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Not More than Three Tissue Kallikreins Identified from Organs of the Guinea Pig

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The large and varied multigene families of tissue kallikreins of rat and mouse are considered to selectively release as many bioactive peptides. In order to determine whether a similar family of enzymes is expressed in the organs of the guinea pig purification studies were performed. Tissue kallikreins from the submandibular gland, coagulating gland/prostate complex and the pancreas were separated by affinity chromatography on benzamidine-Sepharose. Amino-terminal sequences, the patterns of hydrolysis rates of a number of peptide *p*-nitroanilides, inactivation rates by active site-directed irreversible inhibitors, specific kininogenase activities and types of kinin released were used to probe the identity of the isolated enzymes.

Guinea pig tissue kallikreins 1 and 2 have been reported previously. In the present study we have identified a third type, designated tissue kallikrein 1a because of its sequence similarity to kallikrein 1, which differs from the latter in the catalytic properties. The inferred occurrence of not more than two or three independent tissue kallikrein genes in the guinea pig contrasts with the varied family of enzymes expressed by the large number of such genes present in rats and mice. Expression in the guinea pig (and also in humans) of only a small number of tissue kallikreins makes specific processing of a multitude of biologically active peptides by such enzymes unlikely.

Key words: Amino acid sequences / Benzamidine-Sepharose / Irreversible protease inhibitors / Kallidin release / Kininogenases / *p*-nitroanilides.

Introduction

Tissue kallikreins (TKs) characteristically release kallidin (lysyl-bradykinin) from kininogens by site-specific proteolysis. Kinins are potent vasoactive peptides with possible roles in the maintenance of systemic blood pressure and in the pathogenesis of inflammation (Frey *et al*., 1968; Erdös, 1979; Bhoola *et al*., 1992). A large family of enzymes with TK-related sequences has been detected in the mouse with as many as 24 to 25 genes, of which 10 to 14 are functional (Mason *et al*., 1983; Evans *et al*., 1987; Berg *et al*., 1992). A similar large number of 11 to 17 genes has been described in the rat (Wines *et al*., 1991), and in the African rat Mastomys (Bowcock *et al*., 1988). It has been suggested that these enzymes, differing in substrate specificities, are functionally important because of their ability to selectively cleave very many specific polypeptide precursors of bioactive peptides (Mason *et al*., 1983; Bothwell *et al*., 1979).

The question whether an analogous regulatory enzyme system is common to each mammalian species is of considerable functional importance. If this were the case one would have to expect the expression of many TK-like enzymes in each mammalian species. In the guinea pig, however, only two different TKs had hitherto been isolated and extensively characterised, including amino acid sequencing. The TK from the submandibular glands (Fiedler *et al*., 1983) was named guinea pig TK1 (gpK1; Berg *et al*., 1992), and the second one from the prostate (Dunbar and Bradshaw, 1987) was named guinea pig TK2 (gpK2). An earlier attempt to purify TKs from the guinea pig had resulted in the isolation of not more than one enzyme preparation from each of the organs examined (Mayer *et al*., 1989). We report here that the Arg esterase activity (a characteristic of TKs) isolated from guinea pig submandibular glands was resolved by affinity chromatography on benzamidine-Sepharose into three different, but similar TK preparations. In order to estimate the number of different TK gene products occurring in other guinea pig organs, the same purification procedure was used on extracts of guinea pig pancreas and coagulating gland/prostate complex (CPC), known to express TK-related enzymes. Three Arg esterases/amidases were isolated from guinea-pig pancreas, two of which were purified further, whereas only one TK was detected in the coagulating gland/prostate complex.

Extensive characterisation of these TK preparations indicated that besides gpK1 and gpK2, only one additional TK, named gpK1a, occurs in the guinea pig organs studied. The TK family of the guinea pig appears to consist of the products of not more than three distinct genes. The im-

plication of this finding for possible functional roles of TK-like enzymes is discussed.

Results

Isolation of TKs from Submandibular Glands

During isolation of TKs from organs of the guinea pig precautions were taken against loss of additional tissue kallikrein-like activity, for example a possible fourth submandibular kallikrein. Since TKs are synthesised as proenzymes, homogenates were initially tested for an increase in basal activity following prolonged standing, freezing and thawing, or incubation with bovine trypsin. However, no increase of activity was observed. Column eluates, flow-throughs and final washings with 3 M NaCl in the respective buffer for elution of strongly bound enzymes were scrutinised for enzyme activity, mainly with the sensitive ester substrate AcPheArgOEt (Fiedler *et al*., 1983). Column eluates were also screened for Arg amidase activity with the convenient TK substrate D-ValLeuArg-pNA and with SucPheLeuPhe-pNA to detect possible enzyme variants with chymotryptic specificity.

Extracts from submandibular glands of male guinea pigs chromatographed on DEAE-Sephadex (Table 1) yielded only one broad peak of Arg esterase activity. The flow-through and final washings with 3 M NaCl from such columns contained at most 0.01% or 0.02% of the total esterase activity, respectively. On rechromatography, the Arg esterase peak became narrower (Mayer *et al*., 1989). The single peak of Phe amidase activity coincided with the Arg esterase activity. When the material of that peak was eluted from a benzamidine-Sepharose column by an ionic strength gradient, it was resolved into three peaks A, B and C, which exhibited Arg esterase and amidase activities in similar proportions (Figure 1, Table 1). The SucPheLeuPhe-pNA hydrolysing activity co-eluted with Arg esterase and amidase peaks A and especially B (Figure 1).

Rechromatography of peak A on benzamidine-Sepharose (Table 1) removed some contaminating peak B components. During further purification and concentration of the materials of the three peaks A, B and C on hydroxyapatite and DEAE-Sephadex, the ratios of the Arg esterase to amidase and those of the Phe to Arg amidase activities remained essentially constant (Table 1). The fractions showing the highest specific activities were combined. Determination of the Phe amidase activity of the TK preparation previously isolated from guinea pig submandibular glands (Fiedler *et al*., 1983) resulted in a value of 12.9% of the Arg amidase activity, similar to that obtained for the material of peak B.

Isolation of TK from the Coagulating Gland/Prostate Complex (CPC)

Purification on DEAE-cellulose of a TK-like enzyme from the guinea pig coagulating gland (Moriwaki *et al*., 1974) as

Table 1 Isolation of Three Tissue Kallikreins from Submandibular Glands of Guinea Pigs.

