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Cloning and characterization of novel snake venom proteins that block smooth muscle contraction

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In this study, we isolated a 25-kDa novel snake venom protein, designated ablomin, from the venom of the Japanese Mamushi snake (*Agkistrodon blomhoffi*). The amino-acid sequence of this protein was determined by peptide sequencing and cDNA cloning. The deduced sequence showed high similarity to helothermine from the Mexican beaded lizard (*Heloderma horridum horridum*), which blocks voltage-gated calcium and potassium channels, and ryanodine receptors. Ablomin blocked contraction of rat tail arterial smooth muscle elicited by high K^+-induced depolarization in the 0.1–1 μM range, but did not block caffeine-stimulated contraction. Furthermore, we isolated three other proteins from snake venoms that are homologous to ablomin and cloned the corresponding cDNAs. Two of these homologous proteins, triflin and latisemin, also inhibited high K^+-induced contraction of the artery. These results indicate that several snake venoms contain novel proteins with neurotoxin-like activity.

Keywords: snake venom; neurotoxin; helothermine; cysteine-rich secretory proteins; ablomin.

Over the past 30 years, a plethora of toxins have been isolated from poisonous organisms, such as snakes, scorpions, spiders, and micro-organisms. These natural toxins use a variety of approaches to arrest the homeostatic mechanisms of other living organisms, including disruption of intracellular signal transduction and cytoskeleton organization [1–4], and activation or inhibition of blood coagulation factors [5–10]. Toxins that block synaptic transmission, called neurotoxins, are widely distributed in venoms. These toxins include the conotoxins from cone snails, agatoxins from spiders, and scorpion toxins [11–16]. These toxins exert their potentially lethal effects by specifically and potently blocking a variety of ion channels, including those that conduct Na^+, K^+, and Ca^{2+}. Therefore, neurotoxins have been employed as useful tools to investigate the structure and function of these ion channels [17–20]. A large number of neurotoxin families have also been found in the venom of Elapidae snakes. These toxins, the α-neurotoxins [21] (represented by α-bungarotoxin [22,23], α-cobratoxin [24–27], and erabutoxin [28,29]) potently and specifically prevent nicotinic acetylcholine receptor activation. A second family of snake venom neurotoxins, the dendrotoxins, are homologous to Kunitz-type serine protease inhibitors and act primarily by blocking neuronal K^+ channels [30,31]. In contrast to the neurotoxin-rich venom from Elapidae snakes, the venom from other deadly snakes, including Viperidae and Colubridae snakes, contain surprisingly few neurotoxins, although some neurotoxic phospholipases have been discovered [32–36].

In this report, we describe the isolation of a novel protein, ablomin, from the venom of the Japanese Mamushi snake (*Agkistrodon blomhoffi*, a member of the Viperidae family). When applied to arterial smooth muscle preparations from rat-tails, ablomin blocks K^+-stimulated contraction. This effect is similar to that resulting from application of calciseptine, a well-characterized neurotoxin from black mamba (*Dendroaspis polylepis polylepis*). Calciseptine is a known blocker of L-type Ca^{2+} channels, a property that underlies its ability to block K^+-induced contractions of aortic smooth muscle and spontaneous contractions of uterine smooth muscle [37]. Furthermore, we demonstrate that several snake venoms contain ablomin-like proteins, which may constitute a novel venom protein family.

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Abbreviations: CRISP, cysteine-rich secretory protein; HLTX, helothermine; PsTx, pseudechetoxin; CAP, CRISPs Antigen 5 proteins, and Pathogenesis-related proteins.

Note: the nucleotide sequences reported here have been submitted to GenBank database (tigrin, AY093955; ablomin, AF384218; triflin, AF384219; latisemin, AF384220).

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EXPERIMENTAL PROCEDURES

Materials

The lyophilized venom of *A. blomhoffi* was a kind gift from S. Iwanaga (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) [38]. Other snake venoms and venom glands were purchased from the Japan Snake Institute (Gunma, Japan). Superdex 75 pg and 200 pg, SP–Sepharose High Performance, and Q-Sepharose Fast Flow columns were from Amersham–Pharmacia Biotech. The Vydac Protein & Peptide C18 HPLC column and the COSMOSIL 5C18 AR-300 HPLC column were the products of JASCO (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Endoprotease Lys-C was

purchased from Seikagaku Corporation (Tokyo, Japan). Other chemicals were of analytical grade (Sigma–Aldrich, Amersham–Pharmacia Biotech., Wako Pure Chemical Ind. and Kanto Chemical Co.).

Purification of proteins

Tigrin was isolated from the extract of Duvernoy's glands of *Rhabdophis tigrinus tigrinus*. Ten Duvernoy's glands were broken into small pieces after freezing in liquid nitrogen, and then extracted in 30 mL of 50 mM Tris/HCl pH 8.0 for 4 h at 4 °C. After ultracentrifugation, the supernatant was applied onto Q-Sepharose Fast Flow column (1.6 × 10 cm) in the same buffer, and eluted with a linear gradient from 0 to 0.5 M NaCl. A major peak eluted at 0.05 M NaCl, which was subsequently purified by chromatography on Superdex 200 pg (2.6 × 60 cm column).

