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A NEW VASOPEPTIDE FORMED BY THE ACTION OF A MURPHY-STURM LYMPHOSARCOMA ACID PROTEASE ON RAT PLASMA KININOGEN*+

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SUMMARY: A new vasoactive peptide, formed by the action of a Murphy-Sturm lymphosarcoma acid protease on rat plasma kininogen was purified by gel filtration on Sephadex G-50 (fine) and fractions assayed on the isolated rat uterus for smooth muscle stimulating activity. The most active fraction was purified further by CM-cellulose chromatography. High voltage electrophoresis showed the peptide to be one component (M_{gly} 2.49) with an electrophoretic mobility different from bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin. The molecular weight of the peptide was estimated on Sephadex G-25 column to be 1460. The amino acid composition was determined and the carboxyl terminal sequence identified by carboxypeptidase Y treatment to be Pro-Phe-Arg-Leu. Dansyl-Edman procedure yielded an amino terminal sequence of Ile-Ser-Arg-Pro. The peptide produced a dose-dependent contraction of the isolated guinea pig anterior mesenteric vein and relaxed the rabbit superior mesenteric artery contracted by phenylephrine.

The importance of acid proteases as an alternate kinin generating system has been emphasized (1,2). Cathepsin D releases leukokinins from leukokininogen (3), and methionyl-lysyl-bradykinin (MBLK) from bovine kininogen (4). Other acid proteases like pepsin (5) and human neutrophil leukocyte acid proteases (6) also have been shown to cleave a vasoactive peptide similar to MLBK. Very limited information is available about the nature of the vasoactive peptides released by acid proteases. Recent work from our laboratory reported the purification of an acid protease from the rodent Murphy-Sturm lymphosarcoma tumor (7), which hydrolyzed highly purified rat plasma low molecular weight kininogen (8) to generate vasoactive peptides. The present communication reports the purification, physico-chemical and pharmacological characterization and partial sequence of the peptide generated from kininogen.

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MATERIALS AND METHODS

LMW Kininogen used in these studies was purified from citrated rat plasma as described earlier (8). Highly purified acid protease was isolated from Murphy-Sturm lymphosarcoma tumor (7). Trypsin was obtained from Worthington and carboxypeptidase Y was procured from Sigma. The sequenal grade amino acid sequencing reagents such as phenylisothiocyanate, trifluoroacetic acid, PTH-amino acids, dansyl chloride, pyridine, benzene, butylacetate and ethylacetate were purchased from Pierce Chemical Company. Schleicher and Schuell polyamide sheets and 2-(4'-t-butylphenyl)-5-(4'-biphenyl)1,3,4-oxidiazole (butyl PBD) were also from Pierce Chemical Co. Thin-layer cellulose chromatogram sheets were supplied by Eastman. Fluorescamine was obtained from Sigma. All Sephadexes were purchased from Pharmacia. CM-cellulose was procured from Whatman. Captopril (SQ14,225; D-3-mercapto-2-methylpropanoyl-L-proline), a kininase II inhibitor, was a gift from The Squibb Institute for Medical Research, Princeton, N.J.

Amino Acid Analysis: A 1-2 nmole sample of peptide was analyzed for amino acids on a Beckman model 121C amino acid analyzer (9). The samples were hydrolyzed under vacuum at 110°C for 24 hr with 6 N HCl, dried under reduced pressure and dissolved in pH 2.2 buffer immediately before amino acid analysis.

High Voltage Electrophoresis: A cooled flat plate apparatus was used for high voltage electrophoresis. Samples were run on Estar base backed cellulose thin layer plates at 4°C for 70 min at 600 V and 15 mA using pyridine:acetate buffer, pH 4.8 (Pyridine:acetic acid:water, 10:10:480). After the runs the plates were dried for 1 hr in an oven and spots were visualized by spraying thinly with 0.1% ninhydrine, 5% collidine in 95% ethanol, followed by heating at 100°C for about 10 min (10). Ehrlich's stain was used for any tryptophan-containing spots (11). In the preparative runs the spots were visualized by staining with 0.025% fluorescamine in acetone (12). The peptides were recovered by scraping the cellulose from corresponding spots and eluting with 10% acetic acid.

Amino-Terminal Analysis: Dansyl chloride procedure (13) was used. Dansyl amino acids were identified by two dimensional thin layer chromatography on polyamide sheets (14), run in water/90% formic acid (200:3 v/v) in first direction and in benzene/acetic acid (9:1 v/v) in second direction. Appropriate dansyl amino acid standards were run simultaneously on a separate sheet.

