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CONVERSION OF T-KININ TO BRADYKININ BY THE RAT KIDNEY

MARIA A. R. VIEIRA,*† MÔNICA F. MOREIRA,‡ THOMAS MAACK§ and JORGE A. GUIMARÃES‡

*Department of Physiology and Biophysics, ICB, Universidade Federal de Minas Gerais, MG, Brazil; ‡Department of Biochemistry, ICB, Universidade Federal do Rio de Janeiro, RJ, Brazil; and §Department of Physiology, Cornell University Medical College, New York, NY, U.S.A.

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Abstract—Isolated rat kidneys were perfused with T-kinin (TK, Ile-Ser-BK) and bradykinin (BK). HPLC analysis of perfusate samples taken at 2–10 min during the TK perfusion (0.5 nmol/mL initial concentration) showed two peptide peaks, the first one eluting at 14.42 min, the same retention time for standard BK, and the second at 16.20 min, corresponding to that of TK. When BK (0.5 nmol/mL) was perfused, only its corresponding peak was obtained although total BK recovery was reduced quickly, as expected. Using both HPLC analysis and a kinin bioassay on the isolated guinea pig ileum, it was found that 12% of the added TK was converted to BK during the first perfusion cycle (2 min). While the BK recovered (12–14% from the initial TK concentration) was maintained at a similar proportion between the 2nd and the 10th min of perfusion, the rate of TK disappearance, as well as its full recovery from the perfusate, indicated further fragmentation of peptides during kinin perfusion. In the presence of 5 μM DL-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (Mergetpa), an inhibitor of plasma carboxypeptidase N (EC 3.4.17.3), the rate of conversion of TK to BK was not affected. On the other hand, the kininase II inhibitor bradykinin potentiating peptide 9a (BPP_{9a}) increased both the proportion of TK converted to BK and the disappearance rate of TK from the perfusate. In the presence of BPP_{9a}, the rate of BK production increased from 1.5 ± 0.2 to 7.6 ± 0.9 nmol/min. Furthermore, the recovery of BK was reduced during the first 2 min of perfusion to 7.6% and the conversion rate to 0.9 nmol/min when TK was perfused into the kidney in the presence of 10 μM bestatin, a known inhibitor of aminopeptidases. These data indicate that in the kidney TK is converted to BK, probably by aminopeptidase M, thus suggesting that BK is, in fact, an additional and functional kinin, inducing physiological and/or pathophysiological effects in the rat kidney in which TK is the main kinin released.

Key words: T-kinin; bradykinin; kinin conversion; perfused rat kidney; renal aminopeptidase

Kinins are potent vasoactive peptides capable of reducing arterial blood pressure [1, 2], thus being implicated in the pathogenesis of hypertension [3, 4]. This effect is a consequence of their natriuretic and diuretic properties [5, 6]. There are, however, other physiological processes in which the kinins are reported to be involved, including electrolyte fluxes and fluid balance [7, 8]. In addition, some physiopathological processes, such as inflammation [9], may result from other distinct kinin actions. The variety of kinin effects is a result of their specific interactions with distinct receptors on the cell surface [10, 11] and seems to be related to the types of kinins and their origin [10]. Urinary kinins can be generated intrarenally through specific mechanisms, thus yielding significant amounts of these peptides, especially BK‖ (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and LBK, found in human urine [12, 13]. The existence of these peptides in urine and other body fluids is consistent with the presence of the kallikrein-kinin system in the kidney as well as in several other mammalian tissues; in this system, both types of plasma kinin precursors (low- and high-molecular weight kininogens) participate as known substrates for the specific enzymes. In the rat, however, a third type of kininogen (T-kininogen) has been discovered [14–16]. T-kininogen generates, upon certain conditions, TK, a novel kinin possessing the BK sequence (Ile-Ser-BK) [14–16]. Although TK is as potent as BK in contracting the rat uterus [17], it is more potent in causing increases in vascular permeability [18]. It is not known whether TK exerts physiological effects directly or if it requires enzymatic conversion to BK as occurs with MLBK and LBK [19–21]. If TK is converted to BK, its pharmacological action could be due to both TK and BK. We have shown that LBK and a BK metabolite, des-(Arg^9)-BK, are able to stimulate B_1-kinin receptors in the isolated rat kidney, inducing a biphasic response with a vasorelaxant effect followed by a vasoconstrictor phase [22]. Thus, it seems clear that the pathways through which kinins are generated and metabolized by the kidney can influence the physiological response pattern, and thus these

† Corresponding author: Dr. Maria A. R. Vieira, Department of Physiology and Biophysics, ICB, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627, 31270 Belo Horizonte, MG, Brazil. Tel. 55 (31) 448-1248; FAX 55 (31) 441-0835.

