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Rat tissue kallikrein releases a kallidin-like peptide from rat low-molecular-weight kininogen

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1 The kallikrein–kinin system is subdivided into the plasma and tissue–kallikrein–kinin system, with bradykinin (BK) and kallidin (KAL) (Lys^0-bradykinin) as functional peptides. This occurs in both humans and other mammals. Both peptides are released by plasma and tissue–kallikrein. BK, but not KAL, has been detected in rats until now. One can explain this observation by the structural differences found in the sequence of rat high- and low-molecular kininogen containing an Arg-residue instead of a Lys-residue in front of the N-terminus of the BK sequence. Nevertheless, we were able to measure a kallidin-like peptide (KLP), in rat plasma and urine, using a specific KAL antiserum.

2 In order to confirm our data, we isolated low-molecular-weight kininogen from rat plasma and incubated it with purified rat glandular kallikrein. The generated peptide was retained on a high-pressure liquid chromatography column and displaced by an excess of angiotensin I. The KLP-containing fraction was identified with the KLP radioimmunoassay. A specific ion signal with a mass to charge ratio (m/z) of 1216.73 was detected with matrix-assited laser desorption/ionization mass spectrometry.

3 As proposed earlier, the structure of this peptide is Arg^1-KAL, instead of Lys^1-KAL. The structural similarity between the Lys- and the Arg-residue explains the high crossreactivity (80%) of KLP with the specific KAL antibody.

4 The incubation of KLP with angiotensin-converting enzyme yields two molecules with masses of 913.4 and 729.3 containing the sequence H–Arg–Arg–Pro–Pro–Gly–Phe–Ser–Pro–OH and H–Arg–Arg–Pro–Pro–Gly–Phe–OH. The enzymatic cleavage could be inhibited by captopril.

5 The data suggest that in rats, as in other mammals, the tissue kallikrein–kinin system mediates its physiological effects *via* a kallidin-like peptide, which is Arg^1-kallidin (Arg^0-bradykinin).
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Abbreviations: ACE, angiotensin-converting enzyme; ACTH, adrenocorticotropic hormone; Ang I, angiotensin I; Arg, arginine; BK, bradykinin; HMW, high molecular weight; HPLC, high-pressure liquid chromatography; Hyp, hydroxyproline; KAL, kallidin; KKS, kallikrein–kinin system; KLP, kallidin-like peptide; LMW, low molecular weight; Lys, lysine; MALDI, matrix-assisted laser desorption/ionization; p, plasma; RIA, radioimmunoassay; t, tissue; Tyr, tyrosine

Introduction

For functional and biochemical reasons, the kallikrein–kinin system (KKS) is divided into a plasma (p)- and tissue (glandular) (t)-KKS. Both systems are involved in different physiological functions. While p-KKS *via* bradykinin (BK) contributes to blood pressure regulation and to blood coagulation, the t-KKS seems to be of major importance for most peripheral and central kinin action *via* kallidin (KAL). However, there are no reliable data concerning the contribution of BK and KAL to various physiological effects. Technical problems in measurement of kinins resulted in major confusion. Commercially available BK assays apply antibodies, which cannot distinguish between BK and KAL, because they are directed against the identical C-terminus of both peptides (Uchida *et al*., 1986). In addition, antibodies may vary in their specificity and selectivity (Bönner *et al*., 1987). Moreover, there is a rapid degradation of kinins in biological fluids (Pallacani *et al*., 1992), which makes the purification procedure of kinins too complex (Duncan *et al*., 2000). In order to overcome these problems, we have recently developed a sensitive and specific radioimmunoassay (RIA) for BK and for KAL (Hilgenfeldt *et al*., 1995). Structural differences between human and rat kininogen (Figure 1) indicate that KAL (Lys^1-KAL = Lys^0-BK) cannot be generated in rats. Therefore, it was generally accepted that only BK had been found in the urine, blood and tissue of rats (Alhenc-Gelas *et al*., 1981; Campbell *et al*., 1993). Moreover, after enzymatic degradation of rat high-molecular-weight (HMW)