Edman Degradation: Amino acid sequencing was carried out by the Edman-Dansyl procedure (15). PTH-amino acids were identified by thin-layer chromatography on polyamide sheets by the method of Summers et al., (16). The amino-terminal amino acid after each degradative cycle also was identified as a dansyl derivative.

Carboxypeptidase Y Digestion of the Peptide: Peptide sample (20 nmol) in 150 µl 0.1 M pyridine-acetate buffer, pH 5.5 was incubated at 37°C with 10 µl of 0.5 mg/ml solution of carboxypeptidase Y (≈0.1 nmol). Aliquots (15 µl each) were withdrawn at 0,15,30,45,60,75 and 90 min time intervals and mixed immediately with 135 µl 0.2 M citrate-phosphate buffer, pH 2.2, and analyzed for released amino acids on a Beckman model 121C amino acid analyzer.

Enzymatic Hydrolysis of Rat Plasma Kininogen: Purified rat plasma kininogen (116 mg) in a total volume of 2.5 ml citrate phosphate buffer pH 3.0 was mixed with 3.25 units of MSLS tumor acid protease. Three more additions of the acid protease were made at 2 h intervals and finally incubation continued overnight at 37°C. The products of hydrolysis were fractionated by gel filtration on Sephadex G-50 (fine). Each fraction was checked for smooth muscle stimulating activity by bioassay on rat uterus as described previoulsy (8).

Vasoactive Responses on the Guinea Pig Anterior Mesenteric Vein and Rabbit Superior Mesenteric Artery: Anterior mesenteric vein and superior mesenteric artery were removed from guinea pigs and rabbits respectively killed by exsanguination, cut into rings 2 mm wide, and each ring placed on 2 stainless steel hooks in a 10 ml organ bath containing oxygenated Kreb's solution. Anterior mesenteric veins were equilibrated for 1 hr at a constant tension of 0.5 gm before measuring the contractile response of the vein to bradykinin or acid protease-derived peptide. The arterial rings were equilibrated for 2 hr under 1.5 gm resting tension, contracted with 2.5×10^{-6} M phenylephrine and ultimately relaxed in a cumulative manner with increasing concentrations of either bradykinin or acid protease-derived peptide. All contractile responses were recorded electronically via a strain gauge linked to a physiograph Six-B (Narco Bio-Systems, Inc., Houston, Texas).

RESULTS

Smooth muscle stimulating peptides were prepared from the purified rat plasma kininogen by hydrolysis with MSLS-tumor acid protease followed by fractionation of the hydrolysate by gel filtration on a column (1.6x145cm) of Sephadex G-50. Two major peptide fractions, 1 and 2, and four minor fractions, 3,4,5 and 6 were obtained. Most of the biological activity was present in Fraction 5, with a small amount of activity remaining in Fraction 1. Redigestion of all of these fractions with acid protease showed no further production of biologically active peptides. Further, tryptic digestion of aliquots of Fractions 2,3,4 and 6 showed no release of any biologically active peptide, whereas Fraction 1 and 5 showed a five-fold increase in smooth muscle stimulating activity. Fraction 5 was purified further by chromatography on a column (1.5x30cm) of CM-cellulose pre-equilibrated with 0.05 M ammonium formate buffer, pH 3.75. Biologically active peptide was bound to CM-cellulose and was eluted at 0.2 M ammonium formate concentration.

High voltage electrophoresis at pH 4.8 showed that the purified vaso-peptide was comprised of only one component (M_{gly} 2.49), which did stain positively for arginine but not for tryptophan. The electrophoretic mobility of the peptide differed from that of bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin. The peptide eluted on a column of Sephadex G-25 (fine) slightly ahead of bradykinin. A molecular weight of 1460 was obtained from a standard plot.

TABLE 1.
AMINO ACID COMPOSITIONS OF VASOPEPTIDE AND ITS TRYPTIC PEPTIDES

Amino Acid	VP[a]	T-1[a]	T-2[a]	T-3[a]	T-4[a]
Serine	1.8	0	2.0	2.0	0.2
Proline	3.4	0	2.9	3.3	0.2
Glycine	1.0	0.2	1.2	1.3	0.3
Isoleucine	1.0	0	0.8	0.7	0
Leucine	1.1	1.0	0.1	0.2	1.0
Phenylalanine	1.8	0.1	1.8	1.6	0.2
Arginine	1.8	0.1	0.9	1.6	0.8

[a]VP, vasopeptide, T-1 to T-4 tryptic peptides eluted from cellulose plates after electrophoresis.

