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Purification and Characterization of Bitiscetin, a Novel von Willebrand Factor Modulator Protein from *Bitis arietans* Snake Venom

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We have screened 20 snake venoms and purified a novel snake venom protein, named bitiscetin, from *Bitis arietans* venom that specifically binds to human von Willebrand factor (vWF) and induces platelet agglutination. Bitiscetin showed a heterodimeric structure composed of disulfide-linked α (16kDa) and β (13kDa) subunits on SDS-PAGE and showed a basic nature with pI value of 9.1, in contrast to botrocetin (pI 4.6), a vWF modulator isolated from another snake (*Bothrops jararaca*) venom. Bitiscetin-induced platelet agglutination was dependent on vWF and platelet membrane glycoprotein (GP) Ib, but not on Ca^{2+} and GPIIb/IIIa. vWF bound to bitiscetin but not to botrocetin electroblotted to a PVDF membrane after SDS-PAGE and this binding was diminished after reduction of disulfide bonds of bitiscetin. Bitiscetin did not cross-react to anti-botrocetin monoclonal antibodies. These results suggest that bitiscetin directly interacts with vWF and requires the protein conformation for its interaction as well as botrocetin, but its interaction manner with vWF appears to be different from that of botrocetin.

Read *et al.* [1] have reported that several snake venoms have *in vitro* platelet-agglutinating (coagglutinin) activity in the presence of vWF, but there is no information about vWF-binding or vWF-modulating (venom) proteins except for botrocetin purified from *Bothrops jararaca* venom [2-4]. Botrocetin binds to vWF and then the complex induces platelet agglutination *in vitro*, suggesting that botrocetin modulates vWF so that it interacts with platelets [5]. Botrocetin is negatively charged and interacts with positively charged groups of vWF [4, 6], whereas antibiotic ristocetin, another vWF modulator, positively charged, interacts with negatively charged groups of vWF [7, 8]. Berndt *et al.* [7] suggested that neutralizing electrostatic polarity around the GPIb-binding domain of vWF by binding to either ristocetin or botrocetin may induce the conformational change to access GPIb *in vitro.* We have surveyed other botrocetin-like coagglutinins in various viperid, crotalid and elapid snake venoms in order to get more information about the vWF-modulation mechanism by snake venom proteins. In this paper, we describe purification and characterization of a novel basic vWF-modulator termed bitiscetin from *Bitis arietans* venom, which is similar to but clearly distinct from botrocetin.

MATERIALS AND METHODS

Materials. Lyophilized snake venoms from 12 crotalids (*Agkistrodon contortrix, A. halys blomhoffii, A. piscivorus piscivorus, Bothrops alternatus, B. asper, B. atrox, B. jararaca, Crotalus adamanteus, C. atrox, C. horridus horridus, C. ruber, Trimeresurus okinavensis*), 4 viperids (*Bitis arietans, B. gabonica, Echis carinatus, Vipera russelli*), 3 elapids (*Dendroaspis angusticeps, Naja nigricollis, N. naja kaouthia*), and one hydropheid (*Laticauda semifasciata*)

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were purchased from Sigma and Wako Pure Chemicals. Purification of botrocetin (originally termed two-chain botrocetin) and GPIb-BP from *B. jararaca* venom was performed as described previously [2, 9]. Human vWF was purified from factor VIII concentrates, a gift from Hoechst Japan, as described previously [10]. Phenyl Superose HR 5/5, Superose 12 HR 10/30, and Mono S HR 5/5 columns for FPLC were purchased from Pharmacia-LKB. Anti-human platelet GPIb monoclonal antibodies (mAb) (HPL-7 and GUR 20-5) [11, 12] were purchased from Seikagaku Kogyo and Takara Shuzo, respectively. Anti-GPIIb/IIIa mAb (LJ-CP8) [13] and anti-botrocetin mAb (BCI-7) [14] were generous gifts from Dr. Z. M. Ruggeri (Scripps Research Institute, La Jolla, CA). Anti-human vWF mAb (NMC-4) was prepared as described previously [15]. Anti-botrocetin mAbs [16] were generous gifts from Takara Shuzo. Other antibodies were purchased from Dakopatts, MBL and TAGO Immunologicals.