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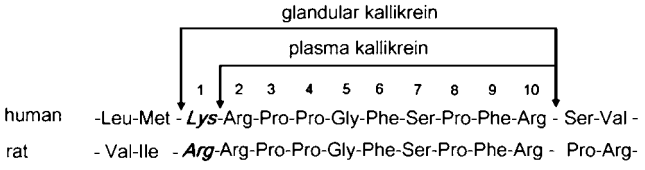

Figure 1 Sequence differences between human and rat kininogen and the cleavage sites of plasma and tissue kallikrein. Due to the structural differences in the N-terminal region of rat kininogen, bradykinin was believed to be the only kinin released from HMW and LMW kininogen by plasma- and tissue (glandular)-kallikrein.

kininogen by rat glandular kallikrein, only BK was found (Kato *et al*., 1985). Also, only BK was found by HPLC and amino-acid analysis of rat urine (Hagiwara *et al*., 1995).

Despite the absence of KAL in rats, we were able to measure a kallidin-like peptide (KLP) in the plasma and urine of rats with the specific RIA for kallidin. Recently, we showed that a KLP is released from rat muscle in correlation with muscle activity (Boix *et al*., 2002).

Therefore, the aim of this paper is to demonstrate the existence of KLP in rat plasma and urine, to show its generation from rat LMW kininogen by rat glandular kallikrein, and to identify its molecular mass and amino-acid sequence.

Methods

Animals

We performed all animal experiments in accordance with the FELASA guidelines for animal experimentation. Male SPF Sprague–Dawley (SD) rats (breeder: Charles River, Sulzfeld, Germany), weighing 300 g, were used for the study. We housed the animals in the Animal Housing Facilities of the Theoretikum of the University of Heidelberg.

Materials

We purchased peptides from Bachem, Heidelberg, Germany. Arg^1-KAL (KLP) was synthesized by Professor N. Metzler-Nolte, IPMB, Heidelberg, Germany. We purchased Arg^1-Tyr^6-KAL (Tyr^6-KLP) from PolyPeptide Laboratories, Wolfenbüttel, Germany. We used insoluble agarose-linked trypsin for further enzymatic experiments (Sigma, Munich, Germany). We purchased ACE from Sigma-Aldrich, Schnelldorf, Germany. CNBr-Sepharose and CM-Sephadex C50 columns were purchased from Amersham Biosciences (Freiburg, Germany).

Separation of KLP from rat urine

We housed rats in metabolic cages for 24 h. The urine was collected in ice-cooled tubes. Subsequently, the urine was centrifuged and the supernatant applied onto a CM-Sephadex column, 5×40 cm, in ammonium bicarbonate buffer, 0.02 M, pH 9.0, at a flow rate of 1 ml min^{-1}.

Isolation of rat LMW kininogen

We collected blood from the carotid artery of SD rats that had been anesthetized with pentobarbital, 60 mg kg^{-1}, containing 1500 IE heparin kg^{-1}. Subsequently, the blood was centrifuged at $3000 \times g$ for 15 min at 4°C. LMW kininogen was prepared according to the method of Uchida & Katori (1979). In short: plasma, 32 ml, was incubated with kaolin suspension, 7.8 mg $\times$ 32 ml^{-1} and 288 ml Tris-HCl, 0.06 M, pH 7.4 for 30 min at 37°C. Subsequently, 16 ml HCl, 1 M, was added, and incubated for 15 min at 37°C. Thereafter, the solution was neutralized with 16 ml 1 M NaOH and clarified by centrifugation, 10 min, at $6000 \times g$. By this procedure most plasma proteins were removed, including HMW kininogen and BK. The supernatant was stored at −20°C. The amount of LMW kininogen was determined by measuring the amount of BK, released by incubation of the solution with an excess of trypsin (2 mg ml^{-1}).

