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Biochemical Properties of a Bushmaster Snake Venom Serine Proteinase (LV-Ka), and Its Kinin Releasing Activity Evaluated in Rat Mesenteric Arterial Rings

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Abstract. A serine proteinase with kallikrein-like activity (LV-Ka) has been purified to homogeneity from bushmaster snake (*Lachesis muta muta*) venom. Physicochemical studies indicated that LV-Ka is a single chain glycoprotein with a molecular mass (Mr) of 33 kDa under reducing conditions which was reduced to 28 kDa after treatment with *N*-Glycosidase F (PNGase F). LV-Ka can be bounded and neutralized by serum α_2-macroglobulin (α_2-M), a prevalent mammalian protease inhibitor that is capable of forming a macromolecular complex with LV-Ka (Mr >180 kDa). Cleavage of α_2-M by the enzyme resulted in the formation of 90-kDa fragments. The proteolytic activity of LV-Ka against dimethylcasein could be inhibited by α_2-M, and the binding ratio of the inhibitor:enzyme complex was found to be 1:1. The Michaelis constant, K_m, and catalytic rate constant, kcat, of LV-Ka on four selective chromogenic substrates were obtained from Lineweaver-Burk plots. LV-Ka exhibits substrate specificities not only for the glandular kallikrein H-D-Val-Leu-Arg-*p*NA (S-2266) but also for the plasmin substrates S-2251 and Tos-Gly-Pro-Lys-*p*NA. Bovine kininogen incubated with LV-Ka generated a polypeptide that dose dependently contracted mesenteric arterial rings from spontaneously hypertensive rats (SHR) in a similar way as bradykinin (BK) does. As it happens with BK, LV-Ka generated polypeptide was inhibited by HOE-140, a bradykinin B_2-receptor antagonist and by indomethacin, a cyclo-oxygenase inhibitor. These results strongly suggest that the polypeptide generated by LV-Ka by cleavage of bovine kininogen is bradykinin. In addition, our studies may help to understand the mechanism of action involved in hypotension produced by envenomation of bushmaster snake.

Keywords: serine proteinase, bradykinin, snake venom, *Lachesis*, mesenteric arterial ring

Introduction

Snake venoms are complex mixtures with reference to their biochemical and pharmacological properties. Venoms of the Viperidae family are a specially rich source of metalloproteinases, serine proteinases, and peptides, which can modify hemostasis and the blood coagulation system of snake bite victims or experimental animals in a complex way. These effects play an important role in giving rise to hemorrhage, hypotension, coagulation, hemolysis, and hemoconcentration. Thus, all of these factors may serve to produce the circulatory shock with internal hemorrhage which is the frequent cause of death in cases of viper and pit viper bites (1, 2). A number of these components, however, cleave some plasma proteins of the victims in a relatively specific manner to give potent effects as either activators or inhibitors of their hemostasis and thrombosis such as blood coagulation, fibrinolysis, and platelet function

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(3 – 5).

The hypotensive nature of snake venoms was first described in the venom of *Bothrops jararaca* by Rocha e Silva et al. (6) and also several kallikrein-like enzymes have been isolated from Viperidae snakes (7 – 9). These kallikrein-like enzymes are the major factors involved in increasing capillary permeability, hypotension, and smooth muscle contraction in envenomed animals. The venom of the large pit viper *Lachesis muta muta* contains an abundance of proteinases and peptides and exhibits very special pharmacological properties. Some of these proteinases are involved in blood coagulation due to a clotting enzyme that acts independently of kinin releasing activity (10); others induce the release of kinin (11), metalloproteinases that are known to produce disruption of the vascular basement membrane resulting in hemorrhage (12) and neurotoxic activity (13).

Recently, a kallikrein-like proteinase named LV-Ka was purified to homogeneity from the venom of the bushmaster snake (14). The enzyme is a single polypeptide chain glycoprotein with Mr of 33 kDa, and belongs to the trypsin family of serine proteinases. The partial amino acid sequence (77%) of the enzyme reveals that it shares structural homology with other serine proteinases isolated from the same source including the plasminogen activator (LV-PA) (15) and the clotting enzyme (16) as well as with other serine proteinases from snake venoms and mammalian kallikrein (8, 17).

In the present study we designed a series of experiments to further characterize its biochemical and pharmacological properties. Thus, we have investigated the effect of α_2-macroglobulin (α_2-M), which is a principal protease inhibitor present in mammalian serum. α_2-M is capable of inhibiting the activity of most proteases from all four classes of the enzymes (aspartic, serine-, metallo-, cysteine proteases). The action of α_2-M on proteinases is exerted by a unique mechanism of molecular trapping followed by rapid clearance of the complex from circulation (18). The proteinase-trapping by α_2-M is initiated by a specific limited proteolysis at the bait region near the middle of the α_2-M subunit chain, followed by conformational changes and entrapment of the enzyme. As a result, the proteinase is sterically hindered to react with large substrates, but it still preserves the activity towards those of low molecular mass (19). Therefore, the inhibition of LV-Ka by plasma proteinase inhibitors may be an important factor in the control of its systemic effects in bite victims. In previous studies we have shown that the metalloproteinase mutalysin II (23 kDa) isolated from the same bushmaster venom was effectively inhibited by α_2-M; however, the large hemorrhagic metalloenzyme mutalysin I (100 kDa) did not have its activity significantly altered after treatment with α_2-M (20).