Platelet agglutination assay. Each venom was dissolved in TBS (150 mM NaCl containing 10 mM Tris-HCl, pH 7.5) at 5 mg/ml, and the supernatant obtained after centrifugation (13,000 $\times$ g, at 4°C for 15 min) was used for platelet agglutination assay. 240 μl of platelet rich plasma (PRP) or normal pooled human serum containing formalin-fixed platelets (3.0×10^8/ml) was incubated in a glass cell at 37°C. After 5 min preincubation with gentle stirring, 10 μl of venom supernatant or other test solution was added, and platelet agglutination was monitored with an aggregometer (Chrono-Log). In some experiments, normal human washed platelets were incubated in 150 mM NaCl, 10 mM PIPES buffer, pH 7.4 containing 10 μg/ml of vWF, in the presence or absence of mAb at 37°C for 5 min, and platelet agglutination was monitored after addition of bitiscetin.

Purification of bitiscetin. 10 mg of *B. arietans* venom was dissolved in 2 ml of 0.85 M $(NH_4)_2SO_4$ containing 50 mM Na-phosphate buffer, pH 7.0 and centrifuged. The supernatant was passed through a cartridge filter and subjected to hydrophobic interaction chromatography on a Phenyl-Superose FPLC column. The column was eluted by a decreasing gradient of $(NH_4)_2SO_4$ from 1.7 to 0 M in 50 mM Na-phosphate buffer, pH 7.0. 500 μl fractions were collected and monitored for platelet agglutination activity in the presence of serum. Fractions showing strongest activity were subjected to gel filtration on a Superose 12 FPLC column equilibrated with 50 mM NH_4HCO_3. Active fractions were subjected to ion-exchange chromatography on a Mono S FPLC column equilibrated with 20 mM Na-acetate buffer, pH 5.0. The column was eluted by increasing concentration of NaCl in 20 mM Na-acetate buffer, pH 5.0. Active fractions were combined, concentrated, and dialyzed against TBS.

SDS-PAGE and Western blotting. SDS-PAGE was performed as described [17] and proteins were visualized by silver staining kit (Wako). IEF was performed in a capillary glass tube using ampholine (Pharmacia-LKB) [18]. The intact or S-pyridylethylated protein was electrophoretically transferred to a PVDF membrane immediately after SDS-PAGE [19] and subjected to western blotting analysis as previously described [20] or amino acid sequence analysis [19] with an ABI protein sequencer Model 494. Protein concentration was determined with BCA protein assay reagent (Pierce) using BSA as a standard.

RESULTS

Screening and purification of bitiscetin from snake venoms. When we screened buffer extracts of 20 snake venoms, only the two (from the crotalid *Bothrops jararaca* and the viperid *Bitis arietans*) showed strong vWF-dependent platelet agglutinating activity. Since *B. jararaca* is the source of botrocetin, we tried to purify the *B. arietans* coagglutinin (named bitiscetin) as described in ''materials and methods''. The coagglutinin activity of *B. arietans* venom was retained on a Phenyl-Superose column and eluted as several peaks at low ionic strength (Fig. 1A). Fractions showing strong activity were pooled and subjected to gel filtration on a Superose 12 column (Fig. 1B) followed by ion-exchange chromatography on a Mono S column (Fig. 1C).

Fig. 2 shows SDS-PAGE of the crude extract of *B. arietans* venom and the active fraction obtained by Mono S column chromatography. The purified bitiscetin showed apparent molecular mass values of ~25 kDa by SDS-PAGE under non-reducing conditions, and ~29 kDa by gel filtration (Fig. 1B). Under reducing conditions, however, it showed two bands of 16 kDa (α subunit) and 13 kDa (β subunit) on SDS-PAGE (Fig. 2A), indicating that it is a heterodimer composed of α and β subunits linked by disulfide bond(s). IEF showed that bitiscetin has a pI value of about 9.1. ~100 μg of bitiscetin was purified from 10 mg of the crude venom. N-terminal 20-residue amino acid sequences of S-pyridylethylated α and β subunits electroblotted on the PVDF membrane was identified to be DPGCLPDWSSYKGHCYKVFK and DEGCLPDCSSYKGHCYKVFK, respectively, and showed 50 and 65% identity with those of botrocetin.