Purification of rat tissue kallikrein from rat submaxillary glands

We homogenized rat submaxillary glands in Tris buffer, pH 8.4, 0.2 M, in an ice bath. The homogenate was centrifuged at $12{,}000 \times g$ for 30 min. The supernatant was separated on an aprotinin CNBr-Sepharose affinity column. Bound kallikrein was eluted with glycine, 0.1 M, pH 5.0. We measured glandular kallikrein amidolytically with the chromogenic substrate S-2266 (D–V–L–R–*p*-nitroanilide) (Haemochrom Diagnostica GmbH, Essen, Germany) on a 96-well microplate in a spectrophotometer (Molecular Devices, Spectra-max 250). The kallikrein-containing fractions were pooled, dialyzed in NH_4HCO_3 buffer, 20 mM, pH 7.4, and lyophilized. The specific amidolytic activity was 0.13 U mg^{-1} protein.

Kinin release from rat LMW kininogen by rat glandular kallikrein

We dissolved lyophilized rat kininogen, 1.0 mg, in Tris-buffer, 200 μl, 60 mM, pH 7.8, containing *o*-phenanthroline, 0.6 g l^{-1}. The solution was digested in Teflon vials with rat glandular kallikrein, 2 mU $\times$ 20 μl^{-1}, at 37°C for 60 min. Subsequently, the sample was incubated at 70°C for 10 min. The reaction was stopped with ethanol, 70% and incubated for 10 more minutes at 70°C. After centrifugation ($13{,}000 \times g$, 10 min) the supernatant was reduced under vacuum in a rotary evaporator, and stored at −20°C.

We performed KLP RIA using the specific KAL antiserum (Hilgenfeldt *et al*., 1995), ^{125}I-Arg^1-Tyr^6-KAL as tracer molecule, and an Arg^1-KAL standard curve ranging from 5 to 1280 pg per vial.

We performed the BK RIA using the specific BK antiserum (Hilgenfeldt *et al*., 1995), ^{125}I-Tyr^8-BK as tracer molecule and BK standard curve ranging from 0.5 to 128 pg per vial.

Separation of KLP on HPLC was performed on a reversed-phase C12 column (Jupiter 4 μ Proteo 90 Å column, 250×4.6 mm, Phenomenex, Hösbach, Germany) and a HPLC system (Latek, Heidelberg, S1990 pump, S5200 sample injector, S7510 vacuum degasser, UVIS 200 spectrophotometer) in an acetonitrile-H_2O gradient, containing trifluoroacetic acid, 0.05%, with the following profile: *t*0–10 min: 10% acetonitrile, *t*10–30 min: 10–50% acetonitrile, *t*30–40 min: 50% acetonitrile, *t*40–50 min: 50–10% acetonitrile, *t*50–60 min: 10% acetonitrile. The volume of the samples separated on HPLC was 100 μl. The volume of the fractions (1 ml) was reduced to 100 μl and analyzed by the KLP RIA. The fraction containing

KLP activity was lyophilized and dissolved in 3 μl 0.1% trifluoroacetic acid. For mass spectrometric analysis, 0.5 μl of sample and 0.5 μl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA, 33% acetonitrile were cocrystallized directly on the MALDI target plate.

We recorded MALDI mass spectra in the positive ion mode with delayed extraction on a Reflex II (Bruker-Daltonik, Bremen) time-of-flight instrument equipped with a SCOUT-26 inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 26.5 kV, the reflector voltage to 30.0 kV and the first extraction plate to 20.6 kV. Mass spectra were obtained by accumulation of 50–200 individual laser shots. Calibration of the spectra was performed externally by a two-point linear fit using angiotensin I at m/z 1296.69 and the oxidized B-chain of bovine insulin at m/z 3494.65.

We performed post-source decay (PSD) analysis in the positive ion reflector mode with delayed extraction by setting an ion gate width of 40 Da around the ion of interest. Data were acquired in 14 segments by decreasing the reflector voltage in a stepwise fashion. For each segment, 100–200 individual laser shots were accumulated. The fragment ion spectrum was obtained by pasting together all segments to a single spectrum using the FAST software provided by Bruker. Fragment ion calibration was performed externally with the fragment masses of the ACTH (18–39 clip).