Since LV-Ka acts on bovine kininogen liberating a polypeptide that induces guinea pig ileum contraction (14), the biochemical and biological characterization of such a polypeptide became the main objective of the present study. Considering that relaxed mesenteric arterial rings isolated from SHR contract repetitively and dose-dependently in response to bradykinin (BK) (21, 22), this preparation was used as a biological test to verify whether the polypeptide liberated by LV-Ka from bovine kininogen could be BK.

Materials and Methods

Materials

Lachesis muta muta venom was obtained from the serpentarium of the Ezequiel Dias Foundation, Belo Horizonte, Brazil; Rats (spontaneously hypertensive) (SHR) were provided by CEBIO from the Federal University of Minas Gerais; Chromogenic substrates: H-D-Pro-Phe-Arg-*p*NA (S-2302), H-D-Val-Leu-Arg-*p*NA (S-2266), and H-D-Val-Leu-Lys-*p*NA (S-2251) were purchased from Kabi Vitrum (Stockholm, Sweden); PNGase F was from New England BioLabs (Beverly, MA, USA); Human fibrinogen essentially plasminogen free, human plasmin, bovine thrombin, N-p-Tos-Gly-Pro-Lys-*p*NA, bradykinin acetate (BK), acetylcholine chloride, L-arterenol hydrochloride (norepinephrine), *N*-α-benzoyl-L-arginine-*p*-nitroanilide (DL-BAPNA) and indomethacin were purchased from Sigma (St. Louis, MO, USA); D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin (HOE 140) was kindly provided by Hoechst-Roussel Pharmaceutical (Frankfurt, Germany). Sephacryl S-200 and DEAE Sepharose CL-6B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Human α_2-macroglobulin was from Calbiochem (San Diego, CA, USA). All other chemicals were of analytical reagent grade.

Purification of Lachesis venom LV-Ka

LV-Ka was purified from *Lachesis muta muta* snake venom by a combination of gel filtration on Sephacryl S-200 and ion-exchange chromatographies on DEAE-Sepharose CL-6B at pH 8.0 and 7.3, respectively, as previously described (14).

The progress of purification was evaluated by determining the following parameters for each fraction: a) amidolytic activities on DL-BAPNA, S-2302, and Tos-Gly-Pro-Lys-*p*NA; b) proteolytic activity on dimethylcasein; c) coagulant activity on human fibrinogen; d) fibrino(geno)lytic activity; e) hemorrhagic activity; f) phospholipase A_2 activity; and g) effect on platelet function. Homogeneity of the proteinase was

demonstrated by SDS-PAGE according to the method of Laemmli (23).

Preparation of anti-LV-Ka antibody

Anti-LV-Ka antibody was raised in one rabbit. Purified LV-Ka (250 μg in 1.0 ml of phosphate-buffered saline, pH 7.4) was emulsified with 2.0 ml of Freund's complete adjuvant. Aliquots of 0.5 ml from this emulsion were injected (subcutaneously) into five sites in the back of the rabbit. A booster injection consisting of 100 μg (800 μl) of LV-Ka and 800 μl of incomplete adjuvant was administered 21 days after the original injection. After 40 days, the rabbit was bled. The IgG fraction of immune rabbit serum was purified by affinity chromatography on protein A-Sepharose (24).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

SDS-PAGE without reduction or after reduction with 4% β-mercaptoethanol was performed by the method of Laemmli (23), using 12% gels and stained with Coomassie blue R-250. After electrophoresis, the proteins were transferred onto nitrocellulose membranes using the Bio-Rad transblot-aparatus. Blotting procedures, using rabbit IgG against LV-Ka, were performed according to the method of Towbin et al. (25). Apparent molecular masses were determined from reduced gels, by comparison with protein calibration mixtures from Pharmacia containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa) or from Oncogene (Boston, MA, USA) containing myosin H-chain (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa).

α_2-M proteinase interaction

Increasing concentrations of human α_2-M (10 – 100 nM) were incubated with a constant concentration of LV-Ka (20 nM) (α_2-M: LV-Ka molar ratios of 0 to 5.0) in 150 μl of 50 mM Hepes, 0.15 M NaCl, pH 7.5. After 5 min at 37°C, proteinase activity on dimethylcasein and on the low molecular weight substrate S-2302 was measured. The stoichiometry of the LV-Ka/α_2-M complex was determined using dimethylcasein as substrate. The concentration of the enzyme, 0.1 μg (20 nM), was approximately 1.5-times higher if compared with the concentration of mutalysin II (13 nM) used in similar experiments (26). At the same time, 50 μl of the incubation mixture was removed and mixed with an equivalent volume of denaturing solution and kept overnight at room temperature for SDS-PAGE (7.5 or 10% gels) under reducing (4% β-mercaptoethanol) or nonreducing conditions. The gels were stained with 0.25% coomassie brilliant blue in ethanol/acetic acid/water (1:2:6) and destained with the same solution. In a similar way, LV-Ka (0.1 μg) was also incubated with 20 μl of human plasma (diluted 1:2) for 15 min at 37°C before testing the proteinase activity on dimethylcasein and on S-2302.