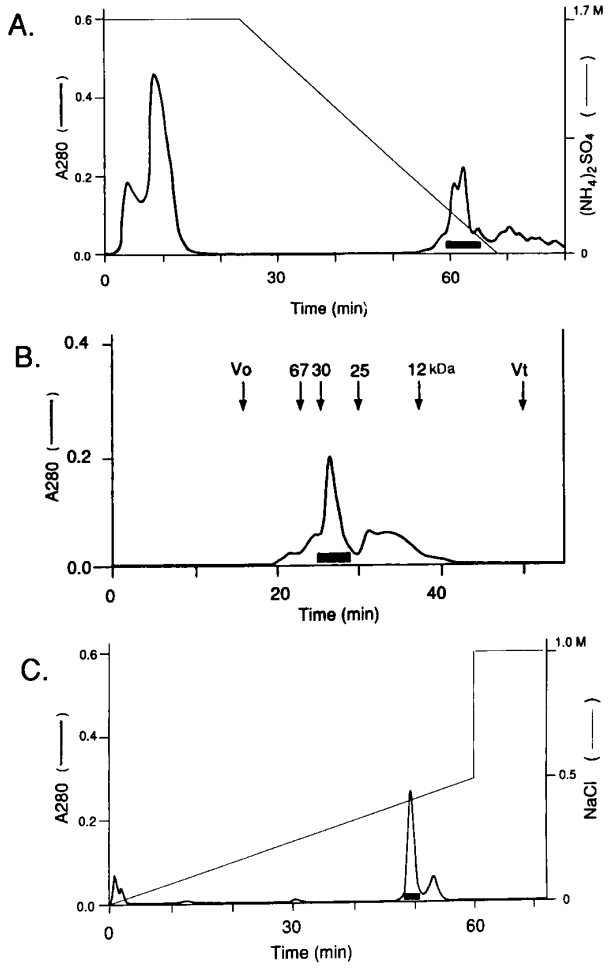

FIG. 1. Purification of bitiscetin from *B. arietans* venom. (**A**) The crude venom of *B. arietans* was subjected to hydrophobic interaction chromatography on a Phenyl-Superose FPLC column. The column was eluted with a decreasing gradient of ammonium sulfate. The bar indicates the fractions having platelet agglutination activity. (**B**) The active fractions were combined and subjected to gel filtration on a Superose 12 FPLC column. The platelet agglutinating activity was eluted around 29 kDa (bar). Arrows with molecular mass indicate elution position of standard marker proteins. Vo and Vt indicate the position of void and total volume fractions, respectively. (**C**) Bitiscetin was finally purified on a Mono S FPLC column. The platelet agglutinating activity was retained and eluted at the position consistent with a protein peak (bar). The active fractions were pooled and concentrated.

Platelet agglutination induced by bitiscetin. Bitiscetin did not coagulate normal human plasma nor aggregate human washed platelets by itself but it agglutinated washed or formalin-fixed platelets in the presence of vWF (Fig. 3B) as well as PRP (Fig. 3A) like ristocetin or botrocetin. A minimum concentration of bitiscetin inducing platelet agglutination in the presence of human vWF was $\sim$1 μg/ml (Fig. 3B). The GPIb-BP from *B. jararaca*, which inhibits platelet agglutination induced by ristocetin or botrocetin in the presence of human vWF [9], also inhibited that induced by bitiscetin (Fig. 3C). Neither anti-GPIIb/IIIa mAb (LJ-CP8), which inhibits fibrinogen binding to platelets, nor EDTA had a significant effect on the agglutination. Commercially available anti-GPIb mAbs (HPL-7, GUR20-5), which block ristocetin-induced