Step	Vol.	A_{280}	$\frac{A_{280}}{A_{260}}$	AcPheArgOEt		$\frac{OEt}{pNA}$	$\frac{Phe}{Arg}$
	ml	units		U	U/A_{280}	U/U	U/U (%)
Extract	125	3370	0.77	4700	1.39	97	7.4
DEAE-Sephadex	4580	1360	0.95	3810	2.80	84	8.4
DEAE-Sephadex	1490	416	1.10	3110	7.48	63	10.8
Benzamidine							
A	61	18.6	1.23	410	22.0	101	2.9
B	81	15.8	1.38	952	60.3	79	13.0
C	215	5.0	0.88	60	12.0	71	< 1
Sum A – C		39.4		1420			
Peak A							
Benzamidine	55	6.6	1.00	355	54	122	2.1
Hydroxyapatite	3.6	0.92	1.49	206	224	104	1.7
DEAE-Sephadex	1.5	0.57	1.84	157	275	106	1.8
Peak B							
Hydroxyapatite	8.5	5.14	1.47	1270	247	91	14.8
DEAE-Sephadex	4.3	2.93	1.85	721	246	84	11.7
Peak C							
(Benzamidine)	(765)	(8.75)	(0.90)	(113)	(12.9)	(65)	(1)
Hydroxyapatite	81	5.8	0.88	78	13	75	
Hydroxyapatite	5.6	7.6	0.90	55	7.2	72	
DEAE-Sephadex	1.7	0.136	1.22	18.8	138	72	

For details see text and Materials and Methods. OEt/pNA, ratio of AcPheArgOEt/D-ValLeuArg-pNA activities; Phe/Arg, ratio of SucPheLeuPhe-pNA/D-ValLeuArg-pNA activities (expressed in %). In the purification of peak C, material from 4 different runs on benzamidine-Sepharose was combined.

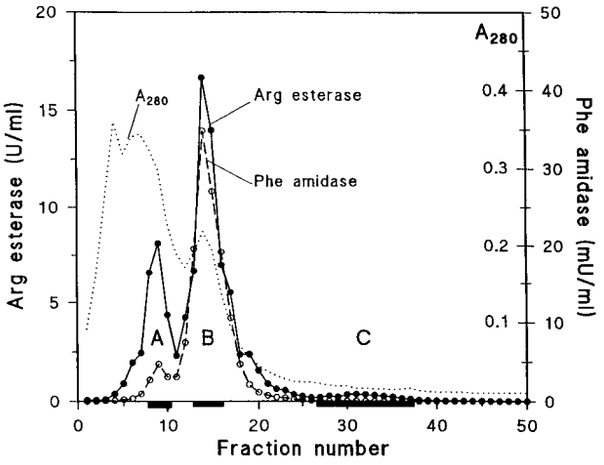

Fig. 1 Resolution of Three Peaks (A–C) of Arginine Esterase from Guinea Pig Submandibular Glands by Gradient Elution from a Benzamidine-Sepharose Column.
For details see Materials and Methods. Fractions pooled are indicated by horizontal bars.

well as one from the prostate (Dunbar and Bradshaw, 1985) has been reported previously. Chromatography of the CPC extracts on DEAE-Sephadex, under comparable conditions, produced a single peak of Arg esterase activity (Table 2), that appeared at a position similar to the Arg esterase activity of the submandibular gland extracts (Mayer *et al*., 1989). Evidently, the Arg esterase from guinea pig coagulating gland and that from prostate could not be separated by DEAE-Sephadex chromatography. The peak of Phe amidase activity coincided again with the Arg esterase peak, but the Phe amidase activity of the combined eluates amounted to only 0.02% of the Arg amidase activity. Single peaks of Arg esterase were again observed on subsequent gel filtration and hydroxyapatite chromatography (Table 2).

When small amounts of a DEAE-Sephadex eluate were applied to a benzamidine-Sepharose affinity column, a single Arg esterase peak (84% recovery) and co-eluting Arg-amidase (98% recovery) and low (0.01%) SucPhe-LeuPhe-pNA activities were obtained. When the column was loaded with a 10-fold higher amount of enzyme (Table 2), almost 50% of the Arg esterase activity was found in a peak A, well ahead of the main esterase in peak B, which was due to overloading of the column. Rechromatography of peak A gave a single esterase peak, B_r, with a similar elution profile as peak B. Both peaks were pooled for the final DEAE-Sephadex step. The low Phe amidase activity of the CPC enzyme resembled that of the submandibular benzamidine-Sepharose peak C (Figure 1, Table 1). Indeed, when a mixture of submandibular and CPC homogenates was chromatographed on DEAE-Sephadex and the single Arg esterase peak subsequently resolved on benzamidine-Sepharose, the peak of the CPC enzyme coincided with the submandibular peak C.

Isolation of TKs from Guinea Pig Pancreas

On chromatography of pancreas extracts on DEAE-Sephadex, Arg-esterase was found mainly in the flow-through (presumably cationic trypsin), and in one comparatively minor peak eluting in a region similar to the AcPheArgOEt esterases isolated from the salivary and coagulating glands of the guinea pig (Mayer *et al*., 1989). On benzamidine-Sepharose ionic strength gradient chromatography, the esterase peak was resolved into three Arg esterase peaks A, B and C (Table 3), in a similar pattern as the three submandibular peaks (see Figure 1, Table 1). The three Arg amidase peaks showed an identical profile to that of the esterase peaks. The Phe/Arg amidase ratios in the fractions of the second main peak B were the highest (Table 3) and similar to the values obtained for the submandibular peak B, while those of the other two peaks resembled the submandibular peaks A and C, respectively (Table 1). The low A_{280}/A_{260} ratios on further purification of peaks A and B (Table 3) were considered to indicate that complete purity had not been attained. The small activity in peak C proved unstable on further purification.

Amino-Terminal Sequences of the TKs

N-terminal amino acid sequencing of the three submandibular TKs suggested that the enzyme preparations were of satisfactory purity, and that no intrachain cleavage had occurred. Due to the sequencing technique used, Cys

Table 2 Isolation of Tissue Kallikreins from 25 g of Frozen Coagulating Gland/Prostate Complex of Guinea Pigs.