Determination of kinetic parameters

The steady-state parameters Michaelis constant (K_m) and catalytic efficacy (kcat) were determined from initial rate measurements at various substrate concentrations in 0.10 M Tris-HCl, pH 8.0 at 25°C using as substrates S-2302, S-2266, S-2251, and N-*p*-Tos-Gly-Pro-Lys-*p*NA. The precise concentration of each substrate was determined by measuring the amount of product released after complete hydrolysis with an excess of trypsin. At least three separate measurements were carried out for each substrate. Reactions were initiated by the addition of appropriate amounts of enzyme, and the formation of *p*-nitroanilide (*p*NA) was continuously monitored by measuring the absorbance at 405 nm. The amount of substrate hydrolyzed was calculated using the measured absorbance at 405 nm by using a molar extinction coefficient of 8800 $M^{-1} \cdot cm^{-1}$ for free *p*NA. K_m and V_m were determined by analysis of double-reciprocal plots of the initial velocity as a function of substrate concentration. Calculations were carried out as described (27).

Biological preparation and biological assay methodology

The experiments were performed in mesenteric arterial rings of male SHR weighing 200 – 300 g. The animals were killed by decapitation and a portion of the superior mesenteric artery about 1 mm in diameter was immediately excised and placed in a Petri dish containing nutritive solution at room temperature and following under a Zeiss magnifying lens, carefully cleaned from its surrounding fat and connective tissues. Then, arterial rings measuring about 2-mm-wide were prepared from this cleaned preparation. In some experiments only one arterial ring was prepared from each artery segment, but in most of our experiments, in order to perform comparative experimental protocols, up to four neighboring rings from the same artery were prepared.

Each SHR arterial ring was suspended in a vertical 10-ml organ chamber, between two stainless steel hooks, and connected to a Grass FT-03 force displacement transducer. In a few arterial rings, the endothelium was mechanically rubbed off in order to allow the evaluation of any effect of endothelium-derived substances in the responses to BK. At the end of the experiments, the integrity of the endothelium or the effectiveness of its

intentional destruction was confirmed by the presence or absence of relaxation in response to 10^{-6} M acetylcholine in a ring precontracted with 2×10^{-7} M norepinephrine.

The organ chambers were filled with Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM glucose) maintained at 37°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. Isometric contraction forces were recorded on a Grass model 7 polygraph. A basal tension of 1.0 g was applied to each ring at the beginning of an experiment. The rings were equilibrated in the organ chambers for 90 min with replacement of nutritive solution each 15 min before any drug addition. Aliquots of LV-Ka/bovine kininogen incubated mixture or pharmacological drugs were added to the muscle chamber in small volumes (5 to 300 μl). Drugs suspected to modify the action of BK were left in contact with the biological preparation in the muscle chamber for 10 to 15 min before the addition of BK or of LV-Ka/bovine kininogen incubated mixture. In order to obtain appropriate repetitiveness of responses to BK and to LV-Ka/bovine kininogen incubated mixture, the arterial rings were left to rest for 20 to 60 min, with washing every 10 – 15 min, between consecutive challenges of the same agonist. The contractile response of each ring to 60 mM KCl at the beginning of an experiment was taken as a standard to evaluate the magnitude of the responses to LV-Ka/bovine kininogen incubated mixture or BK.

Incubation of bovine kininogen with LV-Ka for kinin production

To evaluate the kinin production induced by LV-Ka on bovine kininogen, 600 μl of bovine kininogen (10 mg/ml), buffered in 20 mM Hepes at pH 8.0 was mixed with 3.8 μg of LV-Ka and incubated at 37°C for 20 min. Aliquots of 5 μl and up to 300 μl were then taken from this mixture and added to three different organ chambers to evaluate the effect on mesenteric arterial rings. (In a fourth organ chamber 0.1 ml of BK solution 1.0×10^{-4} M was added for comparison). Indomethacin or HOE-140 (both for final concentration of 10^{-6} M) were added to organ chambers as above described to evaluate the ability of these drugs to modify BK and/or LV-Ka/bovine kininogen incubated mixture effect on mesenteric arterial rings.

Statistical analyses

Each biological experimental procedure was repeated with 3 to 5 animals. Where appropriate, means, standard error of means (S.E.M.) and comparison of means through Student's *t*-test were calculated. Values (in % of response to 60 mM KCl on same arterial ring) given in the Results section are means ± S.E.M.

Results

Purification of LV-Ka

LV-Ka was purified by a three-step method of gel filtration on Sephacryl S-200 and ion exchange chromatographies on DEAE Sepharose CL-6B at pH 8.0 and 7.3, respectively (Fig. 1, 1st – 3rd step). Analysis by SDS-PAGE under non-reducing or reducing conditions gave a single band indicating the homogeneity of the preparation (Fig. 2A, lanes 1 and 2). In addition, IgG anti-LV-Ka cross-reacted with both, crude venom of *Lachesis muta muta* from which it was prepared and the purified enzyme when explored by Western immunoblotting (Fig. 2B, lanes 1 and 2). Using a different purification procedure, another kininogenase (27.9 kDa) was also reported from *Lachesis muta* venom (11). However, the mechanism of action of this enzyme is unclear and information of its structure is as yet unavailable. Furthermore, as can be seen in Fig. 1, 2nd step, four peaks (D, E, F, and G) associated with amidolytic activity on S-2302 were obtained after DEAE Sepharose ion-exchange chromatography of peak 5 (1st step). Although a serine proteinase with plasminogen activating activity (LV-PA) was isolated from peak E (15), peak F contained a LV-Ka enzyme (present study) and peaks D and G are under investigation. Therefore, we believe that LV-Ka is a new enzyme distinct from the kininogenin proteinase previously reported (11).