Cleavage of KLP by ACE

We dissolved KLP, 10 μg in Tris-buffer, 300 μl, 100 mM, pH 7.4, containing gelafundine, 0.1% at 0°C. In all, 3 μl of ACE and 6.3 mU were added. In parallel, the same fraction containing in addition Captopril, 3 μl, 1 mM, was prepared. Both fractions were incubated for 20 min at 37°C in Teflon vials. Thereafter ethanol, 700 μl, was added to stop the enzymatic reaction and, subsequently, both samples were centrifuged at 6000 $\times$ g for 5 min. The supernatant was reduced under vacuum to a volume of 200 μl. We analyzed the generated peptides of both fractions by MALDI.

Results

In rat urine the concentration of KLP vs BK is approximately 10 times higher (KLP: 4.71 ng ml^{-1} vs BK: 0.523 ng ml^{-1}, $n = 10$) when measured with the specific RIA for KLP and BK, respectively. In order to exclude nonspecific binding of the KAL antiserum, we separated rat urine on HPLC in a linear acetonitrile gradient. The fractions were analyzed by the KLP-, and BK-RIA, respectively. Figure 2a displays the spectrum at 215 nm. Figure 2b depicts the KLP and BK levels of the HPLC fractions. Only one KLP peak with a retention time of 21 min was detected. BK eluted with a retention time of 25 min. Other kinin peaks, like Hyp^3-BK or Hyp^3-KLP, which had been described in humans (Duncan *et al*., 2000), were not found. After HPLC of rat urine, the ratio of KLP vs BK, however, was reduced by a factor of 3.

We separated urinary KLP on a CM-Sephadex column in an ammonium-bicarbonate buffer, pH 9.0. The elution profile displays a KLP peak in front, outside the main urinary peak (Figure 3). A BK peak containing a 100-times-lower concentration colocalized with the KLP peak. However, the BK-RIA

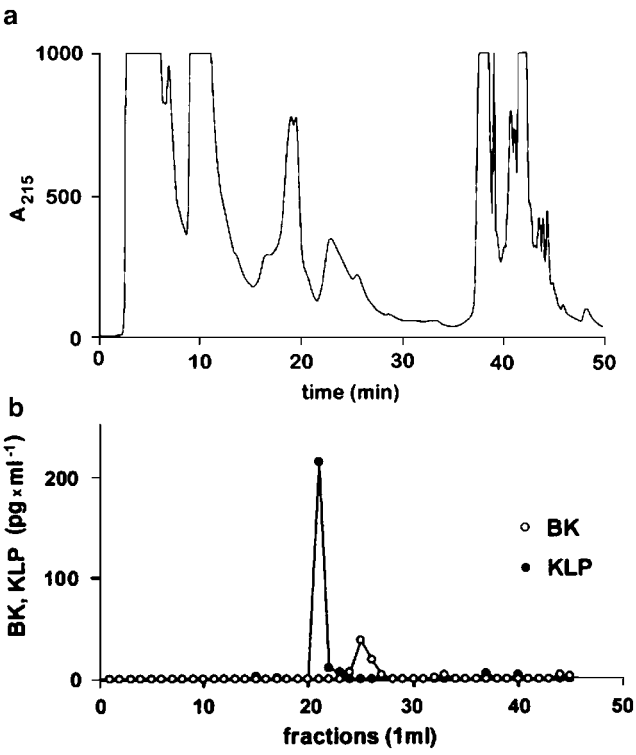

Figure 2 (a) Chromatography of rat urine on a reversed-phase C12 column by HPLC in a linear gradient, 10–50% acetonitrile in H_2O, at 10–30 min. (b) Measurement of BK and KLP in fractions obtained after chromatography of rat urine by HPLC with a RIA for BK (open circles) and KAL (solid circles). Note that both KLP and BK can each be detected in one single fraction (21 and 25).