Step	Vol.	A_{280}	$\frac{A_{280}}{A_{260}}$	AcPheArgOEt		$\frac{OEt}{pNA}$	$\frac{Phe}{Arg}$
	ml	units		U	U/A_{280}	U/U	U/U (%)
Extract	133	2580	1.36	127000	49		0.02
DEAE-Sephadex	385	420	2.11	130000	310		
Sephadex G-100	140	353	2.12	106000	300		
Hydroxyapatite	227	293	2.12	98000	294		0.01
Benzamidine Peak B	2.5	2.47	1.62	650	263		
(Aliquot)	(8.0)	(11.7)	(2.12)	(3460)	(294)		(0.01)
Benzamidine Peak B_r	4.7	1.53	1.60	365	239		
DEAE-Sephadex	2.4	2.12	1.84	574	271	64	

For details see text and Materials and Methods. OEt/pNA, ratio of AcPheArgOEt/D-ValLeuArg-pNA activities; Phe/Arg, ratio of SucPhe-LeuPhe-pNA/D-ValLeuArg-pNA activities (expressed in %).

Table 3 Isolation of Tissue Kallikreins from 50 g of Frozen Guinea Pig Pancreas.

Step	Vol.	A_{280}	$\frac{A_{280}}{A_{260}}$	AcPheArgOEt		$\frac{OEt}{pNA}$	$\frac{Phe}{Arg}$
	ml	units		U	U/A_{280}	U/U	U/U (%)
Extract	161	6070	0.71	53.5	23	11	
DEAE-Sephadex	2030	855	0.68	53.5	0.062	110	
DEAE-Sephadex	560	47.0	0.82	34.5	0.73	100	11
Benza midine							
A	37	5.88	1.20	5.2	0.88	110	6
B	29	1.61	1.00	13.7	8.5	105	14
C	36	0.78	0.85	2.5	3.2	100	0.5
Sum A – C		8.27		21.4			
Peak A							
DEAE-Sephadex	2.7	(0.52)	0.98	1.3	(2.5)	109	3.4
Peak B							
Hydroxyapatite	1.7	0.16	1.02	12.7	76	87	11
DEAE-Sephadex	3.5	(0.05)	0.82	3.7	(74)	79	11

Details are given in the legend to Table 1.

residues appeared as blanks. The results obtained were compared to the two known sequences of guinea pig TKs (Table 4). The principal enzyme preparation (Peak B, Figure 1) obtained in the current study was certainly identical with the submandibular enzyme isolated previously (Fiedler *et al*., 1983), as indicated by the presence of His-20 and Phe-21 and named gpK1 (Berg *et al*., 1992). When material from Peak A (Figure 1) was sequenced, the protein showed His-20 and Phe-21 like gpK1, but Lys-25 like gpK2 (Dunbar and Bradshaw, 1987). Thereafter the sequence remained identical to gpK2 until position 45 and only then differed at Arg-46, Tyr-47 and Ile-49 (Table 4). Resequencing of the previously isolated material from submandibular glands (Fiedler *et al*., 1983) confirmed the presence of Lys instead of the previously allocated Glu at position 25 of gpK1 (Table 4, second sequence). The sequence from position 22 to 45 of gpK1 was the same as that obtained for gpK2. Differences between gpK1 and gpK2 in the first 52 residues were located at only 4 or 5 positions, namely 20, 21, 47, 49, and possibly 46. Submandibular gpK1 and the enzyme from peak A were indistinguishable in all of the 49 positions sequenced (giving due regard to the tentative nature of position 46) (Table 4). Their molecular masses on reducing SDS-PAGE were also similar. In accordance with the nomenclature of TK based on sequences (Berg *et al*., 1992), the newly isolated enzyme from peak A was called gpK1. However, because the two TKs differed in their catalytic properties, the new enzyme was named gpK1a. Sequencing of the third submandibular benzamidine-Sepharose peak C (Table 2) revealed that the 49 amino-terminal residues were exactly the same as in gpK2, isolated previously from the prostate (Table 4).

When the amino terminus of the only Arg esterase/amidase isolated from guinea pig CPC was sequenced, the 52 positions determined were found to be identical to the sequence reported for prostatic gpK2 (Table 4), except that position 44 seemed to be glycosylated.

The sequence of the benzamidine-Sepharose purified pancreatic peak B, the main TK-like Arg-esterase from this

Table 4 Amino-Terminal Sequences of Guinea Pig Tissue Kallikreins gpK1 and gpK2 and of the Newly Isolated Tissue Kallikreins from Guinea Pig Submandibular Glands.

Protein	Assignment	1 10 20 30 40 50
gpK1*		V I GGQECARDSHP W qAAVY**HF**sD I eCGGVL v
gpK1 (resequenced)		V I GGQECARDSHP WQAAVY**HF**SD I KCGGVLVDPQ w VLTAAxx I N**dhYQ**I x L g
Submandibular peak A	gpK1a	V I GGQECARDSHP WQAAVY**HF**SD I KCGgVLVDPQ x VLTAAHx I ND**r**YQ I
Submandibular peak B	gpK1	V I GGQECARDSHP w QAAVY**HF**
Submandibular peak C	gpK2	V I GGQECARDSHP WQAAVYYYSD I KCGGVLVDPQ x vLTAAHx I N**D**s**N**q v
gpK2#		V I GGQECARDSHP WQAAVYYYSD I KCGGVLVDPQ W VLTAAHC I NDSNQV $^{K}_{W}$ L G

Tentative identifications of amino acids are indicated by lower case. Amino acids differing from the sequence of gpK2 are printed in bold.
* (Fiedler *et al*., 1983)
# (Dunbar and Bradshaw, 1987)

gland, showed multiple amino acids which could be easily explained by either intrachain cleavages or/and by insufficient purity. However, amino acids corresponding to the common sequence of submandibular gpK1 and gpK1a were clearly observed up to position 19, together with the characteristic amino acid residues His-20 and Phe-21, but much less Tyr in the latter two positions. These results suggested that the amino-terminal sequence of this pancreatic enzyme resembled that of gpK1 and not that of gpK2.

Active Site-Directed Irreversible Inhibitors as Probes for Enzyme Identity

Active site-directed irreversible inhibitors are useful tools for identifying different proteases if the rates of inhibition of the various enzymes differ significantly. Rate constants of inhibition on the main preparations of guinea pig TKs by several of these inhibitors at pH 7 (chosen to retard their hydrolysis) are listed in Table 5.

Diisopropyl fluorophosphate inhibited all three major guinea pig enzymes at moderately different, slow rates. To facilitate comparison with literature data, the second order rate constant $k_i = k_i'/[I]$ in $M^{-1}\,min^{-1}$ is also given in Table 5. Reaction rates with 4-(2-aminoethyl)-benzene-sulfonyl fluoride (Markwardt *et al*., 1971) were not much different from those of diisopropyl fluorophosphate, and were similar for the three enzymes. D-PhePheArgCH_2Cl, an efficient chloromethyl ketone inhibitor for both human and rat TKs (Kettner *et al*., 1980), only moderately discriminated between the three guinea pig TKs (Table 5).