‖ Abbreviations: ACN, acetonitrile; BK, bradykinin; BPP_{9a}, bradykinin potentiating peptide 9a; CPN, carboxypeptidase N; LBK, lysylbradykinin; Mergetpa, DL-mercaptomethyl-3-guanidino-ethylthiopropanoic acid; MLBK, methionyl-lysyl-bradykinin; TFA, trifluoroacetic acid; and TK, T-kinin.

metabolic routes need to be studied further and characterized more completely. We now report on the conversion of TK to BK in an isolated rat kidney preparation.

MATERIALS AND METHODS

Perfusion of the isolated rat kidney. The technique used was the same as that described by de Mello and Maack [23]. Briefly, male Wistar rats, weighing 280–330 g, were anesthetized with inactin (40 mg/kg, ip; Promonta, Hamburg, Germany), and the right kidney was exposed through an incision in the peritoneal cavity. Nonfiltering kidneys were systematically used. The urinary flow was stopped out by ureter ligation. The right renal artery was cannulated via the superior mesenteric artery to avoid interruption in the perfusion of the kidney. The isolated kidney was transferred to a chamber kept at 37° and perfused with 60 mL of perfusate.

The perfusate consisted of a Krebs–Henseleit bicarbonate buffer (Na^+, 141 mM; K^+, 5.4 mM; Ca^{2+}, 2 mM; Mg^{2+}, 2 mM; Cl^-, 25 mM; HCO_3^-, 25 mM; PO_4^{2-}, 1.53 mM; and SO_4^{2-}, 2.44 mM), containing mannitol (100 mM), bovine serum albumin (2 mg/mL), glucose (1 mg/mL) and creatinine (0.5 mg/mL). This solution was recirculated by the kidney, and the perfusion pressure and renal flow were monitored continuously. A period of 25 min was allowed for equilibrium. After this period, the perfusion pressure and renal perfusate flow were 110 ± 10 mm Hg and 14 ± 2 mL/min, respectively. These parameters remained constant throughout the experimental period.

Kinin perfusion. The kinins (TK, BK) to be perfused were added to the perfusate at zero time just after the equilibration period. Samples of perfusate (5 mL) were collected at 2, 5, 10 and 20 min of perfusion. When the kidneys were perfused with TK in the presence of enzyme inhibitors, the latter were added to the perfusate 5 min before the kinin.

Preparation of samples for HPLC analysis. To concentrate the peptides, each perfusate sample collected was passed through a Sep-Pak C_{18} microcolumn (Waters) preactivated with 100% ACN. Adsorbed peptides were eluted with 4 mL of freshly prepared 40% ACN in TFA solution [0.1% (v/v) in water]. The eluates were vacuum dried overnight in a vac speed concentrator (Savant, USA) and reconstituted in 0.4 mL of the TFA solution. These perfusate samples when concentrated 12.5-fold should contain a theoretical amount of 2.6 nmol of TK if none is hydrolyzed by the kidney enzymes.

HPLC analysis. A high performance liquid chromatography system (Waters Associates, Milford, MA), consisting of two model 6000A pumps, a model 740 Data Module, a model 680 Automated Gradient Controller, and a model 441 UV detector, was employed for HPLC analysis of the peptides. Aliquots (175 μL containing 1.1 nmol of theoretical kinin) from each sample previously reconstituted in 0.1% (v/v) TFA were injected onto a reverse phase column (Waters, C_{18} μBondapak™, 5 μm, 4.6 × 250 mm). All runs were performed using 0.1% (v/v) TFA as solvent A and 100% ACN as solvent B. The column was previously equilibrated with a mixture of 80% solvent A to 20% solvent B, keeping a constant flow of 1.0 mL/min. The elution of the peptides was performed utilizing a linear gradient