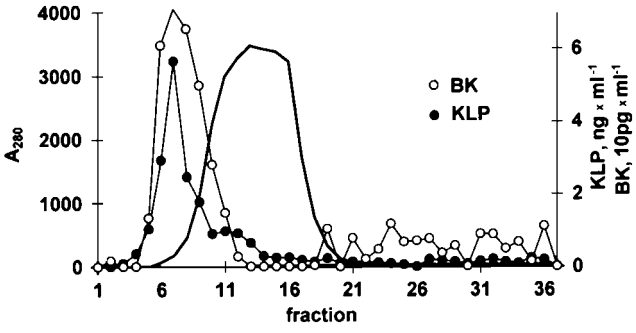

Figure 3 Chromatography of rat urine on a CM-Sephadex ion exchange column in an ammonium bicarbonate buffer, pH 9.0. The solid line depicts the absorption at 280 nm, the closed circles display the amount of KLP measured in the fractions. The open circles depict the amount of BK as measured with the BK RIA. Note that the amount of BK is by a factor of 100 smaller than the amount of KLP (right axis). This suggests that BK peak is primarily caused by the crossreactivity of KLP with the BK antiserum, which is in the order of 2–4%.

detected mainly KLP, which crossreacts with the BK antiserum by 2–4%.

We lyophilized the fraction containing the highest KLP concentration and separated it by HPLC. However, active KLP could not be measured in the effluent (data not shown).

In plasma, following EtOH precipitation, BK levels of 39.8 pg ml^{-1} and KLP levels of 8.58 pg ml^{-1} have been found.

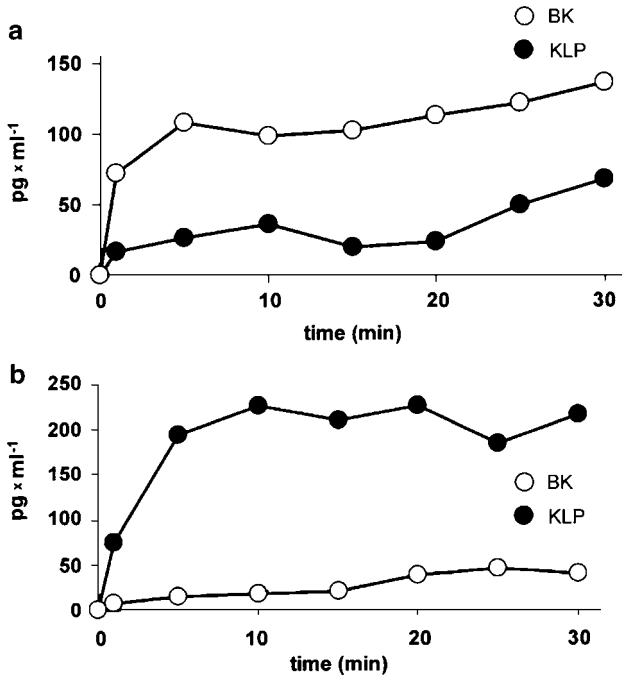

Figure 4 Enzymatic release of KLP (solid circles) and BK (open circles) from rat plasma LMW kininogen, 5 mg ml^{-1} in the presence of trypsin, 2 mU ml^{-1} (a), and from LMW kininogen, 8 mg ml^{-1} in presence of glandular kallikrein, 8 mU ml^{-1} (b) is shown. The reaction was stopped by adding ethanol, final concentration 70%. The samples were centrifuged and evaporated prior to the RIA.