Surprisingly, the Phe chloromethyl ketone ZGlyLeuPheCH_2Cl did not inhibit even the submandibular enzyme with the highest Phe amidase activity, gpK1. Only after removal of the Z residue, the resulting GlyLeuPheCH_2Cl proved to be a rather effective inhibitor for this enzyme and discriminated strongly (by a factor of 8) between gpK1 and gpK1a (Table 5). A reasonable determination of the very low inactivation rate of CPC gpK2 by GlyLeuPheCH_2Cl was only possible by raising the inhibitor concentration to 1 mM. The value in Table 5 for gpK2 at 20 μM inhibitor was calculated by linear extrapolation. Submandibular gpK2 was also inactivated very slowly. Evidently, the inhibition rates of the various TKs parallel their Phe amidase activities.

The unusual inhibition of Arg amidase activities by the Phe compound GlyLeuPheCH_2Cl, and the greatly differing rate constants of inhibition of the three main TKs suggested that this inhibitor may prove useful in characterising also the two guinea pig pancreas enzyme preparations. The limited availability of these enzymes required the use of single samples, and then following rates of inhibition as described under Materials and Methods. Effective inhibition rate constants k' were in the range of $0.5 \times 10^{-3} s^{-1}$ for both the pancreatic peak A enzyme and submandibular gpK1a (both treated with 0.5 mM *p*-amidino-phenylmethanesulfonyl fluoride to eliminate potential tryptic impurities of the pancreatic preparation). Ratios of k' from runs with the two enzymes conducted in parallel were 0.954 ± 0.013 and 1.029 ± 0.022 in two separate experiments. This ratio, being close to 1 (i.e. the k' values for the two enzymes are indistinguishable), was regarded as convincing evidence that the Arg amidase peak A from pancreas represented gpK1a. Comparable results for the peak B pancreatic enzyme and submandibular gpK1 were a k' of about $1.3 \times 10^{-3} s^{-1}$ and a ratio of 0.887 ± 0.040 (n = 4), respectively. As this ratio was also near to 1, it strongly suggested that the Arg amidase activity of the pancreatic peak B was identical with that of submandibular gpK1.

Inactivation constants for submandibular gpK1 obtained with 20 μM GlyLeuPheCH_2Cl were indistinguishable, whether measured with SucPheLeuPhe-pNA or D-ValLeuArg-pNA as substrates (Table 5). The similarity between these constants was convincing evidence that both the Arg and the Phe amidase activities were from a single enzyme. For submandibular gpK1a, these inhibition constants were also similar. The distinctly lower rates found for gpK1 indicate that the Phe amidase activity of gpK1a was not due to contamination with gpK1.

Table 5 Rates of Inhibition of the Three Major Tissue Kallikrein Preparations from the Guinea Pig by Active Site-Directed Irreversible Inhibitors.

Tissue kallikrein			gpK1 (Submandibular)	gpK1a (Submandibular)	gpK2 (CPC)
Inhibitor	Conc.	t_{max}	Inhibitor constant $k_1' \times 10^3$ (s^{-1})		
Diisopropyl fluorophosphate	1 mM	3.5 h	0.089 ($5.3\ M^{-1}\,min^{-1}$)	0.14 ($8.4\ M^{-1}\,min^{-1}$)	0.029 ($1.7\ M^{-1}\,min^{-1}$)
4-(2-Aminoethyl)-benzenesulfonyl fluoride	1 mM	2 h	0.15	0.17	0.30
D-PhePheArgCH_2Cl	5 μM	5 min	4.6	4.5	3.9
GlyLeuPheCH_2Cl	20 μM 1 mM	1 h; 2 h 2 h	1.22 (1.17*)	0.16 (0.19*)	(0.001) 0.050

Conditions: 0.05 M Tris/HCl, 0.1 M NaCl, 0.1 mM EDTA, pH 7.0, 25.0 °C. Activity determinations with D-ValLeuArg-pNA as substrate (or with SucPheLeuPhe-pNA when indicated by *). t_{max}, longest time of reaction. Conc. = concentration.

Table 6 Comparison of the Specific Activities of Tissue Kallikreins with D-ValLeuArg-pNA and Bovine L-Kininogen, and Blood Pressure Lowering Activity in the Guinea Pig.

	gpK1 (sub-mandibular)	gpK1a (sub-mandibular)	gpK2 (sub-mandibular)	gpK2 (CPC)	pK1 (autolysed pancreas)	hK1 (urine)
D-ValLeuArgpNa						
μmol/min/mg protein	3.95	3.55		5.11	4.36	4.0
turnover number (s^{-1})	1.71	1.53		2.24	1.86	1.8
Bovine L-kininogen						
nmol kinin/min/ D-ValLeuArg-pNA-U	68	24	2.0	1.8	447	(900)
μmol kinin/min/mg protein	0.27	0.085		0.009	1.95	3.6
turnover number (s^{-1})	0.12	0.037		0.004	0.83	1.6
Hypotensive effect						
mg Bkeq/mg protein	670	170		60		

Conditions: 0.1 mM D-ValLeuArg-pNA or 4.5 μM bovine L-kininogen, 0.1 M Tris/HCl, 0.1 M NaCl, 0.1 mM EDTA, pH 8.0, 25 °C. Values for porcine pancreatic Aβ-kallikrein B (pK1) were normalised to 230 BzArgOEt-U_{253}/mg protein (Fiedler *et al*., 1983) and for human urinary kallikrein (hK1) to 1100 AcPheArgOEt-U/mg protein (Geiger *et al*., 1980). Data for kinin release by hK1 were obtained with 3.2 μM human L-kininogen in the experiment outlined in Fiedler and Hinz (1992) and those for pK1 were calculated from kinetic constants determined under somewhat different conditions (Fiedler and Hinz, 1992).
BKeq = brodykinin equivalents.

Specific Activities and Substrate Specificities of the Guinea Pig TKs

Specific activities of the three main TK preparations from the guinea pig, submandibular gpK1 and gpK1a and coagulating gland/prostate complex (CPC) gpK2, were determined with the best *p*-nitroanilide substrate, i. e. D-ValLeuArg-pNA. Only in the case of gpK2, isolated from both submandibular glands and CPC, was an unusual increase in activity of about 10% observed during the first 10 min of the hydrolysis of this substrate; thereafter a constant rate (used for calculating the enzyme activity) was reached. This finding provided further evidence of common enzyme activity in the two preparations. For the purposes of comparison, TKs from autolysed porcine pancreas (pK1) and from human urine (hK1) (Berg *et al*., 1992) are shown in Table 6. The data for pK1 and hK1 appear compatible with published results obtained under somewhat different conditions (Geiger *et al*., 1980; Fiedler *et al*., 1978; Olivera *et al*., 1987; Blaber *et al*., 1989). Specific activities measured with D-ValLeuArg-pNA were remarkably similar for all TKs studied.

