-agkistin was determined. Per cent of agkistin binding in the presence of inhibitors was compared to that of saline (23.16% of total binding as 100%) and values are presented as means ± s.e.mean ($n=4$).

Table 1 Antithrombotic activity of agkistin *in vivo* and *ex vivo*

Function	*Control* (in s)	*Agkistin-treated*	P *value*
Bleeding time	58±12 (5)	>600 (5)	<0.001
Fluorescein-induced occlusion time	93±22 (5)	379±31 (5)	<0.001
Clotting time of whole blood	173±29 (4)	188±37 (4)	0.653

The bleeding time *in vivo*, clotting time of whole blood *ex vivo*, and elapsed time for induction of platelet plug formation upon the irradiation of venules *in vivo* (in s) were determined independently after intravenous administration of saline (control) or agkistin (5 μg mouse^{-1}, i.e. 250 μg kg^{-1}) as described in Methods. Values are presented as means±s.e.mean (n). The paired Student's t-test was used for determination of P value.

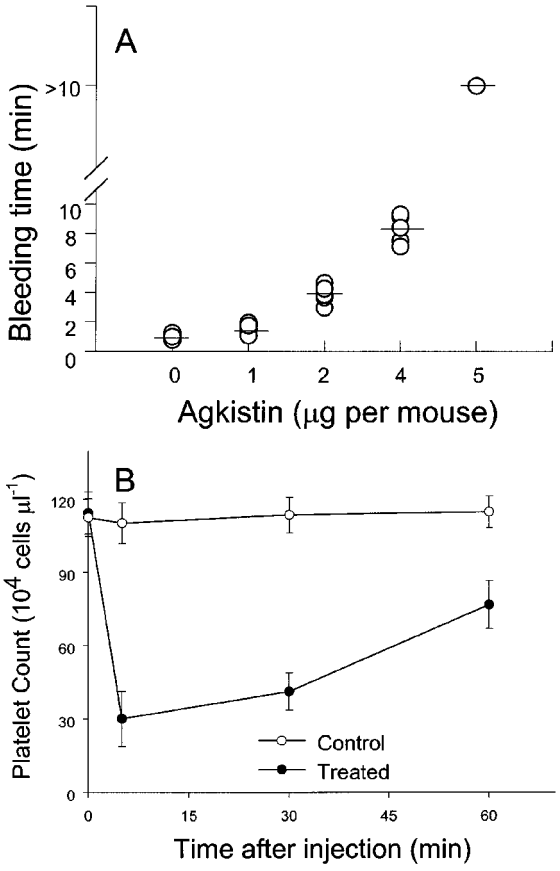

Figure 7 Effect of agkistin on tail bleeding time and platelet counts in mice. (A) Tail bleeding time was measured 10 min after intravenous administration of saline or various doses of agkistin. Bleeding time longer than 10 min was expressed as >10 min. Each bar represents the mean value (n=5). (B) Platelet count was measured at indicated time after administration of saline (○, control) or agkistin (●, 5 μg mouse^{-1}, i.e. 250 μg kg^{-1}). Data are presented as mean±s.e.mean (n=3).

thrombocytopenia effect lasted for at least 60 min. However, there is a tendency of gradual restoration of platelet count 30 min after agkistin administration (Figure 7B).

Discussion

Agkistin, a heterodimer protein purified from the venom of Formosan *Agkistrodon acutus*, specifically inhibited human platelet aggregation elicited by either ristocetin or low-concentration thrombin *in vitro* and exhibited antithrombotic activity in an *in vivo* experimental model. There are several lines of evidence indicating that agkistin exhibited its antiplatelet activity *in vitro* by acting as a GPIb antagonist. Firstly, agkistin is a disulphide-linked heterodimer composed of α and β subunits, and its N-terminal amino acid sequencing of α- and β-subunits are highly homologous to those of C-type lectin GPIb-binding proteins (Figure 1). Especially, its N-terminal sequence is highly homologous to agkicetin except for a few different substitutions at position 15 (R/Q) and 24 (A/E) of α chain, and at position 4 (P/S) of β chain. Secondly, agkistin specifically inhibits ristocetin induced GPIb-dependent platelet aggregation and TXB_2 formation in the presence of vWF with similar IC_{50}s (Figures 2A and 4A). Ristocetin-induced platelet aggregation is mediated through the initial binding of vWF to platelet GPIb, subsequently resulting in the exposure of fibrinogen receptor (Coller & Gralnick, 1977). In contrast to RGD-containing peptides, however, agkistin apparently did not affect platelet aggregation induced by collagen, U46619 and ADP in the presence of fibrinogen (Figure 2B). Thirdly, GPIb is a high affinity-binding site for α-thrombin and it can facilitate the activity of thrombin at a low concentration in activating platelets (Gralnick *et al*., 1994). It is inferred that binding of GPIb may lead to the activation of endogenous phospholipase A_2, and the subsequent formation of TXA_2. Agkistin concentration-dependently prolonged the latent period in triggering aggregation (Figure 3) and inhibited TXB_2 formation (Figure 4) caused by low concentration of thrombin (0.03 u ml^{-1}), but not by high concentration (>0.05 u ml^{-1}). Similarly, agkistin (40 μg ml^{-1}) did not affect platelet aggregation caused by thrombin receptor activating peptide (50 μM, data not shown), a tethered ligand towards thrombin seven-transmembrane receptors (Vu *et al*., 1991). Finally, radio-ligand binding studies clearly indicate that agkistin interacts with platelet membrane GPIb receptor. As shown in Figure 5, the binding of ^{125}I-agkistin to unactivated platelets is specific, reversible, and markedly inhibited by mAbs AP1 and 6D1 raised against the 45 kDa N-terminal vWF-binding domain of human GPIb (Coller *et al*., 1983). However, pretreatment of mAb 7E3 raised against human platelet GPIIb/IIIa (Coller, 1985), snake venom protein trigramin (a GPIIb/IIIa antagonist, Huang *et al*., 1987), ADP or EDTA apparently does not affect this binding reaction (Figure 6). All of above-mentioned results suggest that agkistin binds to human platelet GPIb receptor in a divalent-cation independent manner, and the binding domain

