Volume 232, number 2, 395–398 FEB 05891 May 1988

Identification of [hydroxyproline^3]-lysyl-bradykinin released from human kininogens by human urinary kallikrein

Manfred Maier, Guenther Reissert, Ingrid Jerabek, F. Lottspeich* and Bernd R. Binder

Laboratory for Clinical and Experimental Physiology, Department of Medical Physiology, University of Vienna, Vienna, Austria and **Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG*

Received 30 March 1988

The types of kinins released from purified native, single chain human high and low molecular mass kininogens (HMMKs and LMMKs, respectively) by purified human urinary kallikrein were separated by reverse-phase HPLC and quantitated by the rat uterus bioassay. [Hyp^3]-lysyl-bradykinin, a recently discovered kinin, represented up to 58% of the biological activity released from 4 individual HMMK preparations purified from 4 different healthy volunteers. In contrast, the majority of the biological activity released from LMMKs purified from pooled plasma was identified as Lys-bradykinin and [Hyp^3]-lysyl-bradykinin represented only 6.4 ± 3.8%. These findings indicate posttranslational hydroxylation of human kininogens and suggest a preference of HMMKs for this modification.

Kininogen; Kallikrein; Kinin; [Hyp^3]-lys-bradykinin

1. INTRODUCTION

Kinins are a family of potent vasodilator peptides released by limited proteolysis from their plasma precursor proteins, high molecular mass and low molecular mass kininogens, by enzymes known as kallikreins [1]. It appears that lysyl-bradykinin is the major product released from both human high and low molecular mass kininogens by tissue kallikreins [2], while bradykinin is mainly generated from high molecular mass kininogens by plasma kallikrein [3].

In addition to these well known kinins, however, the presence of modified kinins in human urine [4] and in human ovarian carcinoma ascites [5] has been reported. Furthermore, we and others have isolated another kinin, [Hyp^3]-lysyl-bradykinin, which could be released from human heat inactivated plasma by human urinary kallikrein [6], from Cohn's fraction IV-4 by hog pancreatic kallikrein [6,7] and which could also be identified in ascitic fluid of the rat and mouse [6]. In these reports, the kinin peptides were generated from crude substrate sources. It was therefore the aim of the present study to use highly purified, native human kininogens for characterization of the kinins released by human urinary kallikrein.

Correspondence address: M. Maier, Laboratory for Clinical and Experimental Physiology, Department of Medical Physiology, University of Vienna, Schwarzspanierstr. 17, A-1090 Vienna, Austria

2. MATERIALS AND METHODS

2.1. *Preparation of kininogen substrates*

Low molecular mass kininogen (LMMK) was purified as a native single chain molecule from pooled human plasma [8] and individual human high molecular mass kininogens (HMMKs) were purified as native single chain molecules from the plasma of four single healthy donors [9]. The purity of the proteins was analyzed by SDS gel electrophoresis [10] and nativity was judged by comparing their individual specific activities obtained with the homologous enzyme, urinary kallikrein, with those obtained previously [1]. Each preparation of purified HMWK and LMMK contained 0.65–0.72 and 0.8–0.9 mol of kinin per mol of substrate, respectively.

2.2. *Incubation conditions and sample preparation*

Human urinary kallikrein (20 ng in 10 and 30 μl, respectively), purified from pooled human urine as described [1], was incubated with approx. 150 μg of the purified substrates (170 μl)

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

for 30 min at pH 8.5 and 37°C. Incubation was stopped by precipitation with 800 μl of solution B [11] which gives a final concentration of 64% acetonitrile. After centrifugation at 5000 × g for 15 min the supernatant was lyophilized, resuspended in 250 μl solution A [10] and filtered through a Centricon 10-filter (Amicon B.V., Oosterhout, The Netherlands). The ultrafiltrate was subjected to reverse-phase HPLC using a 250 μl sample loop. Control experiments were performed by incubation of standard kinins with the purified substrates in the absence of urinary kallikrein.