Rates of hydrolysis of a number of peptide *p*-nitroanilides by five different preparations of guinea pig TKs in comparison to pK1, hK1 and bovine trypsin are listed in Table 7, relative to a value of 100 given to the hydrolysis rate of D-ValLeuArg-pNA. The data for bovine trypsin compared favourably with published kinetic constants (Lottenberg *et al*., 1981), and thereby provided validation for the hydrolysis rates determined for the substrates with the TKs. The specificity patterns of the guinea pig TKs appear grossly similar to those of the other TKs (and totally different from that of trypsin). However, characteristic differences useful as identity criteria do exist. The pattern of substrate specificities obtained with pancreatic gpK1 was convincingly identical within experimental error with that for authentic gpK1 from submandibular glands (Table 7). The same was true for submandibular gland gpK2 and for CPC gpK2 (Table 7). Only the SucPheLeuPhe-pNA activity of the submandibular enzyme preparation had a little higher value, a fact that could be easily explained by minor contamination with gpK1. The activity of hK1, reported to have a high Phe activity (Chagas *et al*., 1995) with the latter substrate, was second only to that of gpK2. The guinea pig TKs as well as hK1 showed low activity on the partial kininogen sequence D-SerLeuMet-pNA (Friberger *et al*., 1982). In this respect they resembled porcine TK, where the efficiency of hydrolysis of the Met bond in kininogen could not be simulated by some low molecular mass model compounds (Fiedler and Hinz, 1992; Fiedler and Leysath, 1979; Fiedler *et al*., 1986).

Kinin Release

The type of kinin peptide released and the rates of kinin formation by the guinea pig TK preparations were determined at several reaction times with bovine L-kininogen as substrate (Table 6). With each enzyme a steady, gradually slowing down release occurred when kinin liberation was followed until at least 50% of the kinin content of the kininogen in the sample was hydrolysed. This observation indicated that kinins were not released preferentially from the one of the nicked forms present in the kininogen preparation (Fiedler and Hinz, 1992). Kallidin was always the main product formed. All of the enzyme preparations examined exhibited the characteristic property of a tissue kallikrein, namely the ability to cleave the Met bond of bovine kininogen. des-Arg-10-kallidin was released in parallel and in proportions corresponding to the content of 11% of releasable kinins present in form of des-Arg-370-

Table 7 Rates of Hydrolysis of Peptide *p*-Nitroanilides Expressed Relative to that of D-ValLeuArg-pNA.

p-Nitroanilide	gpK1 (pancreatic)	gpK1 (sub-mandibular)	gpK1a (sub-mandibular)	gpK2 (sub-mandibular)	gpK2 (CPC)	pK1 (autolysed pancreas)	hK1 (urine)	Trypsin (bovine)
D-ValLeuArg	100	100	100	100	100	100	100	100
BzPheLeuArg	8.4	7.0	6.3	15	17	6.8		110
BzLeuLeuArg	34	37	16	50	48	17		160
D-ProPheArg		43	37		84	67	(10)	110
BzProPheArg	4.2	3.5	1.7	26	27	8.7		40
ZPheArg	3.8	3.7	1.8	8.8	9.1	4.2		31
BzPheValArg		0.2	0.1		1.5	1.1	(< 1)	140
ZGlyProArg		0.3	0.2		0.2	1.3		610
TosGlyProArg		0.35	0.2		0.2	1.4	(3.5)	550
ZValGlyArg	0.2	0.2	0.1	0.6	0.5	0.2		310
ZLysArg		< 0.1	< 0.1		0.1	< 0.1		39
D-ValLeuLys		6.1	4.7		4.3	6.6		13
BzLeuLeuLys	0.9	0.6	0.2	9.3	9.4	0.4		37
SucPheLeuPhe	13	14	2.4	0.3	0.01	0.4	4.6	< 0.1
SucPheProPhe	< 0.2	0.1	< 0.05	< 0.1	< 0.05	< 0.05		
SucAlaAlaProPhe	< 0.2	< 0.05	< 0.05	< 0.1	< 0.05	< 0.05		
SucGlyGlyPhe		< 0.05	< 0.05		< 0.05	< 0.05		
PyrPheLeu		< 0.05	< 0.05		0.3	< 0.05		
SucAlaAlaProLeu		< 0.05	< 0.05		< 0.05	< 0.05		
D-SerLeuMet	1.6	1.8	0.6	< 0.1	< 0.05	0.2	0.5	
MeOSucAlaAlaProMet		< 0.05	< 0.05		< 0.05	< 0.05		

Conditions: 0.1 mM substrate, 0.1 M Tris/HCl, 0.1 M NaCl, 0.1 mM EDTA, pH 8.0, 25.0 °C. Data for hK1 in parentheses were obtained at 37 °C (Fiedler *et al*., 1978).

kininogen in the kininogen preparation used. Due to the presence of small amounts of Lys-361-nicked chains in the kininogen preparation (Fiedler and Hinz, 1992), a trace of bradykinin was found in the release experiments with gpK1 and gpK1a. In contrast, both CPC and submandibular gpK2 produced significant amounts of bradykinin which represented about 20% of total measurable kinin. As is evident from controls, this finding was not due to metabolism of kallidin to bradykinin. This result was regarded as an important common property of the gpK2 enzymes from the submandibular and coagulating/prostate organs. However, the additional formation of bradykinin by gpK2 did not indicate a pronounced tendency of this enzyme to cleave the lysyl bond in kininogen. Bradykinin liberation by other TKs at a similar low rate would not be readily observed due to the large amounts of kallidin released.