Ablomin was purified by three successive chromatographic steps. Five hundred milligrams of lyophilized *A. blomhoffi* venom was dissolved in 3 mL of 20 mM imidazole/HCl pH 6.8 containing 0.2 M NaCl, and insoluble materials were removed by centrifugation and filtration (0.22 μm). The filtrate was loaded onto a Superdex 75 pg column (2.6 × 60 cm) and eluted with the same buffer. The ablomin fractions from two gel filtration runs (a total of 1000 mg of snake venom) were pooled and dialyzed against 50 mM Tris/HCl, pH 8.0, and applied to the Q-Sepahrose Fast Flow column (1.6 × 15 cm). The column was eluted with a linear gradient of NaCl from 0 to 0.4 M at a flow rate of 2 mL·min^{-1}. Chromatographic fractions containing ablomin were then dialyzed against 20 mM imidazole-HCl, pH 6.0, and fractionated on a SP–Sepharose High Performance column (1.6 × 11 cm). This column was developed with a linear gradient of NaCl in the imidazole buffer (0–0.4 M, 2 mL·min^{-1}).

For the purification of triflin, 500 mg of the venom of *Trimeresurus flavoviridis* was applied to the SP–Sepharose Fast Flow column (1.6 × 30 cm) with 10 mM phosphate buffer, pH 6.8, and eluted with a linear gradient from 0 to 0.15 M NaCl, as described previously [39]. Fractions containing triflin were detected by Western blotting using anti-tigrin serum. These fractions were pooled and fractionated on Superdex 75 pg (2.6 × 60 cm) in a 50-mM Tris/HCl, pH 8.0, containing 0.2 M NaCl. Finally, triflin was purified by chromatography on a Blue-Sepharose Fast Flow column (1.6 × 15.5 cm) in 50 mM Tris/HCl, pH 8.0, which was eluted with a linear gradient from 0 to 0.5 M NaCl.

For purification of latisemin, 500 mg of the venom of *Laticauda semifasciata* was loaded onto Superdex 75 pg (2.6 × 60 cm) in a buffer containing 50 mM Tris/HCl, pH 8.0, and 0.2 M NaCl. The latisemin fractions were loaded onto the SP–Sepharose Fast Flow column (1.6 × 11 cm) in 10 mM phosphate buffer, pH 6.8, containing 0.05 M NaCl, and eluted with a linear gradient to 0.2 M NaCl. The latisemin fractions were re-chromatographed on a Mono S column (0.5 × 1 cm) in 10 mM phosphate buffer, pH 6.0, with a linear gradient to 0.2 M NaCl, and on Heparin-Sepharose CL-6B columns (1.6 × 14 cm) with 50 mM Tris/HCl, pH 8.0, using a linear gradient from 0 to 0.3 M NaCl.

All purification steps were performed at 4 °C with an FPLC system (Amersham–Pharmacia Biotech).

Amino-acid sequence analysis

Proteins were reduced for 3 h at room temperature with 20 mM dithiothreitol in the presence of 0.5 M Tris/HCl, pH 8.5, 6 M guanidine hydrochloride, and 2 mM EDTA in a volume of 0.5 mL. Three microliters of 4-vinylpyridine were then added, and alkylation was allowed to proceed for 3 h at room temperature. The S-pyridylethylated proteins were separated from the reagents by C18 reverse-phase HPLC, and the amino-acid sequence was determined by sequencing the peptides obtained by digestion with endoprotease Lys-C. All the samples were analyzed on Applied Biosystems protein sequencers (models 473 A and 477).

cDNA cloning of proteins

The cDNAs encoding tigrin, ablomin, and latisemin were obtained using the RT-PCR method. Typically, venom gland total RNA was isolated from the venom gland with ISOGEN^{TM} (Wako Pure Chemical Industries, Japan) according to the manufacturer's protocol. 5′ and 3′ RACE were carried out to determine the nucleotide sequence of the 5′ and 3′ end cDNAs with the SMART^{TM} RACE cDNA amplification kit (Clontech). The amino-acid sequences of peptides derived from purified proteins were used to design degenerate primers. For the first amplification of tigrin and latisemin cDNA, degenerate primers were used for both sense and antisense primers. For ablomin cDNA, PCR was performed with single degenerate primer (sense or antisense) and an primer recognizing an adaptor sequence that had been attached to the 5′ or 3′ end of cDNAs. In the case of triflin, PCR was carried out using habu cDNA library as a template [40] with a degenerate primer and an adaptor primer. The PCR products were subcloned into the pGEM T-easy vector (tigrin and triflin) or pUC19 vector (ablomin and latisemin) and sequenced with the DSQ 2000 L DNA sequencer (Shimadzu, Japan). Primers used this study are described as follows: tigrin, sense 5′-AA(C,T)GT(A,C,G,T)GA(C,T)TT(C,T)AA(C,T)TC(A,C,G,T)GA(A,G)TC-3′ (corresponding to amino acids 1–8 in tigrin) and antisense 5′-(A,G)TT(A,G)CA(A,G)TT(A,G)TT(A,G)TA(A,G)TC(A,G)TC-3′ (corresponding to amino acids 187–193 in tigrin); ablomin, sense 5′-GGCCATTA(C,T)ACTCAG(A,G)T(A,G)G-3′ (corresponding to amino acids 114–120 in ablomin) and antisense 5′-C(C,T)A(C,T)CTGAGT(A,G)TAATGGCC-3′ (corresponding to amino acids 114–120 in ablomin); triflin, antisense 5′-GC(A,G)TG(A,G,T)AT(A,G,T)AT(A,G)TC(A,C,G,T)GTCCA-3′ (corresponding to amino acids 86–91 in triflin); latisemin, sense 5′-GA(A,G)AA(C,T)CA(A,G)AA(A,G)GA(A,G)AT(A,C,T)G-3′ (corresponding to amino acids 11–17 in latisemin) and antisense 5′-G(A,G)CA(A,G)TT(A,C,G,T)GT(A,G)AA(C,T)TC-3′ (corresponding to amino acids 183–189 in latisemin).