2.3. *Separation and identification of kinins by HPLC*

The HPLC system consisted of two 114M pumps with controller, a UV-detector model 160 (all from Beckman Instruments) and a Shimadzu CR-3A integrator. A micro-Bondapak C18 column (3.9 × 300 mm) with guard column was employed and elution was performed under isocratic conditions as described by Proud [11] with slight modifications. The flow rate was 1 ml/min and fractions of 0.5 ml were collected (Gilson fraction collector model 203). The respective retention time, biological activity, determined by the rat uterus bioassay [12], and immunoreactivity [13] of eluted kinins were determined and compared with those of the following standards: Lys-bradykinin, bradykinin, Met-Lys-bradykinin, Ile-Ser-bradykinin, des-Arg^9-bradykinin (Sigma, St. Louis, MO); des-Arg^1-bradykinin (Serva, Heidelberg, FRG) and [Ala^3]-Lys-bradykinin (Peninsula Laboratories, Belmont, CA). The distribution of released kinins was calculated by comparing the biological activity of a particular kinin eluted to the total amount of kinin activity eluted from the column.

2.4. *Determination of amino acid sequence*

Amino acid sequence analysis was performed in a gas-phase sequencer 470A (Applied Biosystems, Foster City, USA). The phenylthiohydantoin derivatives were analysed by an HPLC system which separates all components isocratically [14,15]. The phenylthiohydantoin derivative of 4-hydroxyproline which elutes as a characteristic double peak shortly before alanine was added to the reference mixture.

3. RESULTS

Compared to the elution pattern of standard kinins upon HPLC separation the majority of the biological activity released from purified LMMK corresponded to the position of Lys-bradykinin (table 1) and another peak of biological activity similar to that of the bradykinin standard. In addition, a small peak of biological activity which did not correspond to the retention time and biological activity of any of the standard kinins employed eluted in front of the Lys-bradykinin standard.

Using individual high molecular mass kininogens purified from four different plasma samples as substrate for purified human urinary kallikrein, the elution profile of the kinins released was similar (fig.1). However, this time the majority of the biological activity eluted faster than Lys-bradykinin and corresponded to the small unidentified peak observed when low molecular mass kininogen was the substrate. Compared to the retention times of all standard kinins employed (fig.1), this fast eluting biological activity again did not correspond to any of them. Table 1 summarizes the percentage of kinin distribution from four different individual donors from which HMMKs have been purified and compares them with the data obtained with LMMK purified from pooled plasma of multiple donors.

The fast eluting biological activity of individual experiments, which was also immunoreactive, was pooled and 150–350 ng (biological activity and immunoreactivity) were subjected to amino acid sequence analysis. The initial yield was between 65 and 75%, the repetitive yield amounted to about 95%. The amino acid sequence was similar to Lys-bradykinin (kallidin). However, in cycle 4 of the sequence degradation the characteristic double peak of hydroxyproline was found instead of pro-

Table 1

Percent distribution of biologically active kinins released by human urinary kallikrein

Substrate	HMMK				LMMK multiple
Donor	1	2	3	4	
[Hyp^3]-Lys-BK	55.8 ± 1.6	29.5 ± 7.2	42.8 ± 2.6	58.8	6.4 ± 3.8
Lys-BK	39.6 ± 5.1	60.7 ± 8.9	45.1 ± 2.6	39.6	64.0 ± 13.5
BK	4.1 ± 2.9	6.1 ± 1.4	11.7 ± 0.2	–	29.4 ± 16.3
Met-Lys-BK	–	3.3 ± 3.1	–	–	–

Biological activity of kinins released from high (HMMKs) and low molecular mass kininogens (LMMKs) and eluted from the HPLC column was measured by the rat uterus bioassay. Data are expressed as mean ± SD. BK, bradykinin