Interaction between α_2-M and LV-Ka

The effect of incubating increasing amounts of α_2-M with a constant concentration of LV-Ka (molar ratio 0.25, 0.5, 0.75, and 1.5) for 5 min at 37°C was analyzed by SDS-PAGE. Under reducing conditions, the cleavage of the 180-kDa subunits of human α_2-M to yield the characteristic fragments of 90 kDa resulting from the bait region cleavage were clearly seen (Fig. 3A, lanes 4 and 5); however, under our experimental conditions cleavage of the bait region was not completed. At molar ratios α_2-M:LV-Ka below 0.5/1, no cleavage was detected (lanes 2 and 3). Under nonreducing conditions (7.5% gels) protein bands above 180 kDa (approx. 320 and 410 kDa) corresponding to the α_2-M dimers (half molecule) or the complexes between LV-Ka and α_2-M were detected (data not shown). The stoichiometry of inhibition of LV-Ka with α_2-M is shown in Fig. 3B. Increasing amounts of the plasma inhibitor were mixed with a fixed concentration of enzyme (20 nM) and residual activity on dimethylcasein was determined.

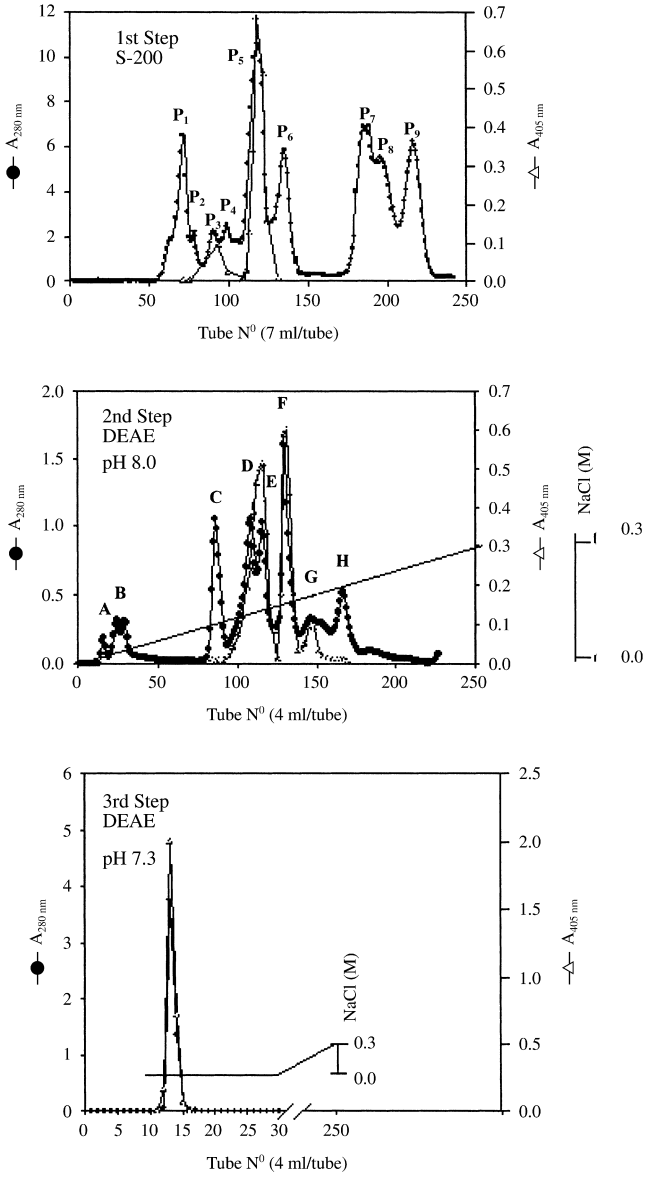

Fig. 1. Purification of LV-Ka. The isolation procedure is described in the text. Closed circle, absorbance at 280 nm; open triangle, amidolytic activity on substrate S-2302, measured by absorbance at 405 nm. 1st step: gel filtration on Sephacryl S-200, elution with ammonium acetate buffer, at pH 7.4; 2nd step: ion exchange chromatography on DEAE-Sepharose CL 6B, equilibrated with 20 mM Hepes buffer, at pH 8.0, of the pooled active fractions from the previous step (peak 5). 3rd step: re-chromatography on DEAE-Sepharose CL-6B, equilibrated with 20 mM HEPES buffer, at pH 7.3, of fraction F from second step. In steps 2 and 3, proteins were eluted with a linear gradient of NaCl (0 – 0.3 M, 500 ml of each) at a flow rate of 14 ml/h.