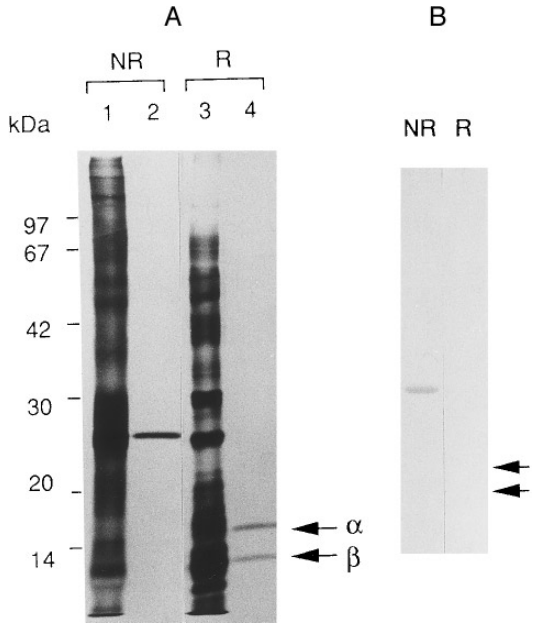

FIG. 2. SDS-PAGE and binding of vWF to bitiscetin electroblotted to a PVDF membrane. (**A**) Purity of bitiscetin was examined by SDS-PAGE followed by silver staining. Approximately 8 μg of crude venom (lanes 1, 3) and 0.8 μg of purified bitiscetin (lanes 2, 4) were subjected to SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Bitiscetin showed a 25 kDa band composed of disulfide-linked α (16 kDa) and β (13 kDa) subunits (arrows). Numbers at left indicate molecular masses (kDa) of marker proteins. (**B**) Purified bitiscetin (1 μg) was electroblotted to a PVDF membrane after SDS-PAGE under non-reducing (NR) and reducing (R) conditions and incubated with human plasma (diluted 1:10), followed by immunoblotting with anti-vWF antibody. Arrows indicate the positions of bitiscetin subunits.

platelet agglutination, showed only a little inhibitory effect on botrocetin- and bitiscetin-induced agglutination (data not shown). Bitiscetin-induced agglutination was completely blocked by anti-vWF mAb NMC-4 (Fig. 3C), which also blocks ristocetin-, botrocetin- or high shear-induced vWF binding to platelet GPIb.

Binding of vWF to bitiscetin. When bitiscetin electroblotted to a PVDF membrane after SDS-PAGE was incubated with human plasma, direct binding with vWF was observed (Fig. 2B). Several other plasma proteins examined showed no significant binding to bitiscetin (Fig. 4) suggesting this binding is highly specific to vWF. No other snake venom examined (including *B. jararaca*) contained bitiscetin-like vWF binding activity after SDS-PAGE except for *Naja naja kaquthia* venom, but this venom had no coagglutinin activity (data not shown). vWF binding activity was diminished when bitiscetin was separated into α and β subunits by SDS-PAGE under reducing conditions (Fig. 2B). Anti-botrocetin mAbs (including BCI-7, which inhibits botrocetin binding to vWF) did not inhibit vWF binding to bitiscetin. No significant binding was observed when BCI-7 or other anti-botrocetin mAbs were incubated with bitiscetin electroblotted on PVDF membrane (data not shown).

DISCUSSION

We isolated a novel coagglutinin (bitiscetin), with vWF-binding and modulating properties, from the venom of the viperid *Bitis arietans.* Read *et al.* [1] described the presence of coagglutinin in *B. arietans* venom as well as four crotalid venoms (including botrocetin from *B.*