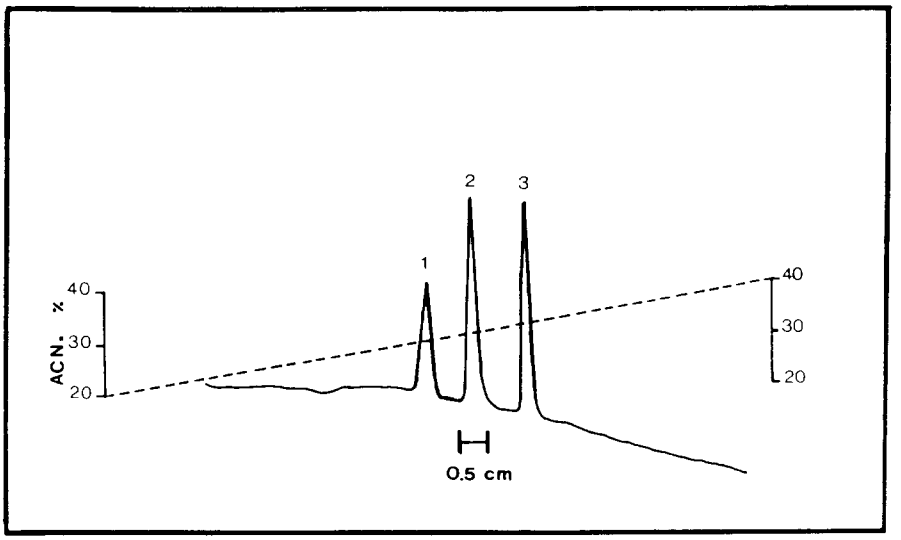

Fig. 1. HPLC fractionation of kinins on a reverse phase column: Elution pattern profile of LBK (0.7 nmol), BK (1.2 nmol) and TK (1.1 nmol). The experimental conditions were as follows: Column: reverse phase, μBondapak C_{18}, 5 μm, 4.6 × 250 mm; solvent A: 0.1% (v/v) TFA in water; solvent B: 100% ACN; column equilibrium: solvent A: solvent B: 80:20; flow rate: 1.0 mL/min; elution: linear gradient, 20–60% of solvent B during 50 min; detection: 214 nm; attenuation: 256 mV. The retention times were: (1) LBK, 12.21 min; (2) BK, 14.47 min; and (3) TK, 16.15 min.

from 20 to 60% of solvent B over 50 min (1.0 mL/min). In this procedure, any other kinin fragments accumulated in the perfusate in a level below 0.08 nmol could not be detected. Fractions of 1.0 mL were collected and vacuum dried for approximately 4 hr. Each fraction was then reconstituted in 0.1 mL of 0.1% (v/v) TFA for further bioassay, when necessary.

Kinin bioassay. The kinins were bioassayed in an isolated guinea pig ileum preparation [24]. The peptide peaks eluted in those fractions corresponding to BK or TK peaks on the HPLC chromatograms were bioassayed, and their concentrations were determined using BK or TK as standard kinins, respectively.

BK and TK were obtained from Peninsula Laboratories (Belmont, CA), and BPP_{9a} and bestatin from the Sigma Chemical Co. (St Louis, MO). The plasma CPN inhibitor Mergetpa was purchased from Calbiochem-Behring (San Diego, CA). Bovine serum albumin was from Boehringer Mannheim (Mannheim, Germany). ACN and TFA were from the Pierce Chemical Co. (Rockford, IL).

Statistical analysis. Results are expressed as means ± SEM and compared by an independent Student's *t*-test.

RESULTS

LBK, BK and TK were well separated on an HPLC reverse phase column, as shown in Fig. 1. With the solvent and gradient systems used, the retention times for LBK (0.7 nmol), BK (1.2 nmol) and TK (1.1 nmol) were 12.21, 14.47 and 16.15 min, respectively. The peptides were selectively eluted from the column when ACN concentration (v/v) reached specific gradient proportion: LBK, 30.50%, BK, 31.50% and TK, 32.75%. Using the same chromatographic conditions, samples obtained at different kidney perfusion times were submitted to HPLC fractionation. Figure 2A shows the chromatographic profile of the perfusate samples obtained after 2–10 min recirculation through the kidney preparation in the absence of added kinin. Two small peaks, which eluted at 13.99 and 15.75 min, may represent perfusate contaminations and did not have any biological activity when bioassayed in the guinea pig ileum preparation. When the kidneys were perfused with BK (30 nmol, 0.5 nmol/mL, N = 3) for 2 and 5 min, a unique peak corresponding to this kinin (14.41 min) appeared in the chromatogram (Fig. 2B). The BK peak had almost disappeared by the 10th min of perfusion. Meanwhile, when the kidneys were perfused with TK (30 nmol, 0.5 nmol/mL, N = 4), two peaks appeared in the chromatograms of samples collected at 2, 5 and 10 min of perfusion (Fig. 2C). The first peak eluted with the same retention time of BK (14.42 min) and the second one (16.24 min) corresponded to the remaining TK added, which had not been converted yet to another kinin and/or fully degraded. Furthermore, significant reduction of intact TK was observed at 5 and 10 min of kidney perfusion, with a slight increase of the BK peak at the 5th min of perfusion. At the 20th min of perfusion with TK, a complete disappearance of both peaks