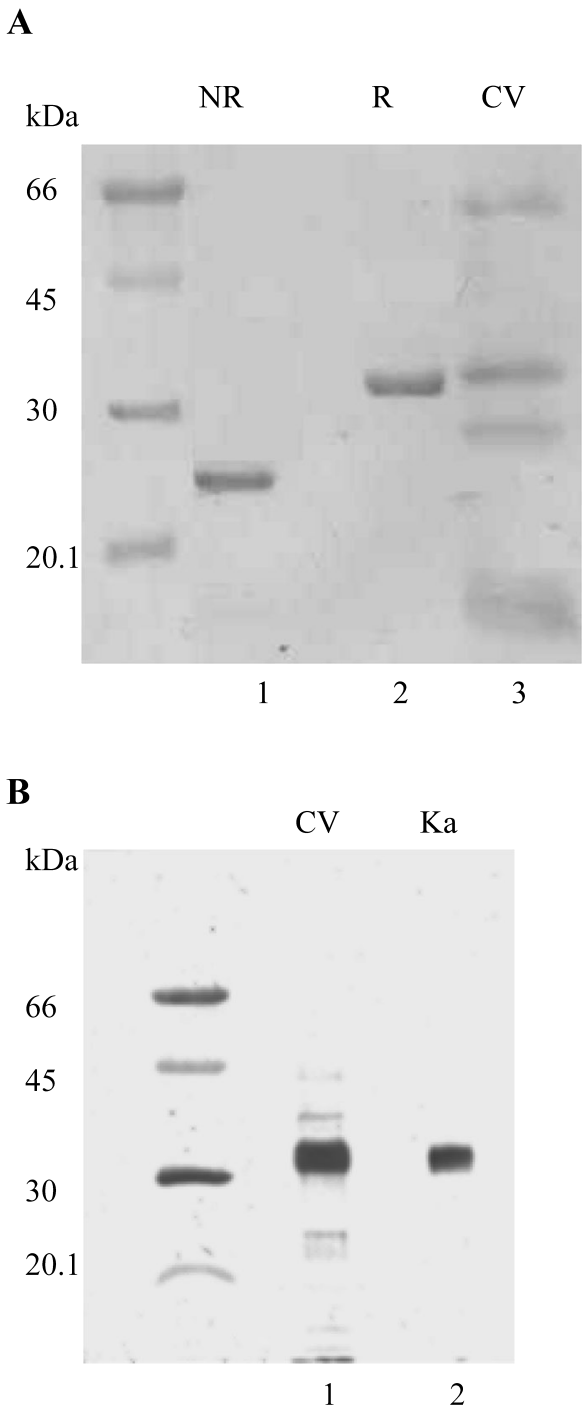

Fig. 2. SDS-PAGE and Western blotting analysis of purified LV-Ka. A: SDS-PAGE electrophoresis of LV-Ka after third step of purification shown in Fig. 1, under non-reducing (NR, lane 1) and reducing (R, lane 2) conditions, crude venom under reducing conditions (CV, lane 3). B: Western immunoblotting of crude venom of *Lachesis muta muta* (CV, lane 1), and purified LV-Ka (Ka, lane 2) with IgG anti-LV-Ka. Molecular mass markers are indicated at the left.

Proteinase activity was totally inhibited and molar ratio inhibitor/enzyme determined from X-intercepts was found to be 1:1. In similar assay procedures, the rate of hydrolysis of the peptide S-2302 by the α_2-M:LV-Ka complex was not significantly affected (data not shown). In addition, preincubation of LV-Ka (0.1 μg) for 15 min

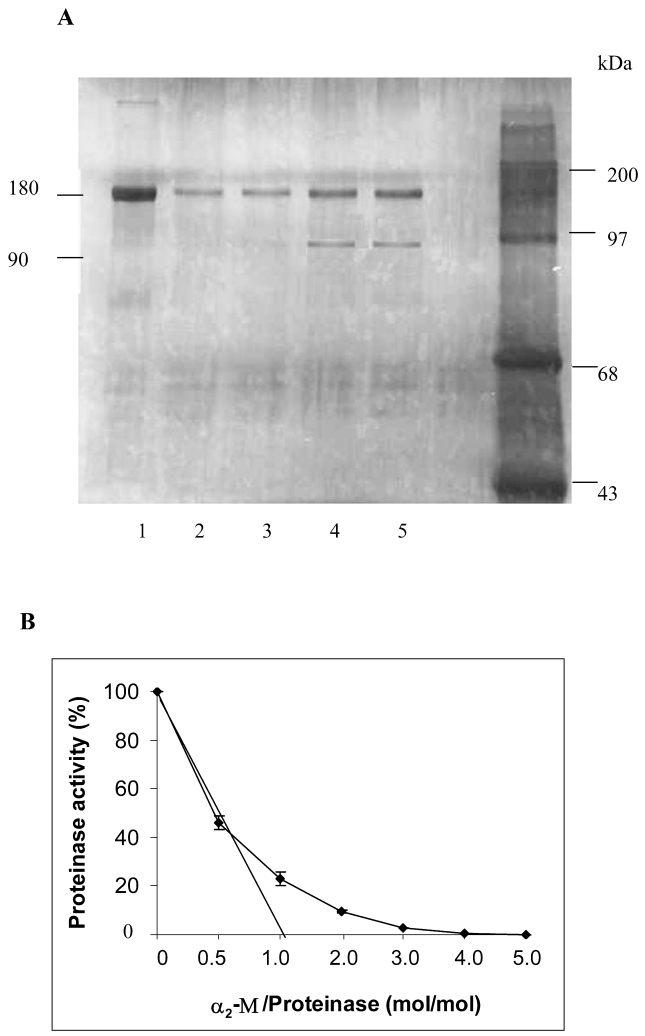

Fig. 3. Cleavage patterns of human α_2-M by LV-Ka and stoichiometry of inhibition of LV-Ka by α_2-M. A: Reduced SDS-PAGE pattern of human α_2-macroglobulin incubated with LV-Ka. Enzyme (0.1 μg) was incubated with increasing concentrations of inhibitor for 5 min at 37°C. Lane 1, α_2-M control. Molar ratios of α_2-M/enzyme were as follows: 0.25 (lane 2), 0.5 (lane 3), 0.75 (lane 4), and 1.5 (lane 5). Molecular mass markers are in the lane on the extreme right. (B) Determination of the stoichiometry of inhibition of LV-Ka by α_2-M. LV-Ka (20 nM) was incubated with various amounts of inhibitor at molar ratios indicated on the abscissa (α_2-M/enzyme = 0 – 5). Proteinase activity in the mixture was measured using dimethylcasein as substrate. The ordinate indicates the remaining activity of the proteinase as a percent of the original activity. Results are shown as means of two separate experiments and are given as means ± S.D., n = 4.

at 37°C with 1:2 diluted human plasma, before testing the proteolytic activity, resulted in more potent inhibition of its activity with respect to the large substrate (dimethylcasein) than to small substrate (S-2302) molecules in a similar way to the results obtained with α_2-M, suggesting the presence of LV-Ka inhibitor(s) in plasma (data not shown).