Contraction measurements on rat-tail arterial smooth muscle

Helical strips of endothelium-free rat-tail arterial smooth muscle were prepared as described previously [41]. All the contraction experiments were carried out at room temperature, and all buffers were pre-oxygenated with 100% O_2.

The strips were held at 75 mg resting tension in Hepes/Tyrode (H-T) solution (137 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5.6 mM glucose/10 mM Hepes, pH 7.4) for 45 min Then, the strips were treated with H-T solutions containing 1 μM prazosin, to block the effect of norepinephrine via α1 adrenergic receptors, for 30 min The strips were then exposed to 60 mM KCl H-T solution for 15 min KCl H-T solution was prepared by replacing the NaCl in H-T solution with equimolar KCl. After washing with calcium-free H-T solution for 5 min, the smooth muscle strips were stimulated with 20 mM caffeine H-T solution. For measuring the effect of the proteins, all the H-T solutions contained the indicated concentrations of proteins.

RESULTS

Identification, isolation and cloning of tigrin and ablomin

During the isolation process of a prothrombin activator from the Duvernoy's gland of Yamakagashi snake (*R. tigrinus tigrinus*), we identified a large quantity of a single chain 30-kDa protein (Fig. 1), which we named tigrin. To permit further study, tigrin was purified two chromatographic steps. The extract from Duvernoy's glands was first separated by anion-exchange chromatography (Fig. 1A), and then the major peak was purified by gel filtration (Fig. 1B). An amino-acid sequence was determined by peptide sequencing and partial cloning, revealing that tigrin was structurally homologous to helothermine (HLTX; 49.0% identity, Fig. 1C) from the venom of the Mexican beaded lizard (*Heloderma horridum horridum*).

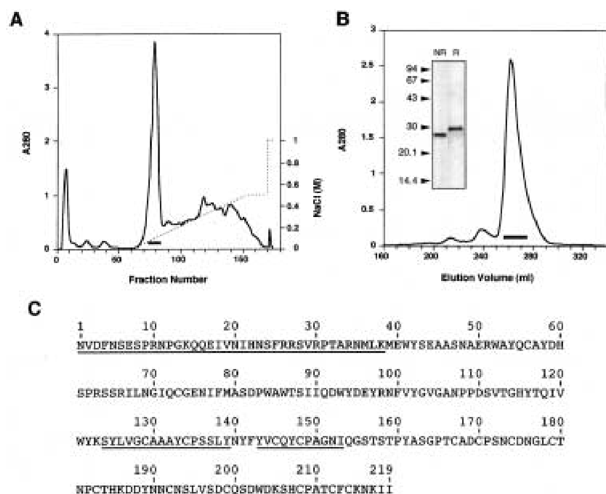

Fig. 1. Isolation of tigrin from the Duvernoy's glands of *R. tigrinus tigrinus*. (A) The extract from Duvernoy's glands of *R. tigrinus tigrinus* was fractionated on a Q-Sepharose Fast Flow column with a linear gradient of NaCl (dotted line). (B) Major peak (bar in A) from Q-Sepharose Fast Flow column was fractionated by gel filtration on a Superdex 200 pg column. The pooled fraction (bar) contained purified tigrin (inset, SDS/PAGE; R, reducing conditions; NR, nonreducing conditions). (C) Primary structure of tigrin. The residues determined by peptide sequencing are underlined.

HLTX is known to alter a variety of ion channel activities, including voltage-gated K^+ channels, voltage-gated Ca^{2+} channels, and ryanodine receptors [42–44]. Because we speculated that HLTX-like proteins would be widespread in snake venoms, we generated an rabbit anti-tigrin serum. We then screened several snake venoms with the anti-tigrin serum using Western blotting or ELISAs. As a result, we detected immunoreactive proteins in the venoms from three snakes: *A. blomhoffi*, *T. flavoviridis*, and *Laticauda semifasciata*. The immunoreactive proteins were then purified by column chromatography, using anti-tigrin serum as a detection reagent.