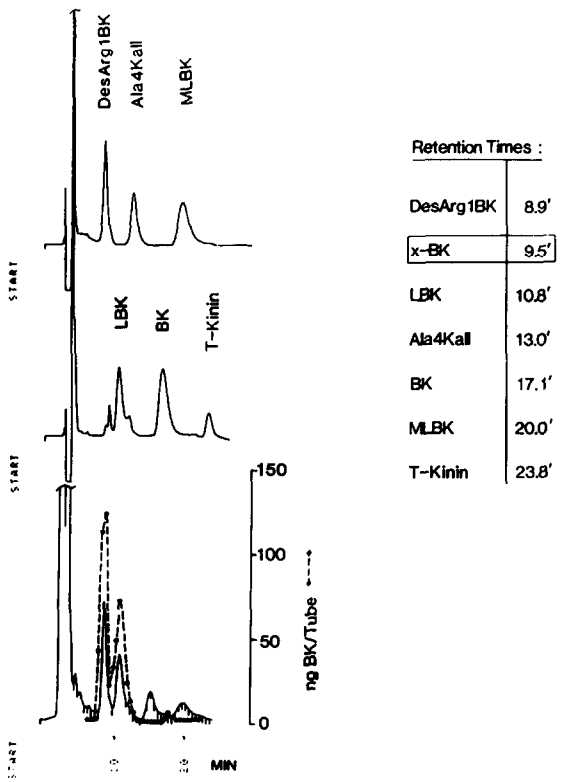

Fig.1. HPLC separation of kinins. Separation of standard kinins (top two panels) and kinins released from high molecular mass kininogen (donor 1) by urinary kallikrein (bottom panel) was achieved by reverse-phase HPLC and isocratic conditions. Fractions of 0.5 ml were collected and the biological activity of kinins determined by the rat uterus bioassay is expressed as ng of bradykinin equivalents per tube (●– –●). The retention times of the kinin standards are given for comparison. DesArg1BK, Des-Arg[1]-bradykinin; LBK, Lys-bradykinin; Ala4Kall, [Ala[4]]-kallidin or [Ala[3]]-Lys-bradykinin; BK, bradykinin; MLBK, Met-Lys-bradykinin; T-Kinin, Ile-Ser-bradykinin; and x-BK, [Hyp[3]]-Lys-bradykinin.

line. Thus, the sequence data, confirmed by FAB-mass spectrometry (not shown), revealed the structure to be Lys-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg.

Synthetic [Hyp[3]]-lysyl-bradykinin was obtained from the Max Planck Institute for Biochemistry (Martinsried, FRG). The synthetic peptide, whose structure was confirmed by amino acid sequence analysis and FAB-mass spectrometry (not shown), had an identical retention time upon HPLC separation to that of the fast eluting activity released from the purified substrates. On a molar basis, both the native and synthetic [Hyp[3]]-lysyl-bradykinin contracted the rat uterus in a similar way to lysyl-bradykinin and exhibited a similar immunoreactivity.

4. DISCUSSION

This communication reports for the first time on the release of [Hyp[3]]-Lys-bradykinin from purified human kininogens by homologous urinary kallikrein. Our findings support and extend those presented by others and ourselves at a meeting [6] and published very recently [7]. However, in these studies a crude substrate source was used. Purified kininogens also permitting the differentiation between high and low molecular mass kininogens, and individual plasma donors for their preparation have not been employed.

The common kinin moiety in human kininogens has the amino acid sequence Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Lys-bradykinin or kallidin). The finding of hydroxyproline in position 3 of the nonapeptide bradykinin does, therefore, not contradict the established sequence nor does it indicate genetic variation, since hydroxylation of proline is an intracellular posttranslational event which requires a specific hydroxylase and ferrous ions, molecular oxygen, α-ketoglutarate and ascorbic acid as cofactors [16]. Hydroxylation of the proline next to glycine in the bradykinin sequence (Pro-Pro-Gly) within kininogens suggests that the specific enzyme is similar or identical to prolyl-4- hydroxylase [16] acting upon kininogens as substrates. In fact, both bradykinin [17] and lysyl-bradykinin [18] can serve as substrates for prolyl hydroxylase in vitro, whereby hydroxylation is limited to only one of the three prolines in these molecules, the one in position 3.

Upon incubation with purified, native single chain human low molecular mass kininogen, human urinary kallikrein releases primarily Lys-bradykinin (table 1). However, [Hyp[3]]-Lys-bradykinin and bradykinin are released from this substrate preparation as well. In contrast, upon incubation with individual native single chain human high molecular mass kininogens obtained from four different donors, urinary kallikrein releases considerably more [Hyp[3]]-Lys-bradykinin, less Lys-bradykinin and a small amount of brady-

kinin (fig.1, table 1). In each single preparation of high molecular mass kininogen the biological activity and immunoreactivity of [Hyp^3]-Lys-bradykinin represented more than 25% of the total kinins released. The observed difference in [Hyp^3]-Lys-bradykinin content between high molecular mass kininogen and low molecular mass kininogen is unclear and can only be speculated upon. As deduced from the biosynthesis of collagen, the residue hydroxylated must be located in a peptide with a nonhelical conformation and long peptide substrates are more readily hydroxylated than are short peptides [16]. It is, therefore, interesting that high molecular mass kininogen is nearly double the size of low molecular mass kininogen due to its larger carboxy-terminal light chain and may possess multiple domains, each forming a loop structure [19]. However, since human low molecular mass kininogen has been purified from a pool of human plasma the amount of [proline^3]-hydroxylation might also depend on the individual donors contributing to the plasma pool.