The initial rate of kinin release from bovine L-kininogen by the most active guinea pig enzyme, gpK1 (Table 6), appeared similar in value to the 462 μg kallidin × min^{-1} × mg^{-1} protein determined previously with the purified TK from submandibular glands under different experimental conditions (Fiedler *et al*., 1983). By comparison, the rate of kinin release by gpK1a was about three-fold lower. The rate of kinin liberation from bovine L-kininogen determined for CPC gpK2 was similar to that obtained for submandibular gpK2 (Table 6), amounting to as little as 3% of the specific activity of gpK1. Inspite of similar specific activities measured with D-ValLeuArg-pNA, the initial rate of kinin release from bovine L-kininogen by gpK1, the most active guinea pig enzyme, was 7 fold lower than the rate measured for pK1 (Fiedler and Hinz, 1992) and 13-fold lower than that obtained for hK1. The latter value was similar to published kinetic constants (Pierce and Guimaraes, 1976; Geiger *et al*., 1977; Maier *et al*., 1983).

When each TK was injected into the circulation of guinea pigs, the decrease in blood pressure considered to be due to kinin release from endogenous kininogen was greatest for submandibular gpK1, which had shown the fastest rate of kallidin release from bovine kininogen (Table 6). The present result for this enzyme is in reasonable agreement with the value of 300 μg bradykinin equivalents/mg protein determined previously for guinea pig submandibular TK (Fiedler *et al*., 1983). Effects on the blood pressure diminished with decreasing kininogenase activity (Table 6). However, even gpK2 retained notable activity.

Discussion

The two guinea pig TKs, gpK1 (predominant in submandibular glands) and gpK2 (predominant in the CPC), are remarkably similar in their amino-terminal sequences: only 4 or 5 of the first 52 positions were found to differ. In this region, rapidly kinin-liberating 'true' TKs differ from TK-like enzymes from the male sexual tract in humans (as compiled in Schedlich *et al*., 1987) by as many as 17 or 20, in the dog (Chapdelaine *et al*., 1991; Gauthier *et al*., 1994) by

16, and in the rat (compilation in Wines *et al*., 1991) by 12 or 13 amino acid residues. The unusual sequence similarity cautioned against inferring the identity of a guinea pig TK preparation from results of amino-terminal sequencing alone.

The newly found third TK of the guinea-pig, termed gpK1a, is indistinguishable from gpK1 by amino-terminal sequencing. However, the nature of its enzymatic properties, as evidenced by the pattern of substrate specificities, rate of inhibition by GlyLeuPheCH_2Cl and relative kininogenase and blood pressure activities clearly indicate that gpK1a is a distinct enzyme. Sequencing excluded intrachain splits as a cause of the differences. Whether gpK1a is a product of post-translational modification, resulting from a loss of a C-terminal peptide or differences in amidation or glycosylation, or whether it is an allotypic variant or a product of a third TK gene may be clarified only by a complementary study of the TK gene locus of the guinea pig.

Identity of the whole protein part of gpK2 detected in submandibular glands with authentic gpK2 from CPC was supported, in addition to their similar elution pattern from benzamidine-Sepharose, by their similar specificity patterns, their unusual time-dependent substrate activation by D-ValLeuArg-pNA and their similar relative kininogenase activities, characterised by the release of significant amounts of bradykinin besides kallidin.

The very low content of TKs in guinea pig pancreas prevented complete purification and thorough characterisation of the two enzymes isolated from this source. The elution pattern from benzamidine-Sepharose, amino-terminal sequencing, patterns of substrate specificity and rates of inhibition by the Phe chloromethyl ketone provided sufficient evidence that the two enzymes (Peaks A and B) isolated from the pancreas are identical with gpK1a and gpK1, respectively.

Concerning the physiological roles of the three guinea pig TKs, gpK1 is the enzyme most efficiently releasing kallidin and lowering the blood pressure of the guinea pig. Therefore, this enzyme evidently is the 'true' TK in this animal. The occurrence of gpK1 also in the pancreas is in accordance with this view. gpK1a, which also occurs in the pancreas, had a similar efficiency in releasing kallidin, as well as lowering blood pressure. This enzyme may therefore well represent a variant of 'true' TK without an independent physiological role.

No other TK-like enzyme besides gpK2 was found in the CPC. This suggests that in CPC or in its secretions the biological functions of a TK-like enzyme would be mediated by gpK2 alone. One possible function could be the release of kinin from kininogen. A 'true' TK able to release kallidin, and originating from the prostate, has been identified in human seminal plasma (Fink *et al*., 1985). Although the kallidin-releasing and guinea pig blood pressure regulating activities of gpK2 were comparatively low, due to the large amounts of this enzyme occurring in these glands it could generate significant amounts of kinin and compensate for the absence of gpK1. Another TK-like enzyme in human semen is prostate-specific antigen (PSA; Christensson *et al*., 1990) which is unable to release kinin, but cleaves semenogelin at certain Tyr and Leu bonds. The predominant Arg, negligible Phe and low Leu specificities of gpK2 (Table 7) argue against a task analogous to PSA. The very low activities of all guinea pig TKs on ZLysArg-pNA exclude these enzymes from the role of cleaving at pairs of basic amino acid residues.

Large TK multigene families have been identified only in the rat (Wines *et al*., 1991), mouse (Evans *et al*., 1987; Berg *et al*., 1992), and the African rat *Mastomys* (Bowcock *et al*., 1988), which are closely related species. Similar multigene families comprising around 10 members have been reported for the trypsins of man (Emi *et al*., 1986), rat (Craik *et al*., 1984) and mouse (Stevenson *et al*., 1986). Evidently, the existence of such families of secretory serine proteinases does not necessarily imply that each of its members has a distinct or specific physiological function. The occurrence of not more than three and possibly even two products of independent TK genes in the guinea pig resembles the profile in man, where only three TKs are considered to exist (Baker and Shine, 1985; Schedlich *et al*., 1987; Riegman *et al*., 1992). Not more than 2 – 3 genes for such enzymes have also been reported in dog (Chapdelaine *et al*., 1991) and hamster (Howles *et al*., 1984). As pointed out also by Isackson and colleagues (Isackson *et al*., 1987), the existence in several species of only a few TKs does not support the suggestion that these enzymes convert many biologically important peptides into active compounds (Mason *et al*., 1983).