Using this procedure, we isolated a novel snake venom protein (named ablomin) from the venom of the Mamushi snake (*A. blomhoffi*) through three purification steps. First, the crude venom of *A. blomhoffi* was separated by gel filtration on a column of Superdex 75 pg (Fig. 2A). Fractions containing ablomin were identified using with SDS/PAGE, based upon an M_r that was initially determined by Western blotting (Fig. 2B). These fractions were further separated by anion-exchange chromatography on Q-Sepharose Fast Flow column (Fig. 2C). Ablomin was eluted at the concentration of 0.2–0.3 M NaCl (bold line in Fig. 2C). This fraction was then subjected to cation-exchange chromatography on SP–Sepharose High Performance (Fig. 2D). The purified ablomin migrated with a M_r of 26 kDa on SDS/PAGE under nonreducing conditions and 29.7 kDa under reducing conditions (Fig. 2D, inset). From this purification, we obtained 7 mg of purified ablomin from 1 g of crude venom. The N-terminal and partial amino-acid sequences of this protein were determined by peptide sequencing of enzymatically digested peptides (underlined in Fig. 3). Based on the obtained partial amino-acid sequence, we cloned ablomin cDNA from the venom gland of *A. blomhoffi* by RT-PCR using degenerate primers. The cloned ablomin cDNA was 1336 base pairs in length, encoding a 19-residue putative signal peptide, starting at nucleotide 66, and a 221-residue mature protein (molecular mass 24 932 Da), starting at nucleotide 123 (Fig. 3). As expected, ablomin was quite homologous to HLTX, with 52.8% of the deduced amino acids identical to the corresponding residues in HLTX. (Fig. 4).

The effects of tigrin and ablomin on rat tail arterial contraction

We examined the effects of ablomin and tigrin on high K^+- or caffeine-induced contraction using helical strips of endothelium-free rat-tail arterial smooth muscle. Ablomin remarkably inhibited contraction evoked by treatment with high K^+, but not that evoked by treatment with caffeine (Fig. 5A). In contrast, tigrin did not affect both contraction evoked by either treatment (Fig. 6B). The block of contraction by ablomin was concentration-dependent to 1 μM (Fig. 5B) and completely reversible after a 45-min washout of protein (data not shown). Inhibition by ablomion was reduced at a concentration of 3 μM (Fig. 5B). High K^+-treatment of the artery induces membrane depolarization and activates voltage-gated channels, leading to smooth muscle contraction [45,46]. In contrast, caffeine exposure causes transient contraction by activating ryanodine receptors of the sarcoplasmic

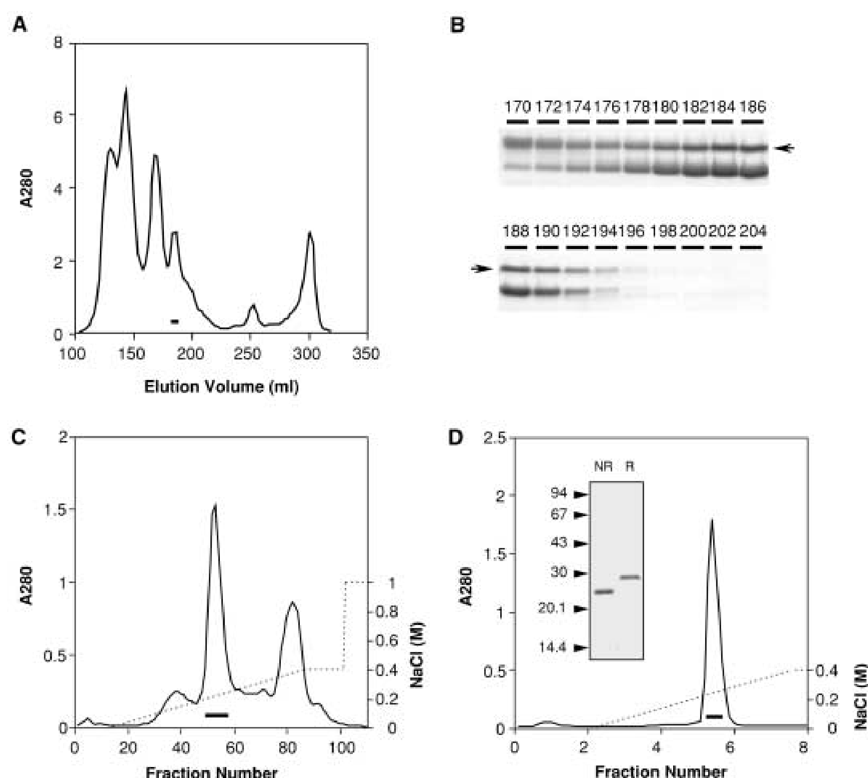

Fig. 2. Isolation of ablomin from the venom of *A. blomhoffi*. (A) The venom of *A. blomhoffi* was fractionated on a column of Superdex 75 pg. (B) SDS/PAGE of continuous fractions eluted from Superdex 75 pg column under reduced condition. The numbers above are elution volume (mL) of the fractions, and the arrows show ablomin. The slightly larger protein, which eluted at 170–174 mL, was determined to be a serine protease-like venom protein by protein sequence analysis. (C) Ablomin fractions (182–192 mL in elution volume) were subjected to a Q-Sepharose Fast Flow column and eluted with a linear gradient of NaCl (dotted line). Two-milliliter fractions were collected. The fractions indicated by bar were pooled as ablomin. (D) The ablomin fraction from c was subjected to a SP-Sepharose High Performance column and developed with a linear gradient of NaCl (dotted line). Two-milliliter fractions were collected. The pooled fraction (bar) contained purified ablomin. The result of SDS/PAGE of the purified ablomin is shown in inset (NR, nonreduced; R, reduced). Seven milligrams of ablomin were obtained from 1 g of crude venom. For detailed purification procedures, see 'Experimental procedures'.