Kinetic studies

Table 1 presents the kinetic parameters of LV-Ka on several chromogenic substrates with L-Arg or L-Lys at the P_1 position. Among the compounds tested, S-2266, a substrate for glandular kallikreins was the more effective substrate for the enzyme in regard to catalytic efficiency kcat. As judged by specificity (kcat/K_m), the parameters of the enzyme on S-2266 was 1.58- and 3.57-fold higher than that on S-2251 and Tos-Gly-Pro-Lys-*p*NA (substrates for plasmin), respectively. The lower activity on the substrate for plasma kallikrein, S-2302, indicate a marked influence of the residues in the P_2 and P_3 positions (nomenclature of Schechter and Berger, 28).

Biological assays

Either relaxed endothelium-preserved (n = 7) or relaxed endothelium-denuded (n = 4) mesenteric arterial rings from SHR contracted to the addition of LV-Ka /bovine kininogen incubated mixture to the organ chamber with no difference between them. Responses of SHR mesenteric arterial rings to BK and to LV-Ka /bovine kininogen incubated mixture were repetitive whenever appropriate intervals were allowed between consecutive challenges. Consecutive challenges with BK resulted in similar responses (Fig. 4A, n = 3) after about 20-min intervals. However, repetitiveness with LV-Ka/bovine kininogen incubated mixture (Fig. 4B, n = 3) were obtained only after intervals of up to 60 min between challenges (until the foam generated by bovine plasma residue was completely washed out). The amplitude of contractions to 300 μl of the incubated mixture were 95 ± 5% (n = 5) of the contractions observed in response to the addition of 60 mM KCl to the same arterial ring. Such magnitudes were also similar to contractions in response to 10^{-6} M of BK (Fig. 5A, n = 3). Responses to smaller volumes of the incubated mixture were dose-dependently smaller, as in the one example shown in Fig. 5A.

These contractile responses were not due to either of the components alone because no contractile response was observed upon the addition of LV-Ka alone (n = 3) or to bovine kininogen alone (n = 3) added to the organ chamber (not shown). HOE-140 (10^{-6} M) completely inhibited contractions to 300 μl of incubated mixture (Fig. 5B, n = 3) and to 10^{-6} M BK (n = 3, not shown). Identically, the addition of indomethacin (10^{-6} M) to the organ chamber 10 min prior to the addition of 300 μl of incubated mixture inhibited completely contractions caused by this sample (Fig. 5C, n = 3)) or prior to the addition of 10^{-6} M BK (n = 3, not shown). The inhibitory effects of HOE-140 and of indomethacin were specific for the experimental situation since mesenteric arterial rings under such inhibitory effects still contracted in

Table 1. Kinetic parameters of LV-Ka on different chromogenic substrates

Substrate	$V_m \times 10^{-2}$ ($\mu M \cdot s^{-1}$)	$K_m \times 10^{2}$ (μM)	kcat $\times 10^{-2}$ (s^{-1})	kcat/$K_m \times 10^{-4}$ ($\mu M^{-1} \cdot s^{-1}$)
H-D-Val-Leu-Arg-*p*NA (S-2266)	13.27 ± 2.39	1.34 ± 0.28	10.02	7.48
H-D-Val-Leu-Lys-*p*NA (S-2251)	3.95 ± 0.64	1.38 ± 0.18	6.53	4.73
N-p-Tos-Gly-Pro-Lys-*p*NA	2.24 ± 0.27	0.81 ± 0.20	1.69	2.09
H-D-Pro-Phe-Arg-*p*NA (S-2302)	3.95 ± 0.64	1.66 ± 0.33	2.98	1.80

Data for LV-Ka are given as means ± S.D. (n = 3).