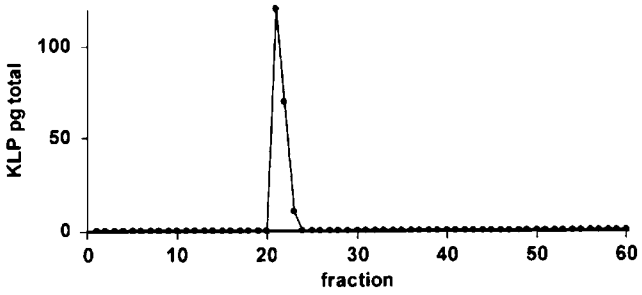

Figure 5 Measurement of KLP in fractions obtained after displacement of bound KLP from the reversed-phase C12 column with an excess of angiotensin I ($1\,\mu g \times 100\,\mu l^{-1}$). After enzymatic degradation of LMW kininogen with rat glandular kallikrein, four portions of 100 μl each were separated on the HPLC column with the acetonitrile gradient and measured with an RIA for KAL.

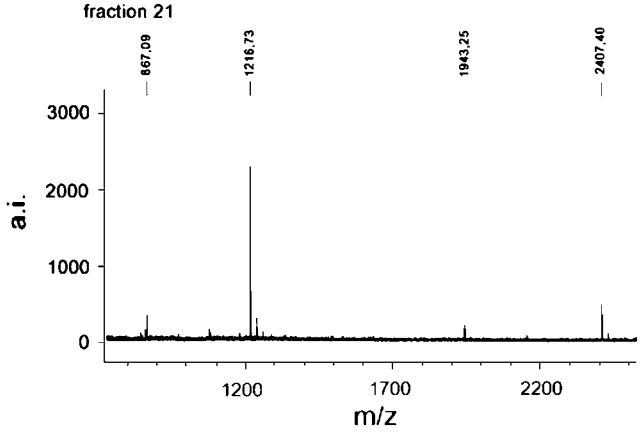

Figure 6 MALDI-TOF mass spectrum of fraction 21 from HPLC. The most intense ion signal at m/z 1216.73 corresponds to KLP.

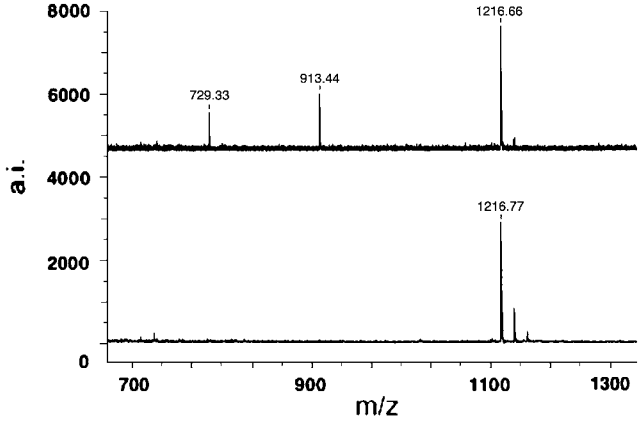

Figure 7 MALDI-TOF mass spectrum of the peptides generated from KLP by ACE (upper panel) and by ACE and the ACE inhibitor captopril (lower panel).

In order to document the formation of KLP in rat plasma, partially purified rat LMW kininogen was incubated with rat glandular kallikrein in the presence of *o*-phenanthroline. In a parallel series, LMW kininogen was incubated with trypsin. The samples were analyzed with the BK- and KAL-RIA. Incubation of LMW kininogen with trypsin generated BK (open circles) vs KLP (full circles) at a ratio of approximately 5 : 1 (Figure 4a). Incubation of LMW kininogen with rat glandular kallikrein generated KLP (full circles) vs BK (open circles) at a ratio of approximately 10 : 1 (Figure 4b).

The fraction obtained by incubation of LMW kininogen with rat glandular kallikrein was separated on HPLC. However, the fractions of the effluent did not contain measurable amounts of KLP. This result was similar to that obtained by the separation of purified urine on HPLC. Even four times running subsequent separations did not yield active KLP fractions in the effluent. This raised the possibility that KLP might stay bound on the column. Thus, AngI was applied to the column to displace KLP from the matrix. The collected fractions were concentrated and analyzed with the KLP RIA (Figure 5). Fraction 21 containing the highest KLP immunoreactivity was examined by MALDI mass spectrometry. As shown in Figure 6, a distinct ion signal at m/z 1216.73 was detected, which is in accordance with the calculated monoisotopic mass of KLP. Sequence analysis by MALDI–PSD MS confirmed the following proposed sequence of KLP: H–Arg–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH.