reticulum (SR). The specific effect of ablomin on high K^+-induced contraction therefore suggests that this blockage was caused by the inhibition of voltage-gated channels, rather than interaction with contraction-related proteins such as ryanodine receptors, myosin, or calmodulin that are found in the cytoplasm [47]. In the rat-tail artery, the intracellular Ca^{2+} concentration is well correlated with contraction force, and contraction evoked by application of high extracellular K^+ is completely dependent on the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels [45,46,48]. In this regard, rat-tail arterial smooth muscle cells predominantly express L-type Ca^{2+} channels among several subtypes of Ca^{2+} channels [49]. For these reasons, we hypothesize that ablomin may target voltage-gated Ca^{2+} channels on smooth muscle. Further investigation is required to determine the target protein(s). In previous studies, HLTX

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   1                                                              AC     2
   3  GCGGGGCACGATTTTCTGAAAGCAACAAAGAAGTTATCTTTACGTTTATTCACAACAATA    62
  63  GAAATGATTGTCTTCATTGTCTTGCCAATTCTTGCTGCAGTGCTGCAACAGTCTTCTGGA   122
 -19     M  I  V  F  I  V  L  P  I  L  A  A  V  L  Q  Q  S  S  G     -1
 123  AATGTTGATTTTGATTCTGAGTCACCCAGGAAACCAGAAATACAAAACGAGATTGTTGAC   182
   1  N  V  D  F  D  S  E  S  P  R  K  P  E  I  Q  N  E  I  V  D     20
 183  TTGCACAATTCTTTAAGGAGATCTGTGAATCCAACTGCTAGCAACATGCTAAAAATGGAA   242
  21  L  H  N  S  L  R  R  S  V  N  P  T  A  S  N  M  L  K  M  E     40
 243  TGGTATCCTGAAGCTGCTGCTAATGCAGAACGTTGGGCGTACAGATGTATTGAGGATCAC   302
  41  W  Y  P  E  A  A  A  N  A  E  R  W  A  Y  R  C  I  E  D  H     60
 303  AGTTCACCCGATTCAAGAGTTCTTGAAGGAATAAAATGTGGTGAAAATATATATATGTCA   362
  61  S  S  P  D  S  R  V  L  E  G  I  K  C  G  E  N  I  Y  M  S     80
 363  CCTATTCCTATGAAATGGACTGACATTATTCACATTTGGCATGATGAATACAAAAATTTC   422
  81  P  I  P  M  K  W  T  D  I  I  H  I  W  H  D  E  Y  K  N  F    100
 423  AAGTATGGCATTGGAGCAGACCCACCAAATGCTGTTAGTGGCCATTTCACTCAGATAGTT   482
 101  K  Y  G  I  G  A  D  P  P  N  A  V  S  G  H  F  T  Q  I  V    120
 483  TGGTACAAAAGTTACCGTGCTGGTTGTGCTGCTGCCTATTGTCCTTCATCGGAATACAGC   542
 121  W  Y  K  S  Y  R  A  G  C  A  A  A  Y  C  P  S  S  E  Y  S    140
 543  TACTTCTATGTTTGTCAGTACTGCCCAGCAGGAAACATGAGAGGTAAAACTGCTACTCCA   602
 141  Y  F  Y  V  C  Q  Y  C  P  A  G  N  M  R  G  K  T  A  T  P    160
 603  TATACATCAGGGCCACCTTGTGGGGATTGTCCTTCGGCTTGTGACAATGGACTATGCACA   662
 161  Y  T  S  G  P  P  C  G  D  C  P  S  A  C  D  N  G  L  C  T    180
 663  AATCCTTGCACACAAGAAGATGTGTTCACGAACTGCAATAGCTTGGTCCAACAAAGTAAC   722
 181  N  P  C  T  Q  E  D  V  F  T  N  C  N  S  L  V  Q  Q  S  N    200
 723  TGCCAGCATAATTATATAAAGACAAATTGCCCTGCTTCTTGCTTCTGCCACAATGAAATA   782
 201  C  Q  H  N  Y  I  K  T  N  C  P  A  S  C  F  C  H  N  E  I    220
 783  AAATAGTGGATCTCTCATTCAATATTTGTTATCTCTGCCTGAAAAATCTAACTTCCTATT   842
 221  K  ***