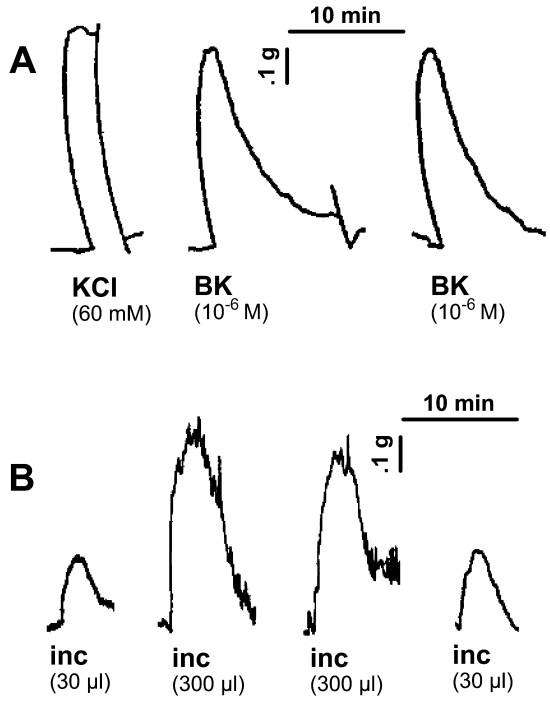

Fig. 4. Responses induced by BK and LV-Ka/bovine kininogen incubated mixture in SHR mesenteric arterial rings. A: Repetitive response induced by BK (10^{-6} M) in a same SHR rat mesenteric arterial ring. B: Repetitive responses induced by 30 and 300 μl of LV-Ka/bovine kininogen incubated mixture (inc) in a same (but distinct from panel A) SHR mesenteric arterial ring. Repetitiveness of responses was observed in at least 3 preparations for each agonist.

response to norepinephrine (10^{-7} M, n = 5) and to KCl (60 mM, n = 5) (Fig. 5: B and C).

Discussion

LV-Ka, a serine proteinase possessing kinin-releasing activity without coagulant activity, was isolated from bushmaster (*Lachesis muta muta*) venom. Its molecular mass (33 kDa) was determined by SDS-PAGE under reducing conditions; however, when submitted to *N*-deglycosylation with PNGase F, its Mr fell to 28 kDa.

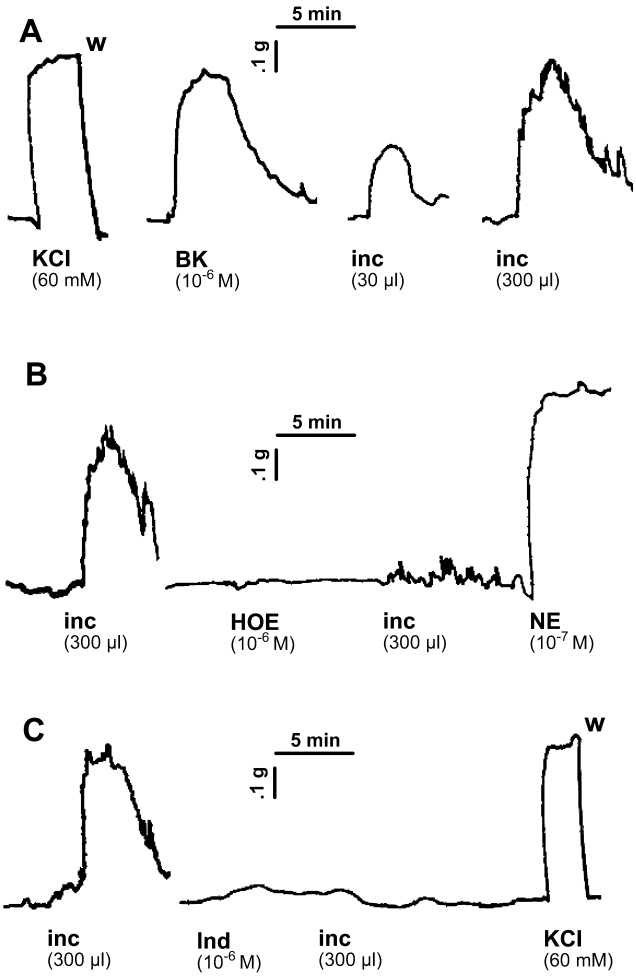

Fig. 5. Responses of mesenteric arterial rings from SHR to BK and LV-Ka/bovine kininogen incubated mixture and inhibition of contractile responses to LV-Ka/bovine kininogen incubated mixture by HOE-140 and indomethacin. A: Contractions of mesenteric arterial rings from SHR to KCl (60 mM), to BK (10^{-6} M), and to 30 or 300 μl of LV-Ka/bovine kininogen incubated mixture (inc). B: Inhibition of contractile response to 300 μl of LV-Ka/bovine kininogen incubated mixture (inc) by 10^{-6} M HOE-140 (HOE). C: Inhibition of contractile response to 300 μl of LV-Ka/bovine kininogen incubated mixture (inc) by 10^{-6} M indomethacin (Ind). For comparison, contractions in response to 10^{-7} M norepinephrine (NE) and to 60 mM KCl in presence of antagonists are shown in panels B and C, respectively. W = wash.

LV-Ka is a glycosylated protein, with a carbohydrate content of approximately 15% based on SDS-PAGE. Apparently, the glycans seem to be needed for protein stabilization as well as for its catalytic function (14). As shown in Fig. 2B, rabbit IgG raised against LV-Ka detected specifically a 33 kDa band in the crude venom (lane 1) as well as the purified protein (lane 2). In addition, anti-LV-Ka polyclonal antibodies cross-reacted with a LV-PA isolated from the same bushmaster venom, when tested by ELISA assay and immunodiffusion (data not shown).