of agkistin on platelet is near to vWF-and thrombin-binding site (i.e. N-terminal domain of GPIbα). Therefore, we conclude that agkistin belongs to the GPIb antagonist family that binds to its specific receptor-GPIb, resulting in blockade of vWF-GPIb interaction.

The platelet GPIb-V-IX consists of four chains, GPIbα (145 kDa) and GPIbβ (25 kDa) are linked by a disulphide bond while GPIX (22 kDa) is strongly non-covalenly linked in a 1 : 1 ratio and GPV (82 kDa) weakly associated with the complex in a 1 : 2 ratio (Lopez *et al*., 1998). The binding sites of both vWF and thrombin to GPIb reside between N-terminal α chain leucine-rich glycoprotein and O-linked carbohydrate region (Vincente *et al*., 1990). Regarding the binding sites of agkistin, the estimation of 61,183 per platelet is higher than that probed by GPIb mAb or other venom GPIb-binding proteins ranging from 21,500 – 47,000 (Fujimura *et al*., 1996), but is similar to that probed by crotalin, another GPIb antagonist recently discovered in our laboratory (Chang *et al*., 1998). Therefore, the varied number of binding sites among mAbs and GPIb-binding protein may be explained as following, the small molecular mass of venom GPIb-binding proteins may access the surface canalicular system of platelets as compared to the bulky, bivalent structure of the mAb.

Many efforts have recently been devoted to characterize the interaction and platelet GPIb at a molecular level with an aim of developing inhibitors that could be useful in the prevention of thrombosis. A recombinant vWF fragment AR535C was found to enhance thrombolysis with t-PA in rabbit thrombosis model (Gurevitz *et al*., 1998). A bolus of Fab fragment raised against GPIb was reported to effectively prevent the deposition of platelets onto the collagen surface in a balloon model of arterial thrombosis (Cauwenberghs *et al*., 2000). In the present study, we evaluated the *in vivo* antithrombotic activity of agkistin in a mouse model including the determination of tail bleeding time and occlusion time of inducing platelet plug formation of mice (Table 1). However, neither whole blood clotting time, PT nor APTT were affected by agkistin *ex vivo* when it was administered intravenously at 5 μg mouse^{-1} (i.e. 250 μg kg^{-1}), suggesting that agkistin apparently has no anticoagulant properties and does not affect the coagulation cascade. From the *in vivo* and *ex vivo* experiments, we suggest that agkistin exerts its antithrombotic activity both *in vivo* and *in vitro* by acting as a GPIb antagonist, blocking vWF-mediated primary platelet adhesion to subendothelium. In contrast to crotalin, intravenous administration of agkistin reduced platelet counts (Figure 7). However, this result is consistent with the previous reports that echicetin and *jajaraca* GPIb-BP caused transient thrombocytopenia in mice (Peng *et al*., 1993; Fujimura *et a*., 1995). At present, the tendency of these GPIb antagonists in causing thrombocytopenia is a precaution to be taken, however, the exact mechanism remains to be investigated. The study on the structure-activity relationship of these GPIb antagonists may help us to understand the detail molecular mechanisms of the vWF-GPIb interaction, hoping to further develop a new class of these potential anti-thrombotic agents. Furthermore, we recently found that agkistin exerted a potent anti-angiogenic effect as evidenced by examining chick CAM model through blockade of vWF-endothelial GPIb interaction (Yeh *et al*., 2000). Taken together, these GPIb antagonists (i.e. agkistin and crotalin) are valuable tools for developing a new strategy of anti-thrombotic and anti-angiogenic drugs for clinical use.

We appreciate very much the generous supply of monoclonal antibodies from Dr R. Montgomery (AP1) and Dr B. Coller (6D1 and 7E3). We are grateful for the financial supports from National Science Council of Taiwan (NSC84-2331-B002-112BC) and National Health Research Institute (NHRI-GT-EX89B920L).

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(Received August 11, 2000
Revised October 23, 2000
Accepted November 27, 2000)