Materials and Methods

Materials

DEAE-Sephadex A-50, Sephadex G-100 and benzamidine-Sepharose were obtained from Pharmacia. Hydroxyapatite was Bio-Gel HTP from Bio-Rad. Diisopropyl fluorophosphate was obtained from Serva and *p*-amidino-phenylmethanesulfonyl fluoride and D-PhePheArgCH_2Cl from Calbiochem. 4-(2-Amino-ethyl)-benzenesulfonyl fluoride was a generous gift from Dr. J. Stürzebecher. D-ValLeuArg-pNA and D-ProPheArg-pNA were purchased from Kabi. D-ValLeuLys-pNA, D-SerLeuMet-pNA, BzLeuLeuArg-pNA and BzLeuLeuLys-pNA were provided by Kabi as generous gifts. BzPheLeuArg-pNA and ZValGlyArg-pNA were purchased from Medor, BzPheValArg-pNA from AB Bofors, TosGlyProArg-pNA, NAD and yeast alcohol dehydrogenase from Boehringer Mannheim, SucAlaAlaProPhe-pNA from Novabiochem, and BzProPheArg-pNA, ZGlyProArg-pNA and bradykinin from Sigma. The other *p*-nitroanilides and AcPheArgOEt · HCl were obtained from Bachem. GlyLeuPheCH_2Cl · HBr was prepared from ZGlyLeuPheCH_2Cl (purchased from Paesel & Lorei) analogous to the synthesis of PheCH_2Cl · HBr (Segal *et al.,* 1971).

Porcine pancreatic Aβ-kallikrein B (Fiedler *et al*., 1981; Kamada *et al*., 1990) treated with neuraminidase was obtained as generous gift from Bayer AG, Elberfeld. Human urinary kallikrein was kindly provided by Dr. R. Geiger (Geiger *et al*., 1980). Trypsin was bovine trypsin, TPCK-treated, from Merck. Isolation and properties of the bovine L-kininogen used are those outlined by Fiedler and Hinz (1992).

Determination of Enzyme Activity during Purification

One unit (U) of enzyme activity is defined as the amount hydrolysing 1 μmol of substrate per min. Arg esterase activity was measured as previously described by Fiedler *et al*. (1978). The molar absorbance of NADH at 366 nm was taken from Ziegenhorn *et al*. (1976).

Arg amidase activity was assayed with 0.1 mM D-ValLeuArg-pNA (Friberger *et al*., 1982) and Phe amidase activity with 0.1 mM SucPheLeuPhe-pNA at 25.0 °C in 0.1 M Tris/HCl, 0.1 mM EDTA buffer, pH 8.0. In the Phe amidase assay, the cuvette also contained 1% (v/v) dimethyl sulfoxide in which the 10 mM stock solution of substrate had been prepared. A_{405nm} was measured on a Kontron Uvikon 860 spectrophotometer. For the molar absorbance of *p*-nitroaniline a value of 9825 M^{-1} cm^{-1} was used (Lottenberg and Jackson, 1983). Microtiter plate assays for screening of column eluates with one of these *p*-nitroanilide substrates (in a 5-fold higher concentration) were run at room temperature in an analogous way. Each well contained a final volume of 200 μl, to which 1% of bovine serum albumin was added. Absorbance at 405 nm was read at intervals on a Dynatech MR 700 microplate reader.

To determine the protein concentration, A_{280nm} was measured. One A_{280} unit was the amount of material showing an absorbance of 1 (at 1 cm light path) when contained in 1 ml of solution. A_{260} and A_{320} were also measured as an indicator for the contribution of non-protein material to A_{280} or a check for turbidity, respectively.

Isolation of TKs from Submandibular Glands

All operations were performed at 4 – 6 °C. 31 g of submandibular glands (which had been stored frozen) from male guinea pigs were homogenised in 100 ml water in a Waring blendor and centrifuged. The supernatant was chromatographed on a 3.4 × 22 cm column of DEAE-Sephadex A 50 with 8.0 l of a linear gradient from 0 to 0.6 M NaCl in 50 mM Tris/HCl, pH 7.5, at 75 ml/h. Fractions with AcPheArgOEt esterase activity (beginning at 1.8 l from the start) were pooled, dialysed and lyophilised. They were rechromatographed in the same way, except using a somewhat steeper gradient of only 5.0 l. As in the preceding step, care was taken to pool all detectable esterase activity (beginning after 1.6 l). After dialysing twice against two 10 l-changes of water and two 5 l-changes of 50 mM Tris/HCl, pH 7.5, the material was applied at 20 ml/h to a 2.3 × 10 cm column of benzamidine-sepharose. After washing with 200 ml of the dialysis buffer, elution was started with a linear gradient (1.1 l) of 0 – 0.6 M NaCl in 50 mM Tris/HCl, pH 7.5, collecting 20 ml-fractions. These were assayed for AcPheArg-OEt-, D-ValLeuArg-pNA- and SucPheLeuPhe-pNA-hydrolysing activities as described below. Fractions of the three activity peaks A – C were pooled as indicated in Figure 1. Peak A was rechromatographed on benzamidine-Sepharose as before.

Fractions A, B and C were separately chromatographed on hydroxyapatite and DEAE-Sephadex as exemplified here for peak B. The material was dialysed against 4 1l-changes of 1 mM potassium phosphate, pH 7.0, and applied at 7 ml/h onto a 1.6 × 12 cm column of Bio-Gel HTP. After washing with 1 column volume of the 1 mM buffer, elution was performed with 250 ml of a linear gradient from 1 to 100 mM potassium phosphate, pH 7.0. The single peak of activity against the three substrates appearing after 60 ml in the eluate was pooled and dialysed against two 250 ml-changes of 50 mM Tris/HCl, pH 7.5. The final chromatography was performed at 2.5 ml/h on a 0.62 × 10 cm column of DEAE-Sephadex A-50 with 75 ml of a linear gradient from 0 to 0.6 M NaCl in 50 mM Tris/HCl, pH 7.5. The single peak of activity appeared at about 30 ml. Fractions with the highest A_{280}/A_{260} ratio and the highest specific activities were combined and stored at – 20 °C in aliquots.

Isolation of TK from Coagulating Gland/Prostate Complex

25 g of frozen CGC was homogenised with 125 ml water, centrifuged and chromatographed on DEAE-Sephadex with 5.0 l of a linear gradient exactly as described for submandibular glands. Fractions with Arg esterase activity (beginning after 2.2 l) were pooled, dialysed against 40 l water and lyophilised. The material was dissolved in 14 ml 50 mM Tris/HCl, pH 7.5, dialysed against 1.5 l of this buffer and applied to a 3.6 × 140 cm column of Sephadex G-100 equilibrated with the same buffer containing 0.2 M NaCl. Elution was performed at 30 ml/h. The single peak of Arg esterase activity was dialysed against 4 × 15 l 1 mM potassium phosphate, pH 7.0, and loaded onto a 5.4 × 12 cm Bio-Gel HTP column. The column was eluted at 90 ml/h with 2.7 l of a linear gradient from 1 to 100 mM potassium phosphate, pH 7.0. Again, the single Arg esterase activity peak beginning at 0.6 l was pooled.