As a general rule, venom proteinases possess much lower hydrolytic activity on several of the chromogenic substrates, indicating the relatively narrow substrate specificities of venom enzymes. Furthermore, the multifunctional character of LV-Ka with respect to having BK releasing and plasminogen activating activity was reflected by its substrate specificity for the kallikrein substrates S-2266 and S-2302 and for the plasmin substrates S-2251 and Tos-Gly-Pro-Lys-*p*NA. Other multifunctional serine proteinases have also been reported from snake and lizard venoms (17, 29, 30). The experimentally determined K_m, kcat, and kcat/K_m parameters reflects, respectively, affinity, catalytic efficiency, and specificity. As shown in Table 1, the K_m values can be found within the same level of magnitude for the four peptide substrates with similar affinity. Based on kcat, the enzyme preferred L-Arg in P_1, hydrophobic residues such as L-Leu in P_2 and D-Val in P_3. Thus, the results on S-2266, a substrate for glandular kallikrein, was the more effective for the enzyme in regard to kcat and specificity (kcat/K_m). In accordance with their structural and biochemical properties, the serine proteinases from snake venoms are composed of approximately 234 amino acid residues and are highly homologous in sequence. Thus, these venom enzymes seem to have diverged from glandular kallikrein and that their functions changed during the process of evolution (31).

Fractionation and characterization of snake venom components is an important step in understanding the mode of action of the toxins present in the venoms. Envenomation by bushmaster is characterized by severe local and systemic effects, of which one is a marked fall in blood pressure (32). Fall in systemic blood pressure is usually attributed either to an impairment in cardiac output or more commonly to peripheral vasodilatation. Histamine, liberated from tissue mastocytes and BK released from blood plasma are the agents most commonly responsible for vasodilatation induced by envenoming of viper and pit viper snakes. Fractionation of *Lachesis muta muta* venom showed that at least one of its components (LV-Ka) liberates a substance from bovine plasma that is able to contract intestinal smooth muscle (14). BK was suspected to be the substance liberated by LV-Ka from bovine plasma because this polypeptide is the one most commonly generated from plasma kininogen, but no conclusive characterization of such contracting substance was done at that time. Therefore, an appropriate biological preparation was used in the present study to allow an adequate characterization of the polypeptide liberated by LV-Ka from bovine plasma.

Here we demonstrated that the venom proteinase LV-Ka was effectively inhibited by plasma α_2-M, a prevalent mammalian protease inhibitor that is capable of forming a macromolecular complex with LV-Ka. The interaction of the venom enzyme with human α_2-M as evidenced by cleavage of 180-kDa subunits is shown in Fig. 3A. As expected, LV-Ka cleaved the subunits to produce the fragments characteristic (90-kDa) of bait region cleavage. Untreated α_2-M displays the characteristic Mr 180-kDa band (Fig. 3A, lane 1). Proteolysis of the bait region induces a conformational change in α_2-M, which entraps the proteinase within the α_2-M molecule. Thus, the entrapped proteinase is sterically hindered, and its ability to react with large substrates is drastically decreased, although it retains almost full activity against small substrates (18, 33). Furthermore, our finding that one molecule of human α_2-M is able to bind and inhibit one molecule of LV-Ka as determined in a standard proteinase activity assay (Fig. 3B) is supportive of a relatively fast rate of complex formation between the enzyme and inhibitor. In agreement with the literature, the cleavage of α_2-M seems to be specific for the bait region and no further degradation was observed. Also, the cleavage at the bait region between Arg^{696}-Leu^{697} is the most common peptide bond in α_2-M cleaved by proteinases (34). At this time we have not determined which is the peptide bond in α_2-M cleaved by LV-Ka; however, based on other reports (34, 35), it is expected that LV-Ka cleaves the same peptide bond.

BK has a direct action on some smooth muscles, including vascular smooth muscle, via its action on two receptor subtypes: B_1 and B_2 (36 – 38). Activation of these receptors leads to relaxation, contraction, or a biphasic response, depending on the organ or species under study (36, 38, 39). Vasodilatation with a fall in blood pressure is the typical in vivo response to systemic infusion of BK (40), but when blood vessels are studied in vitro, the responses to BK are not the same under different circumstances. Thus, in different isolated vessels, this polypeptide was shown to induce relaxation as well as contraction (41). In isolated mesenteric arterial rings from Holtzman rats (21) and SHR (22), BK always induces dose-dependent contractions. Such

an effect was demonstrated to be mediated by BK-induced liberation of thromboxane A_2/endoperoxide from the ring tissue. The contractile action of BK on mesenteric arterial rings from SHR is inhibited by HOE-140, a BK B_2 antagonist, and by indomethacin, a cyclo-oxygenase inhibitor (21, 22).

In the present study, relaxed mesenteric arterial rings from SHR contracted repetitively and dose-dependently to the addition of LV-Ka/bovine kininogen incubated mixture in a very similar way as they did to BK. Moreover, either HOE-140 or indomethacin inhibited the contractile response to the LV-Ka/bovine kininogen incubated mixture to the same degree as the response to BK was inhibited by these antagonists. Such observations strongly suggest that BK and/or some other B_2 agonist is liberated by LV-Ka from bovine plasma and that the liberated kinin acts on the B_2 receptor of mesenteric arterial rings, liberating thromboxane A_2 /endoperoxide to induce contraction.

Due to technical difficulties, no dose-response curves were constructed either for BK-receptor interaction or for the LV-Ka/bovine kininogen incubated mixture-receptor interaction. However, although not shown in our results, in some experiments a tenfold increase in LV-Ka/bovine kininogen incubated mixture added to the organ chamber resulted in an increased contractile response similar to those observed for a tenfold increase in BK concentration. This suggests that both dose-response curves are similar and reinforces a conclusion that BK is the substance generated in the LV-Ka/bovine kininogen incubated mixture.

This finding of a kallikrein-like enzyme in bushmaster venom capable of liberating BK from blood plasma seems to explain, at least partially, local pain and sytemic hypotension observed in patients bitten by this snake.

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