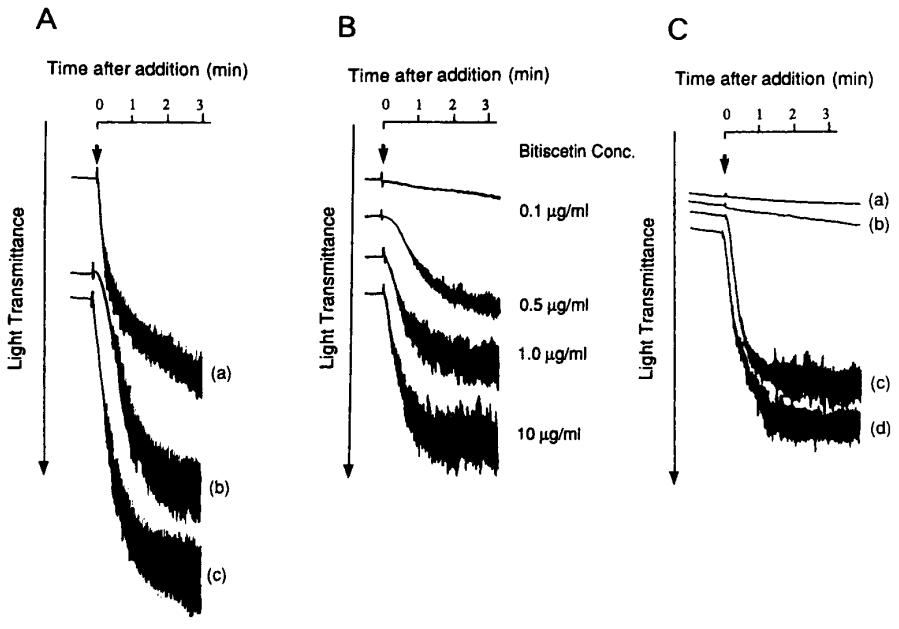

FIG. 3. Platelet agglutination induced by bitiscetin and vWF. (**A**) PRP (3×10^8 platelets/ml) was incubated with 9.2 μg/ml of botrocetin (a), 1 mg/ml of ristocetin (b) and 4.5 μg/ml of bitiscetin at time 0 (arrow). (**B**) Bitiscetin at various concentrations was added to formalin-fixed platelets (3×10^8/ml in TBS) in the presence of vWF (5 μg/ml) at time 0 (arrow). (**C**) Bitiscetin (5 μg/ml) was added to washed normal platelets (3×10^8/ml) containing vWF (10 μg/ml) in the presence of 10 μg/ml of *B. jararaca* GPIb-BP (a), anti-vWF mAb (NMC-4) (b), anti-GPIIb/IIIa mAb (LJ-CP8) (c) or none (control) (d) at time 0 (arrow).

jararaca). The effective concentration of bitiscetin for platelet agglutination is ~1 μg/ml (~40 nM), comparable to that of botrocetin [2, 3]. Like botrocetin, bitiscetin induced platelet agglutination dependent on GPIb and vWF but not on Ca^{2+} and GPIIb/IIIa.

Bitiscetin, like botrocetin, is a heterodimer of disulfide-linked α and β subunits, but the pI values of the two proteins are quite different. Botrocetin has an acidic nature with pI 4.6 and binds to anion exchange column such as DEAE-Sepharose at pH 7.4 [2, 4], but, bitiscetin did not bind to the column under the same conditions due to its highly basic nature (pI9.1). Therefore, a cation exchange column like Mono S was useful for purification of bitiscetin. Positively charged ristocetin promotes platelet agglutination by neutralizing the negatively charged groups of vWF and GPIb [8]. In contrast, botrocetin binds to the inside of the A1-loop region, especially three uncontinuous basic regions of vWF [6]. The fact that bitiscetin is basic suggests that it may interact with the negatively charged flanking region of the A1-loop like ristocetin to change the electrostatic conditions [7]. We showed that vWF binds specifically to bitiscetin electroblotted to a PVDF membrane after SDS-PAGE, but not to botrocetin and either subunit of bitiscetin. These results suggest that bitsicetin interacts with vWF in a different manner from botrocetin and the conformation stabilized by intra- and inter-subunit disulfide bonds is important for the binding with vWF. The relatively lower homology of N-terminal amino acid sequence between bitiscetin and botrocetin, together with no immuno-cross-reactivity between them, suggests that bitiscetin may be not a simple homologue of botrocetin in a different species. Further characterization of bitiscetin including the determination of binding sites with vWF are necessary to understand the modulation mechanism of bitiscetin.

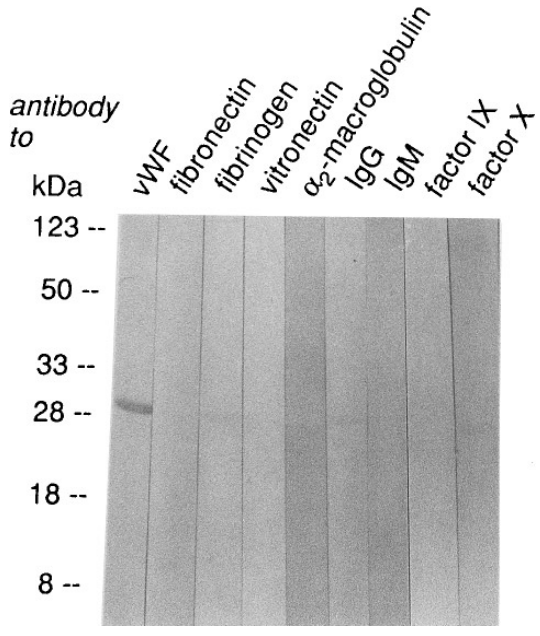

FIG. 4. Binding of plasma proteins to electroblotted bitiscetin. Bitiscetin (2.8 μg) was electroblotted to a PVDF membrane after SDS-PAGE under non-reducing conditions followed by incubation with human plasma (diluted 1:20). The binding of plasma proteins was detected with the specific antibody followed by horseradish peroxidase conjugated 2nd antibody.

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