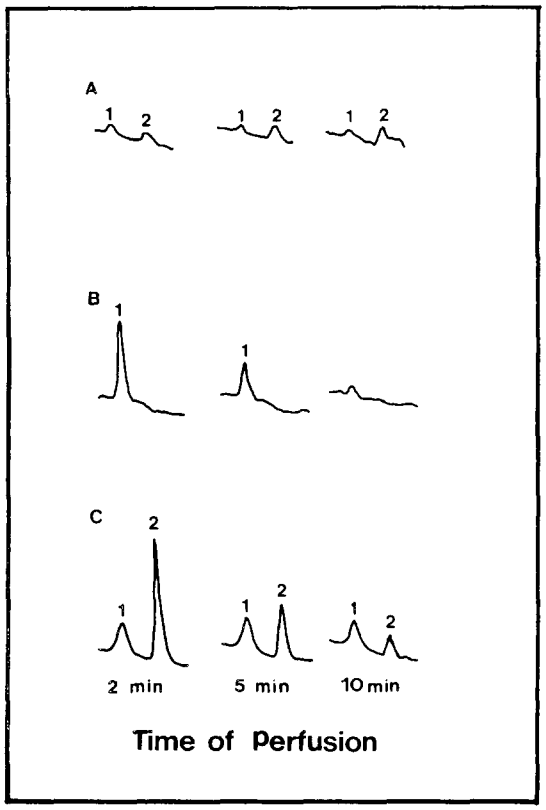

Fig. 2. Typical HPLC chromatographic elution profile of samples collected during kidney perfusion with no kinin (A); with BK (0.5 nmol/mL, N = 3) (B); and with TK (0.5 nmol/mL, N = 4) (C). The chromatographic conditions were the same as described in Fig. 1. The retention times were: A_1, 13.99 min, A_2, 15.75 min; B_1, 14.41 min; and C_1, 14.42 min, C_2, 16.24 min.

was observed (not shown). This pattern was not modified when TK was perfused in the presence of Mergetpa (5 μM, N = 4), a plasma carboxypeptidase inhibitor (compare Fig. 3A and 3B). This inhibitor did not affect either the renal conversion of TK to BK (Fig. 3B) or its disappearance from the perfusate. On the contrary, when TK was perfused through the isolated kidney preparation in the presence of BPP_{9a} (5 μM, N = 3), a kininase II inhibitor, an increase in peak 1 corresponding to BK elution was observed at the 2nd and 5th min of perfusion (Fig. 3C). Accordingly, the peak corresponding to TK (peak 2, Fig. 3A) was diminished considerably at 2 min and even more at 5 min of perfusion (compare Fig. 3A and 3C). However, during the entire experimental period, this inhibitor was not able to prevent further degradation of either TK or BK resulting from the TK conversion (Fig. 3C). The data thus obtained indicated that, in addition to some peptide degradation, BK is one of the major metabolites formed during TK recirculation through the kidney.