The physiological significance of posttranslational hydroxylation of human kininogens is unknown. However, since the amount of hydroxyproline acquired into the procollagen chains is required for formation of the typical triple helix structure of functional collagen [16], hydroxylation of proline in kininogen precursors might similarly influence the conformation and function of the secreted proteins. in this context, it could be speculated that such a hydroxylation might also influence the versatile functions of kininogens in serving as a cofactor in contact activation of the Hageman factor-dependent pathways [20] or as thiol protease inhibitors [21,22] in addition to being the kinin precursors.

Acknowledgements: This work was supported in part by the Fund for the Promotion of Scientific Research, Project no. P6357. The authors thank Dr W. Schaefer from the Max Planck Institute for performing FAB-mass spectrometry and Dr R. Mertz for solid-phase synthesis of [Hyp^3]-Lys-bradykinin. The excellent secretarial assistance of Mrs H. Hitschmann is thankfully acknowledged.

REFERENCES

[1] Maier, M., Austen, K.F. and Spragg, J. (1983) Proc. Natl. Acad. Sci. USA 80, 3928-3932.
[2] Fiedler, F. (1979) in: Bradykinin, Kallidin and Kallikrein, Supplement (Erdoes, E.G. ed.) pp. 103-161, Springer, Berlin.
[3] Nakayasu, T. and Nagasama, S. (1979) J. Biochem. 85, 249-258.
[4] Mindroiu, T., Scicli, G., Perini, F., Carretero, O.A. and Scicli, A.G. (1986) J. Biol. Chem. 261, 7407-7411.
[5] Wunderer, G., Walter, I., Mueller, E. and Henschen, A. (1986) Biol. Chem. Hoppe-Seyler 367, 1231-1234.
[6] Presented independently by Maier et al., Sasaguri et al. and Matsumura et al. at the International Congress 'Kinin 1987-Tokyo' on Nov. 29-Dec. 3, 1987 and published in the abstract book of this meeting.
[7] Sasaguri, M., Ikeda, M., Ideishi, M. and Arakawa, K. (1988) Biochem. Biophys. Res. Commun. 150, 511-516.
[8] Maier, M., Austen, K.F. and Spragg, J. (1983) Anal. Biochem. 134, 336-346.
[9] Higashiyama, S., Ishiguro, H., Kunimatsu, M., Sawaki, K. and Sasaki, M. (1986) Biochemistry 25, 1669-1675.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
[11] Proud, D., Togias, A., Naclerio, R.M., Crush, S.A., Norman, P.A. and Lichtenstein, L.M. (1983) J. Clin. Invest. 72, 1678-1685.
[12] Orange, R.P. and Austen, K.F. (1976) in: Methods in Immunology and Immunochemistry (Williams, C.A. and Chase, M.W. eds) vol. 5, pp. 145-149, Academic Press, New York.
[13] Scicli, A.G., Mindroiu, T., Scicli, G. and Carretero, O.A. (1982) J. Lab. Clin. Med. 100, 81-93.
[14] Lottspeich, F. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1829-1834.
[15] Lottspeich, F. (1985) J. Chromatogr. 326, 321-324.
[16] Prockop, D.J., Kivirikko, K.I., Tuderman, L. and Guzman, N.A. (1979) N. Engl. J. Med. 301, 13-23.
[17] Rhoads, E. and Udenfriend, S. (1969) Arch. Biochem. Biophys. 133, 108-111.
[18] McGee, J.O., Rhoads, R.E. and Udenfriend, S. (1971) Arch. Biochem. Biophys. 144, 343-351.
[19] Takagaki, Y., Kitamura, N. and Nakanishi, S. (1985) J. Biol. Chem. 260, 8601-8609.
[20] Wiggins, R.C., Bouma, B.N., Cochrane, C.G. and Griffin, J.H. (1977) Proc. Natl. Acad. Sci. USA 24, 4636-4640.
[21] Ohkubo, I., Kurachi, K., Takasawa, T., Shiokawa, H. and Sasaki, M. (1984) Biochem. 23, 5691-5697.
[22] Mueller-Esterl, W., Fritz, H., Machleidt, W., Ritonja, A., Brzin, J., Kotnik, M., Turk, V., Kellermann, J. and Lottspeich, F. (1985) FEBS Lett. 182, 310-314.