The amino acid composition of the vasopeptide is shown in Table 1. The molar ratio values reported in the Table were calculated assuming that the peptide contained one residue of isoleucine. A molecular weight of 1456 calculated from its amino acid composition is in agreement with the value obtained by gel filtration. On high voltage electrophoresis of the tryptic digest of the vasopeptide, two major (M_{gly} 0.92 and 2.57) and two minor (M_{gly} 1.89 and 3.11) ninhydrin-positive spots were obtained. Except for T-1 (M_{gly} 0.92) all other spots also were visible with Sakaguchi reagent. The amino acid composition of each fragment, after elution from cellulose plates, also is shown in Table 1.

The carboxyl terminal sequence of the vasopeptide, determined by treatment with carboxypeptidase Y, is Pro-Phe-Arg-Leu. Stepwise application of the dansyl-Edman procedure gave the sequence of amino terminal as Ile-Ser-Arg-Pro, but the sequence could not be persued further because of very low yields of remaining peptide. As a result of the sequence studies the partial sequence proposed for the peptide is: Ile-Ser-Arg-Pro(Pro,Gly,Phe,Ser)-Pro-Phe-Arg-Leu.

Vasoactive Responses of the Acid Protease-Derived Kinin-Like Peptide: Since, in general, small arterial vessels are relaxed and veins are constricted

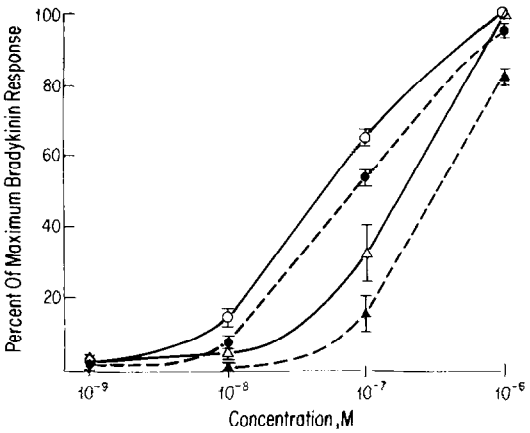

Figure 1. Dose-response of bradykinin (o,Δ) and the acid protease-derived vasoactive peptide (●,▲) on the guinea pig mesenteric vein in the absence (o,●) or presence (Δ,▲) of D-3-Mercapto-2-methyl-propanoyl-L-proline (Captopril). Contractions were elicited in a cumulative manner. The contraction of the vein was maximal with 10^{-6}M bradykinin and all responses were measured as a percentage of this maximal contraction. Each point represents the average of six values. The bars represent the standard error of the mean (S.E.M.)

by bradykinin, the vasoactive response of the purified peptide was investigated on guinea pig anterior mesenteric vein and rabbit superior mesenteric artery preparations. The acid protease-derived peptide produced a dose-dependent contraction of the guinea pig anterior mesenteric vein (Fig. 1). The response was not significantly different from that obtained with synthetic bradykinin. The presence of 4.6×10^{-5} M captopril, a kininase II inhibitor, caused an identical shift in the dose-response curve for both bradykinin and vasopeptide. Responses at 10^{-6} M concentration of bradykinin and the vasoactive peptide were essentially identical in the presence or absence of captopril.

Rabbit superior mesenteric artery, contracted with 2.5 μM phenylephrine, could be relaxed only at a concentration of 10^{-6} M bradykinin (Fig. 2), whereas the vasoactive peptide, at a concentration of 10^{-7} M, relaxed maximally two of the three arterial ring preparations. The third ring, which showed the most resistance to relaxation by bradykinin, was relaxed maximally only at a vasoactive peptide concentration of 10^{-6} M.