To further characterize the kinin-converting process, another series of experiments using the above peptides and inhibitors were performed and

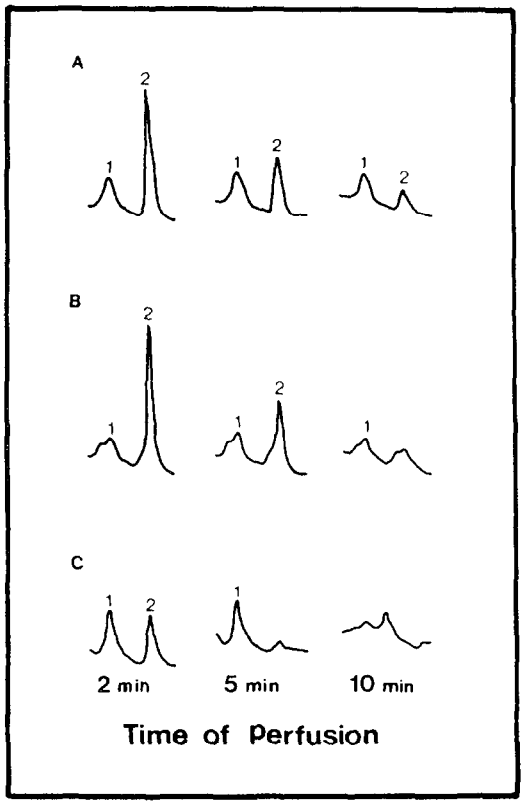

Fig. 3. HPLC chromatographic elution profile of samples collected at different times of kidney perfusion with TK (0.5 nmol/mL) in the presence of inhibitors. (A) TK alone (N = 4); (B) TK plus Mergetpa (5.0 μM, N = 4); and (C) TK plus BPP_{9a} (5.0 μM, N = 3). The retention times were: A_1, 14.42 min, A_2, 16.24 min; B_1, 14.49 min, B_2, 16.33 min; and C_1, 14.44 min, C_2, 16.20 min. The experimental conditions were the same as described in Fig. 1.

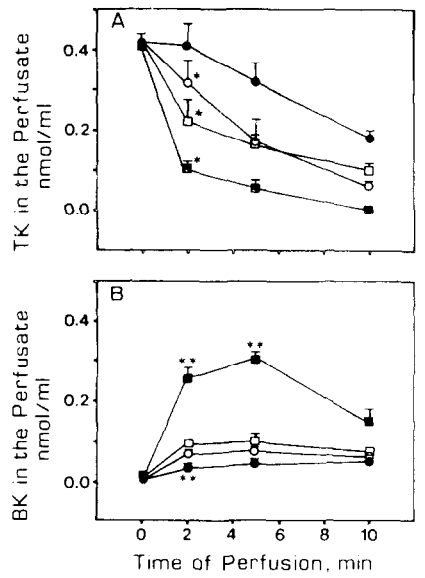

Fig. 4. Residual activity of (A) TK (0.5 nmol/mL) and (B) BK (0.5 nmol/mL) in the isolated rat kidney perfusate, as determined by bioassay on the guinea pig ileum. Samples were taken at the perfusion times indicated. Key: (○—○) alone (N = 4); (●—●) TK plus bestatin (10 μM, N = 3); (□—□) TK plus Mergetpa (5.0 μM, N = 4); and (■—■) TK plus BPP_{9a} (5.0 μM, N = 3). Values are means ± SEM. Statistical significance:* $P < 0.05$, compared with zero time of perfusion; and **$P < 0.05$ compared with experiments performed with TK alone.