 843  AAAATCATGGTATCTTCTAGTATCAGGAAATTCTTACTTGACGTTTGATTTCATATACTT   902
 903  TGCATGAATGTCCTATGAATGCCTAAGGGAAACATCAGCAGGAGTAGAGGCTAGGGATGA   962
 963  AAACTGTAAGTTCAAAGGGTCACAGGAAAAATCACAAGCCTGTGTAGCATCAAAAAATTT  1022
1023  TGATTAAAAGAAAATACTGAAGGCCTGAAAATAACATCACCAAAATTCTTTGTACTCCAA  1082
1083  CTATACCACAAGGATTTCTTAAATGCTTATATTAAAACTAATCTAGAACTGATCTGATGC  1142
1143  CATTCATGCAAAATATATGCCAGGATTCGGTGTTTTTAATGTTTTAATTGGTTATGGTTC  1202
1203  ATAGTTTGATATGATTTTATATGATTTTTAATTTTTGATTGGGAGCCACCCAGAGTTGAT  1262
1263  TGAAAGATGGGCAGCTTTATAAATGTAATAAATAATTAAATAAGCAAACAAATAAATAAA  1322
1323  TTCTGTAGAAGAGT-poly(A)
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Fig. 3. Nucleotide and deduced amino-acid sequence of ablomin. The amino-acid sequence is shown in single-letter code beneath the nucleotide sequence. Nucleotide and amino acid (bold) number are shown in the column at both sides. Translation is depicted as starting at nucleotide 66. The putative signal peptide is dotted underlined (from −19 to −1 in amino acid number). The underlined shows the deduced amino-acid sequence from enzymatic-digested S-pyridylethylated peptides. N-terminal was determined by the sequencing of intact ablomin. The putative poly adenylation signal is boxed.

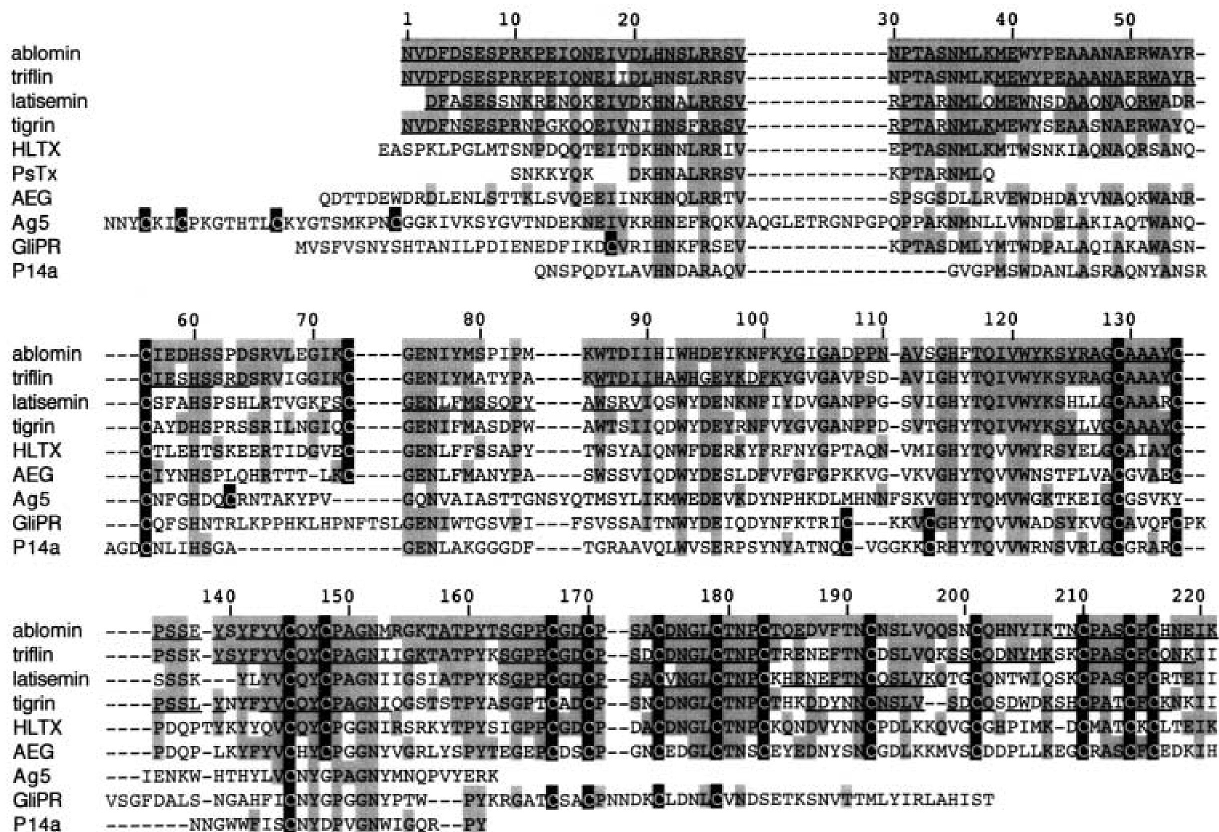

Fig. 4. Sequence alignment of ablomin and structurally related proteins. The residues conserved between the related proteins are shadowed. Gaps (–) have been inserted to maximize similarity. All cysteine residues are shown with black shading; gray shading shows identity. The number of residues corresponds to that of ablomin. The underline in ablomin, triflin, latisemin, and tigrin shows the amino-acid residues determined by peptide sequencing. HLTX; helothermine, PsTx; pseudechetoxin, AEG; rat acidic epididymal glycoprotein (protein D/E), Ag5; hornet antigen 5, GliPR; human glioma pathogenesis-related protein, P14a; tomato pathogenesis-related protein P14a. GenBank accession numbers, ablomin; AF384218, triflin; AF384219, latisemin; AF384220, tigrin; AY093955, HLTX; U13619, AEG; M31173, Ag5; Q05108, GliPR; JC4131, P14a; P04284. Note that the complete sequence of PsTx has not been published [50].