Incubation of KLP with ACE yields two smaller peptides with molecular masses 913.4 and 729.3 Da, and with the amino-acid sequence H–Arg–Arg–Pro–Pro–Gly–Phe–Ser–Pro–OH and H–Arg–Arg–Pro–Pro–Gly–Phe–OH (Figure 7, upper panel). This degradation could be abolished in presence of the ACE inhibitor captropril (Figure 7, lower panel).

Discussion

In humans, as in most other mammals, the KKS is subdivided into p- and t-KKS. Both systems are involved in different physiological functions (for a review, see Bhoola *et al*., 1992). The action of both systems is mediated by at least four main biochemically different kinins. BK is separated from plasma HMW kininogen by plasma kallikrein (EC 3.4.21.34). It acts on the constitutively expressed B_2-receptor, as well as KAL, which is released from LMW kininogen by tissue kallikrein (EC 3.4.21.35). Carboxypeptidase (kininase I) generates des-Arg^{10}-KAL and des-Arg^{9}-BK, which bind to the inducible B_1-receptor. The affinity of both kinins on this inducible receptor, however, is substantially different in humans: des-Arg^{10}-KAL > des-Arg^{9}-BK = KAL > BK (Regoli *et al*., 1998).

In rat B_1-receptor, the K_i for des-Arg^{10}-KAL is 1.6 nM vs des-Arg^{9}-BK, which is 15 nM (Jones *et al*., 1999).

Although there is evidence for a clear distinction of both systems concerning their regulation and physiological properties (Hilgenfeldt *et al*., 1998), it is a general practice to address most kinin actions to bradykinin and to use the term 'kinin action'. Part of this confusion has been caused by technical problems in measuring BK and KAL independently. To the best of our knowledge, there is no commercially available kinin antiserum that is able to distinguish between KAL and BK, as KAL shares an identical amino-acid sequence with BK and contains an additional Lys-residue at the N-terminus. Kato *et al*. (1985) found differences in the N-terminus of the kinin sequence of rat kininogen compared to human and bovine kininogen (Figure 1) (Arg vs Lys in position 0). Thus, it became clear that KAL (Lys^{1}-KAL = Lys^{0}-BK) is not present in rat HMW and LMW kininogen. Although Arg^{0}-BK (Arg^{1}-KAL) had been mentioned in their study, they were unable to confirm its presence. They concluded that BK is the only kinin released from rat HMW kininogen by both rat plasma- and glandular-kallikrein. These findings have been supported by others, which were unable to detect KAL or KLP in rat plasma and urine (Alhenc-Gelas *et al*., 1981; Campbell *et al*., 1993; Hagiwara *et al*., 1995). The release and characterization of the kinin generated from LMW kininogen by cleavage of rat glandular kallikrein have not been performed.

Contrary to this general notion, we were able to detect a KLP in rat urine, as well as in plasma. Moreover, we were able to separate the proposed KLP from LMW kininogen by rat glandular kallikrein. In a recent paper, KLP could be detected in rat muscle tissue (Boix *et al*, 2002). These findings have been obtained applying a highly specific and selective antiserum for KAL. KAL contains three basic amino acids, which are located at both ends. Three Pro-residues in the sequence of KAL favor a ring-like structure and the formation of a basic cluster. This structural feature enables the antiserum a simultaneous binding of both free ends, confirming the molecular integrity of KAL (Hilgenfeldt *et al*., 1995). This antiserum displays only a very low crossreactivity with BK (<0.1%). Given this high selectivity, we supposed that we were measuring a kallidin equivalent, which should be Arg^{1}-KAL. Synthetic Arg^{1}-KAL showed a crossreactivity of 80% with the KAL antiserum. Structural similarity between KAL and the proposed KLP may then explain this crossreactivity. The only difference between KAL and KLP is an N-terminal Lys- vs an N-terminal Arg-residue. Both are basic amino acids, which are similar in length and charge.