the recovered kinin was bioassayed. In this series, bestatin, an aminopeptidase inhibitor [25], was also used. The kinins recovered from the perfusate after HPLC fractionation were bioassayed. The results (Fig. 4) confirmed that Mergetpa did not alter either the disappearance of TK (Fig. 4A) or its conversion to BK (Fig. 4B). BPP_{9a}, however, significantly increased the conversion of TK to BK (Fig. 4B), which followed a fast disappearance of TK (Fig. 4A) where no TK activity was detected at the 10th min of perfusion. On the other hand, bestatin (10 μM, N = 3) partially blocked kidney metabolism of TK, as represented by a slow consumption of TK (Fig. 4A) and inhibition of BK formation (Fig. 4B) during most of the perfusion time with TK. During the first 2 min, 0.032 ± 0.003 nmol/mL of BK appeared in the perfusate. This conversion represented 60% of that observed when TK was perfused alone (0.051 + 0.040 nmol/mL, Fig. 4B). As expected, the disappearance of TK in the presence of bestatin was significantly slower. At the 10th min of perfusion with bestatin, 11.1 ± 1.5 nmol of TK was still recovered, corresponding to 43.6 ± 4.4% of the added peptide (Table 1, N = 3), while in the perfusion with TK alone the remaining TK at the same time was only 3.9 ± 0.8 nmol of the peptide added or 15.1 ± 0.3% of the initial concentration. Quantitative data showing the proportion and rate of TK degradation and conversion are shown in Table 1. When the residual activity of TK was measured at a short perfusion time (2 min) and compared with that recovered as BK, it was verified that most of the TK that disappeared from the perfusate was converted to BK. Recovered TK at 2 min of perfusion was 75.3 ± 13.4% in the perfusate from kidneys perfused with TK alone, while the activity that appeared as BK was 12.1 ± 1.2%, showing that TK was preferentially converted to BK. This result points out the crucial role of enzymes such as kinin-converting aminopeptidase M and/or dipeptidyl-amino-peptidase in the rat kidney, since these enzymatic routes are responsible for the direct conversion of TK to BK observed in the kidney. Although Mergetpa did not have a detectable effect on BK formation, it accelerated TK inactivation (Table 1). Surprisingly, BPP_{9a} accelerated initial TK disappearance (from 3.2 to 9.5 nmol/min) and increased the rate of conversion of TK to BK (from 1.5 to 7.6 nmol/min) (Table 1). These data seem to be due to the protection of TK from degradation, since most of the TK that disappeared from the perfusate appeared as BK. At 2 min of perfusion, the residual TK was 25.1 ± 1.3% of the amount added, and the recovered BK activity was 60.6 ± 6.9%. This recovery should be compared

Table 1. Estimation of residual activities of kinins (TK and BK) during kidney perfusion with TK under different experimental conditions

Perfusion with	Time of perfusion (min)	Residual TK (%)	TK disappearance rate (nmol/min)	BK recovered (%)	BK production rate (nmol/min)	Other fragments formed* (%)
TK alone	2	75.3 ± 13.4	3.2 ± 1.7	12.1 ± 1.2	1.5 ± 0.2	20.7 ± 5.5
(N = 4)	5	41.1 ± 3.8	3.0 ± 0.2	14.5 ± 1.9	0.8 ± 0.1	42.9 ± 1.8
	10	15.1 ± 3.8	1.0 ± 0.3	12.4 ± 1.1	0.3 ± 0.3	72.4 ± 1.3
TK + Mergetpa	2	53.2 ± 12.7	6.6 ± 2.0	15.6 ± 0.5	2.0 ± 0.6	31.2 ± 12.5
(N = 4)	5	40.8 ± 13.7	3.0 ± 0.7	18.4 ± 2.2	0.9 ± 0.1	31.5 ± 12.2
	10	24.4 ± 4.6	1.9 ± 0.1	13.0 ± 1.0	0.3 ± 0.2	63.6 ± 3.9
TK + BPP_{9a}	2	25.1 ± 1.3	9.5 ± 0.2	60.6 ± 6.9	7.6 ± 0.9	14.3 ± 5.6
(N = 3)	5	13.5 ± 4.3	4.4 ± 0.2	73.4 ± 2.6	3.7 ± 0.1	13.0 ± 6.9
	10			37.1 ± 5.9	0.9 ± 0.1	62.9 ± 5.9
TK + Bestatin	2	97.5 ± 12.4	0.3 ± 0.4	7.6 ± 0.7	0.9 ± 0.9	
(N = 3)	5	75.9 ± 11.3	1.2 ± 0.6	10.6 ± 0.6	0.5 ± 0.3	
	10	43.6 ± 4.4	1.4 ± 0.1	12.5 ± 1.8	0.3 ± 0.4	43.9 ± 6.2

The kinins were bioassayed in the guinea pig ileum after their fractionation by HPLC.
Values are means ± SEM.
* Other fragments formed were calculated by 100 − (% residual TK + %BK recovered), these fragments could not be detected at a level below 0.08 nmol.

with the 12.1% observed in the perfusion with TK alone. The data in Table 1 confirmed that bestatin, besides inhibiting TK conversion to BK, also protects TK from being inactivated by other kidney enzymes. At the 10th min of perfusion, 43.6% (as compared with 15.1% in the absence of bestatin) of the added TK was still present in the perfusate, while no increase in BK generation was observed.