has been shown to block ryanodine receptors on SR, in addition to voltage-gated Ca^{2+} channels (including L-, N-, and P-type) [43,44]. In our current study, we used intact arteries for measuring the activity of the protein, which would presumably preclude access to cytoplasmic proteins. In the previous study, HLTX was applied to purified SR membranes and membrane-permeabilized ventricular trabeculae [43] Therefore, these experimental differences are likely to account for the differences in specificity and mechanism of action.

Isolation and characterization of homologous proteins, triflin and latisemin

In subsequent experiments, we isolated two other homologous proteins and cloned them by PCR. Triflin (221 amino-acid residues, molecular mass 24 798 Da) was purified from the venom of the Habu snake (*T. flavoviridis*), and latisemin (217 amino-acid residues, molecular mass 24 272 Da), was purified from the venom of the Erabu sea snake (*Laticauda semifasciata*) (Figs 4 and 6A). In typical purification procedures (see Experimental procedures), we obtained 4 mg of triflin and 2 mg of latisemin from 500 mg of crude venom, respectively. The predicted amino-acid sequences of triflin and latisemin are homologous to that of ablomin (83.7 and 61.5%, respectively). Like ablomin, these proteins were capable of blocking contraction of the artery induced by high K^+ (Fig. 6B). These findings indicate that proteins homologous to ablomin are found in several snake venoms and represent new snake venom proteins with neurotoxin-like activities.

DISCUSSION

Snake venom neurotoxins, represented by α-neurotoxins and dendrotoxins, are thought to be found mostly in Elapidae snake venoms [21,31], and only a few snake venom neurotoxins have been isolated from Viperidae snakes [32–36]. Recently, Brown *et al.* isolated a 24-kDa cyclic nucleotide-gated ion channel blocker (designated pseudechetoxin; PsTx) from the venom of the Australian King Brown snake (*Pseudechis australis*, Elapidae) [50]. The N-terminal amino-acid sequence of PsTx has some identity to those of the proteins in this study, although the complete amino-acid sequence of PsTx has not yet been reported (Fig. 4). These facts strongly imply that other proteins that are homologous to ablomin may possess distinct biological activities. In this regard, tigrin, which did not affect smooth

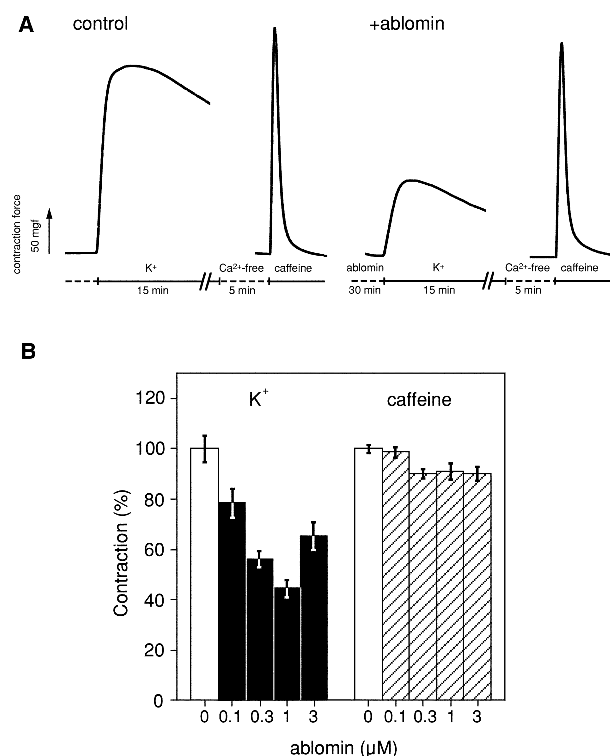

Fig. 5. Ablomin specifically inhibits high K^+-stimulated contraction of rat-tail arterial smooth muscle. (A) Blockage of high K^+-induced contraction by ablomin. Smooth muscle strips were pretreated with 1 μM ablomin, then exposed to stimulants (60 mM K^+ or 20 mM caffeine). To rule out the possible effect of norepinephrine, the H-T solutions contained 1 μM prazosin to block α1 adrenergic receptors. (B) Concentration dependency of ablomin on high K^+- or caffeine-stimulated contraction by ablomin ($n = 4$, mean ± SEM, asterisk, $P < 0.05$).