The attempts of the isolation of KLP, the determination of its molecular mass and its amino-acid sequence were started when we measured this peptide for the first time in 1995. Major difficulties arose for two reasons: instability and high polarity. KLP is rapidly degraded by various enzymes. Even in the presence of inhibitor cocktails (Nussberger *et al*., 1998), KLP was lost (data not shown). In a recent study, Duncan *et al*. (2000) collected blood in guanidine thiocyanate and 1% trifluoroacetic acid to prevent BK generation and degradation. In our study, the KLP degradation could be inhibited by ethanol precipitation.

The second more severe problem is caused by the physicochemical characteristic of KLP. As already mentioned, KLP, like KAL, is a basic peptide with the strong ability to bind to any charged surface or molecule. This feature causes a rapid decline of detectable amounts of KLP, even when an enzymatic degradation could be excluded, like after ethanol precipitation (data not shown). KLP disappeared after freezing, because it had been linked to lipids, which became insoluble by freezing and thawing. The high affinity of basic peptides to phospholipids was confirmed in short synthetic peptides (Abruzova *et al*., 2000). In order to minimize or avoid this binding, we used at first siliconized metabolic cages, siliconized plastic and siliconized glassware. This reduced the loss of KLP during purification. Then, Teflon vials were used for preparation and analytical methods.

The most intriguing challenge was linked with the observation that the recovery of KLP on HPLC declined with increasing purity. At least KLP was completely lost when fractions with a KLP content of 5% or higher were separated on HPLC. The isolation of measurable amounts of KLP in crude urine samples on HPLC can be explained by displacement of KLP from the column matrix by an excess of other urinary peptides.

Thus, the final separation step on HPLC was achieved by saturating the column matrix with KLP and the displacement of KLP from the matrix by applying an excess of AngI. This point is remarkable, because the use of HPLC was demanded prior to the analysis of different kinins in blood and urine. As long as no selective antisera are available, HPLC is thought to enable the precise identification and quantitative analysis of single kinin peptides and their metabolites (Nussberger *et al*., 1998; Duncan *et al*., 2000). Using a HPLC-based RIA, Duncan *et al*. (2000) reported KAL levels of 0.7 fmol ml^{-1} in humans. This is approximately 100 times less than the KAL levels we found in a study without HPLC (Hilgenfeldt *et al*., 1995). Although these authors found more abundant KAL than BK peptides in human urine, the KAL levels in blood were often near or below the detection limit. In a proceeding paper (Liu *et al*., 2005), we investigate the release of KLP from isolated rat hearts.

The data suggest that KLP, rather than BK, is involved in the cardioprotective action of ischemic preconditioning, which could be abolished by the KAL antiserum and by the B_2-receptor antagonist Icatibant. In spite of the outstanding physiological and pathophysiological importance of KLP, the low interest in analyzing KLP may be due to its poor recovery. Considering our data, it can be assumed that the methods applied for kinin analysis require a substantial revision, which is currently under investigation in our laboratory. The physicochemical similarity between KAL and KLP may be a likely explanation that KLP has not yet been found by others.

In this context, there is a biochemical similarity concerning the degradation by ACE. It could be shown that KLP is digested by ACE to KLP (1–8) and KLP (1–6), which seems to be the smallest KLP fragment generated by ACE. This enzymatic degradation could completely be blocked by the ACE inhibitor captopril.

In conclusion, this is the first report documenting the generation of a kallidin equivalent, KLP, in rats with the sequence: H–Arg–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH and the mass of 1216.73 Da, which has been verified by MALDI mass spectrometry. Due to a basic cluster, which is formed by three Arg-residues, KLP has the property to bind to any negatively charged residue. The isolation was obtained on a HPLC column after displacement of bound KLP with an excess of angiotensin I. KLP, like KAL, is cleaved by ACE to KLP (1–8) and KLP (1–6).

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