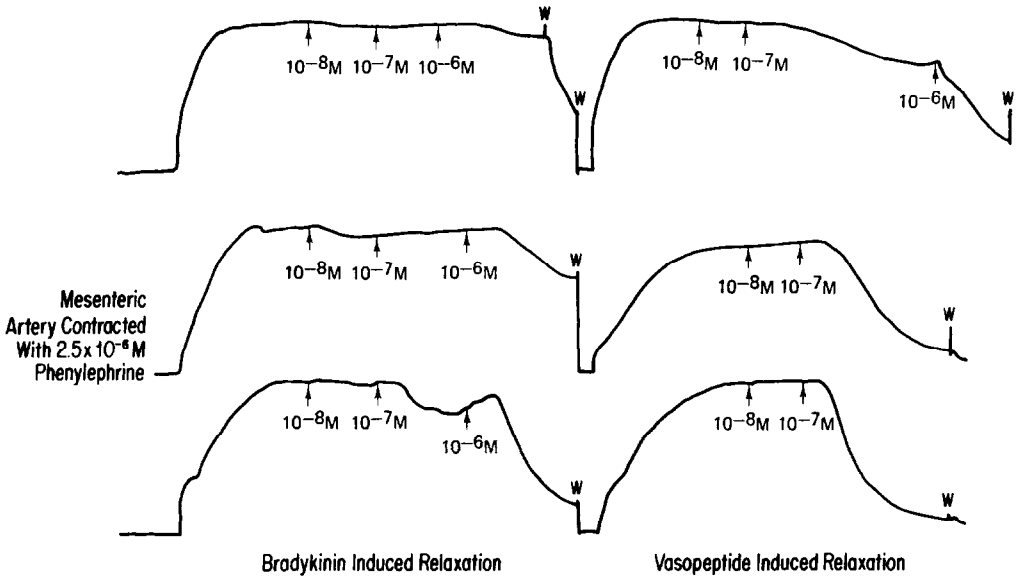

Figure 2. Transverse rings of the superior mesenteric artery from the rabbit maintained a steady contraction with 2.5×10^{-6}M phenylephrine. After the phenylephrine-induced contraction reached it's maximum, increasing concentrations of bradykinin or the acid protease-derived vasoactive peptide were added until the arterial ring was relaxed to baseline tension. The concentration of these agents was not increased above 10^{-6}M. W represents removal of perfusing medium from tissue bath and wash.

DISCUSSION

Although previous attempts have been made to characterize the nature of the vasoactive peptides released from kininogen by acid proteases like pepsin (5), cathepsin D (4) and human neutrophil leukocyte protease (6), the present work concentrates on the physicochemical characterization of the peptide released by the action of a tumor acid protease on a highly purified rat plasma kininogen. The vasoactive peptide released by MSLS tumor acid protease is similar, but not identical to bradykinin, lysyl-bradykinin or methionyl-lysyl-bradykinin. This conclusion was drawn from its difference in size as well as electrophoretic mobility on high voltage electrophoresis. The amino acid composition of the vasoactive peptide differs from bradykinin in having an additional residue each of leucine, isoleucine and serine. The peptide released also differs from leukokinin released from leukokininogen by cathepsin D (3).

From the partial sequence of the peptide it appears that the peptide released is similar to bradykinin but has additional leucine residue at its carboxyl terminal and isoleucine and serine at its amino terminal. Both amino and carboxyl terminal residues conform to the specificity of carboxyl protease (17). This data also are consistant with tryptic digestion of the peptide which released two major fragments consisting of leucine and the remaining fragment respectively. The two minor fragments obtained may have resulted from the cleavage of Phe-Arg or Phe-Ser bonds by chymotrypsin contamination in the trypsin preparation used. Further, a five-fold increase in smooth muscle stimulating activity after tryptic digestion may be due to the removal of leucine leading to the generation of more active vasopeptide.

Based on indirect evidences previous investigators have shown that the kinin released from bovine kininogen by acid proteases is identical to methionyl lysyl-bradykinin (4-6); however, no attempt was made to isolate and further characterize it. The structural difference observed in the present study may relate to species difference since highly purified rat plasma kininogen was used. Subsequent to our preliminary communication (18), Kobayashi and Ohata (19) reported the isolation of four uterine-contractile substances from rat plasma kininogen incubated with a kinin-forming acid protease from rat stomach which were distinct from bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin.

Since the acid protease-derived kinin elicited vasoactive responses both on guinea pig anterior mesenteric vein and rabbit superior mesenteric artery, the peptide has been referred to as a vasoactive peptide. The vasopeptide showed no significant difference from that of bradykinin on the guinea pig mesenteric vein, but was more potent in relaxing the precontracted rabbit superior mesenteric artery. The difference in the response observed between the mesenteric vein and the artery is not clear. It has been proposed that the responses of bradykinin on guinea pig anterior mesenteric vein and the rabbit superior mesenteric artery both are mediated by B_2 receptors (20). The presence of two types of kinin receptors had been proposed earlier by

Wilkens and Back (21). Larger kinin peptides have been found to be more potent than bradykinin in increasing vascular permeability (22). Since the presence of captopril affected bradykinin and vasopeptide identically, the difference may not be attributed to the greater resistance of longer kinin derivative to inactivation by kininase II. Whether the observed difference in the response of vasopeptide on guinea pig mesenteric vein and rabbit mesetneric artery is due to the simultaneous presence of B_1 receptors in the latter cannot be answered and needs further study.

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