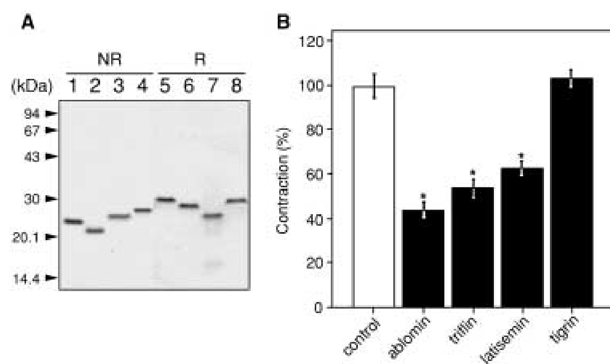

Fig. 6. Several ablomin-like snake venom proteins block high K^+-induced contraction in smooth muscle. (A) SDS/PAGE of snake venom proteins homologous to ablomin under nonreducing (lane 1–4) and reducing (lane 5–8) conditions. The positions of molecular mass markers are shown on the left. Lanes 1 and 5 show ablomin (26 and 29.7 kDa), lanes 2 and 6 show triflin (23 and 29 kDa), lanes 3 and 7 show latisemin (28 kDa each), and lanes 4 and 8 show tigrin (28 and 30 kDa). The numbers on the left are relative molecular mass of standard molecular marker proteins. (B) The effect of ablomin homologous proteins on high K^+-induced smooth muscle contraction. Smooth muscle strips were pretreated with each protein at 1 μM, then stimulated with 60 mM K^+ as described in Fig. 5 ($n = 4$, mean ± SEM, asterisk, $P < 0.05$).

muscle contraction assay (Fig. 6B), possibly possesses other biological activity, such as other neurotoxin-like activity. The differences between structure and activity of these proteins led us propose possible interaction site(s), although we could not confirm any potential active residue(s).

The effective concentration for smooth muscle contraction force herein is in almost the same range (K_i of 0.21 μM for ablomin obtained from double-reciprocal plots) as that of a snake venom L-type Ca^{2+} channel blocker calciseptine (IC_{50} = 0.23 μM on rat aorta depolarization-induced contraction) [37]. However, the blockage was not complete even at the concentration of 1 μM (Fig. 5B). Higher concentrations of ablomin exposure (3 μM) did not induce further inhibition, but rather reduced the extent of inhibition (Fig. 5B). This decrease in the inhibition of contraction at higher concentrations was also found with 3 μM triflin treatment. In contrast, treatment with dihydropyridines, the definitive blockers of L-type Ca^{2+} channels, decreased the arterial smooth muscle contraction to < 20% of the control. Because of the structural similarity with HLTX, it is possible that ablomin and two homologous proteins target multiple ion channels. The possible multiplicity of targets may underlie the partial inhibition seen in our experiments, but further experiments are needed to fully elucidate the factors that cause partial inhibition.

The structural alignment appears to show that these proteins are classified into the CAP family of proteins (CRISPs, Antigen 5 proteins, Pathogenesis-related proteins). The primary structural characteristic of the CRISPs is a high content of cysteine residues [16]. Although the functions of most of the CRISP family proteins are unknown, some are thought to play roles in the immune system and sperm maturation [51,52]. Antigen 5 proteins are components of the venoms of wasps and ants, and they are thought to be involved in the provocation of an acute and localized inflammatory response [53]. The pathogenesis-related proteins of plants, e.g. tomato P14a in Fig. 4, are produced during the defence reaction of plants against pathogenic infection and environmental stress [54]. However, no distinct target molecules have yet been proposed for these family proteins. Although the identity of the CAP proteins vs. ablomin are relatively low, i.e. 42.7% for acidic epididymal glycoprotein, 26.9% for antigen 5, 27.0% for glioma pathogenesis-related protein, and 29.5% for P14a (Fig. 4), ablomin and homologous proteins possess a highly conserved GHF(Y)TQI(V/M)VW sequence among the CAP family proteins at position 114–121. Recently, the NMR structure of tomato pathogenesis-related protein P14a was reported and putative active sites of pathogenesis-related proteins (two histidines and glutamates) have been proposed [55,56]. These residues are also completely conserved in the proteins, which we isolated (His60, Glu75, Glu96, and His115 in ablomin in Fig. 4). These facts strongly suggest these proteins have a three-dimensional structural similarity with other CAP family proteins, indicating their possibility as models for elucidating the virtually unknown functions of CRISP (CAP) family proteins.

In conclusion, we have isolated several novel snake venom proteins. One of these proteins, ablomin, which was purified from the venom of *A. blomhoffi*, blocks high K^+-induced contraction of arterial smooth muscle. We also cloned the cDNA encoding ablomin from the venom gland of *A. blomhoffi* and determined its complete sequence.

Furthermore, we demonstrated that ablomin homologous proteins with similar neurotoxin-like activities, named triflin (*T. flavoviridis*) and latisemin (*L. semifasciata*), are distributed in other Viperidae and Elapidae snake venoms. To date, HLTX is the only neurotoxin that has been classified into the CRISP family of proteins so far. Our present results strongly suggest that HLTX-like proteins are widely distributed in several snake venoms.

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