DISCUSSION

Although T-kininogen has been reported to be the major type of kininogen in rat plasma, little is known about the actions of the biologically active peptide originating from T-kininogen cleavage, the undecapeptide named TK [14]. Nevertheless, this kinin has been reported to be the major kinin formed in plasma of rats with adjuvant arthritis [26]. As far as renal hemodynamics is concerned, the kinins are supposed to exert a major function, although the mechanisms by which kinins influence renal physiology and their fate after exerting their actions are unknown. It has been reported by Palmieri *et al.* [27] that the C-terminal conversion of BK to des-Arg^9-BK is the only detectable pathway for BK metabolism involving plasma membranes of cultured endothelial cells. The conversion of N-terminal elongated kinins such as LBK and MLBK to BK by aminopeptidases present in human urine has been widely recognized [19, 20]. Our results show that the isolated rat kidney perfused with TK generates BK. This kinin conversion, attributed to the action of a vascular renal enzyme similar to aminopeptidase M and/or dipeptidyl-amino-peptidase, was observed by HPLC analysis of the perfusate samples taken during perfusion with TK. The role of aminopeptidase P in this context can be ruled out since this enzyme can degrade BK but does not metabolize N-terminally extended kinins such as TK [28]. Two peptide peaks eluting with retention times identical to those of BK and TK were seen even at a single passage of TK through the kidney (2 min of perfusion). The appearance of a peak that eluted with the same retention time as BK suggested that TK was converted quickly to BK. This observation was confirmed when the fraction corresponding to the BK peak was bioassayed in the guinea pig ileum preparation. These data are consistent with the observation that kinins, like LBK and MLBK, can be converted to the nonapeptide BK by urine, plasma and vascular endothelium aminopeptidases [19–21]. In addition, we have shown in a previous work [22] that LBK seems to be more resistant to inactivation by kidney peptidases. It was suggested that binding of LBK to the kinin-converting aminopeptidase would protect the peptide from being inactivated. The observed conversion of TK to BK now reported can result from a similar process and indicates that BK is somehow playing a physiological role in the rat kidney. The action of aminopeptidase M on TK could be facilitated by a greater resistance of TK to kininase II (see below). A similar resistance to this peptidase is shown by Lys-Lys-BK [29]. This could represent an important physiological phenomenon since BK is the most potent kinin with regard to its ability to contract several isolated smooth muscle preparations [10] and/or to induce vasodilation after intraarterial infusion under different experimental conditions [6, 30]. The formation of other kinin fragments during the kidney perfusion could not be seen in the chromatographic procedure used, because although possibly formed, they did not accumulate above the level of 0.08 nmol, the amount necessary for the analysis, clear detection and identification of the kinins.

T-kininogen has been reported to be the major type of kininogen in rat plasma, so the active peptide

TK would be the main kinin released locally under certain conditions, e.g. inflammation [18, 31]. Inflammatory exudates contain enzymes that are able to cleave the N-terminal portion of kinins. In this condition, the increase in the vascular permeability could be due, at least partially, to the BK originated by conversion from TK. The fact that bestatin, an inhibitor of aminopeptidases [25], partially inhibited the conversion of TK to BK during rat kidney perfusion corroborates the idea that TK could be converted to BK by renal enzymes. This inhibitor has been described as preventing BK generation from kinins more elongated at the N-terminal position. It has been reported that bestatin inhibits both the conversion of angiotensin II to angiotensin III [32] and of LBK to BK [21] by aminopeptidase M. On the other hand, the plasma CPN inhibitor Mergetpa did not affect the amount of the kinin converted to BK by rat kidney. Although an enzyme with kininase I-like activity has been identified in the kidney [33], little is known concerning the renal enzymes that are able to cleave the amino acid arginine from the C-terminal portion of kinins. Surprisingly, the kininase II inhibitor BPP_{9a} [34] increased the amount of TK converted to BK during rat kidney perfusion. This observation is consistent with the finding that some kinin analogs, such as Lys-Lys-BK [28], des-Arg^9-BK and des-Arg^9-[leu^8]-BK [35], are resistant to inactivation by this enzyme. The increase in the amount of TK converted to BK during perfusion in the presence of BPP_{9a} seems to result from the fact that this inhibitor prevents the degradation of BK, the product of aminopeptidase action on TK.

It was concluded that rat kidney contains enzymes that are able to convert TK to BK, both of which may be responsible for the pharmacological actions of kinins. The enzymes could have a modulating influence on kinin activity if TK and BK are shown to exert different qualitative or quantitative differences in their functions.

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