An 8.0 ml aliquot dialysed against the starting buffer was chromatographed on a 0.7 × 3 cm column of benzamidine-Sepharose with 10 ml of the starting buffer followed by 30 ml of a linear gradient from 0 to 0.6 M NaCl in 50 mM Tris/HCl, pH 7.5, at 1 ml/h. The first artifact peak A of Arg esterase was rechromatographed in the same way after regenerating the column with 15 ml each of 0.5 M NaCl in 0.1 M Tris/HCl, pH 8.5, and 0.5 M NaCl in 0.1 M NaAc, pH 4.5, and equilibrating with 50 mM Tris/HCl, pH 7.5. The combined Arg esterase peaks B and B_r were finally chromatographed on a small column of DEAE-Sephadex as described for the submandibular enzyme.

Isolation of TKs from Pancreas

50 g of frozen guinea pig pancreas was homogenised with 125 ml water, centrifuged and chromatographed on DEAE-Sephadex as described. Fractions of the relatively broad peak of Arg esterase activity were pooled, dialysed against 40 l water followed by 8 l of 50 mM Tris/HCl, pH 7.5, and rechromatographed in the same way. After similar dialysis, the material from the only peak of Arg esterase was put onto a 1.3 × 7.5 cm column of benzamidine-Sepharose, washed with 65 ml 50 mM Tris/HCl, pH 7.5, at 6.5 ml/h and eluted with 250 ml of a linear gradient from 0 – 0.6 M NaCl in the same buffer. The main Arg esterase peak B was chromatographed on hydroxyapatite (scaled down by a factor of 5) and DEAE-Sephadex as described for submandibular TK peak B, while peak A was rechromatographed on DEAE-Sephadex only.

Determination of Specific Activities and Substrate Specificities

Submandibular gpK1 and gpK1a and CPC gpK2 showed similar high A_{280}/A_{260} ratios of 1.84 and 1.85. Determination of protein concentrations from A_{280} seemed therefore justified. Values for previously isolated TK from guinea pig submandibular glands (A_{280}/A_{260} = 1.85, a molar absorption coefficient ϵ_{280} of 35 200 M^{-1} cm^{-1} and M_r = 25 900 for the protein; Fiedler *et al*., 1983), were considered to apply to the present preparation of gpK1. Since gpK1a closely resembled gpK1, the same constants were used. The molar absorbance ϵ_{280} of CPC gpK2 was calculated from 6 Tyr residues and 5 disulfides and assuming 5.5 Trp [as an average of 5 Trp of the Lys form and 6 Trp of the Trp form of the enzyme with a protein M_r of 26 300 (Dunbar and Bradshaw, 1987)] per molecule, as an average of 39 800 M^{-1} cm^{-1} (individual values 39 950 or 39 580) by means of two sets of published absorbance increments (Edelhoch, 1967; Mach *et al*., 1992). This procedure was found to be valid in the case of TKs (Fiedler *et al*., 1983).

Activity measurements with *p*-nitro-anilide substrates were conducted in 0.1 M Tris/HCl, 0.1 M NaCl (to approximate physiological ionic strength), 0.1 mM EDTA (as precaution against inhibi-

tion by contaminating heavy metal ions; Fiedler *et al*., 1978,1981), pH 8.0, 25.0 °C, as standard conditions. The reactions in 1 ml volume were followed on a Kontron Uvikon 860 spectrophotometer at 405 nm. ϵ_{405} = 9935 M^{-1} cm^{-1} (Lottenberg and Jackson, 1983) was used under these conditions. TK activities were assayed with 0.1 mM D-ValLeuArg-pNA from a 10 mM stock solution in water. Tests were usually run for 15 or 20 min. When the necessary dilutions of the solutions of guinea pig TKs were made with Tris buffer or in polypropylene vessels, activity losses were observed. Therefore, enzyme solutions were diluted in Pyrex glass vessels with 1 mM potassium phosphate, pH 7.0.

For the determination of substrate specificities, 1 mM stock solutions in water were prepared from the Lys- and Arg-*p*-nitroanilides, except BzPheLeu(and Val)Arg-pNA, BzLeuLeuArg-(and Lys)-pNA and ZPheArg-pNA which were 0.5 mM and were warmed to 37 °C prior to use to redissolve any crystallised material. 10 mM Phe-, Leu- and Met-*p*-nitroanilides were dissolved in dimethyl sulfoxide, and assays with these compounds contained 1% (v/v) dimethyl sulfoxide. Substrate concentrations were checked by the absorbance at 405 nm after complete hydrolysis by bovine trypsin or chymotrypsin and adjusted to 0.1 mM in the assays. Water and buffer were filtered through 0.45 μm cellulose nitrate membrane filters (Sartorius).

Determination of N-Terminal Amino Acid Sequences

Amino acid sequence analyses were performed on a 477A pulsed liquid phase sequencer equipped with an on-line 120A PTH analyser (both Applied Biosystems, Weiterstadt) according to the instructions of the manufacturer.

Determination of Inhibition Constants

Rates of inhibition of TK by active site-directed irreversible inhibitors were determined at 25.0 °C in 0.05 M Tris/HCl, 0.1 M NaCl, 0.1 mM EDTA, pH 7.0, under conditions $[I] >> [E]_0$. After starting the reaction by addition of inhibitor in the specified concentration, samples were withdrawn at intervals and assayed for residual enzyme activity with D-ValLeuArg-pNA and occasionally with SucPheLeuPhe-pNA. To increase the sensitivity of the latter assay, the Tris/NaCl buffer pH 7.0 just mentioned and 0.5 mM substrate [and consequently 5% (v/v) dimethyl sulfoxide] were used. After graphical inspection of the data, inhibition constants k_i' were calculated according to the basic first order rate equation, $[E] = [E_0] \cdot e^{-k' \cdot t}$ or an appropriate expansion by nonlinear regression.

Rates of inhibition by 0.5 mM $GlyLeuPheCH_2Cl$ were also directly followed spectrophotometrically in 1 ml cuvettes in the presence of 0.1 mM D-ValLeuArg-pNA substrate under otherwise identical conditions for about 1.5 half lives, when less than 5% of the substrate was hydrolysed. Effective inhibition rate constants k' were calculated for constant [S] and [I] by nonlinear regression according to $[P] = [P]_0 + v_0 (1 - e^{-k' \cdot t})/k'$ (Gray and Duggleby, 1989).

Determination of Kinin Release and Blood Pressure Decrease

Kinin release from bovine L-kininogen (4.5 μM) was followed at 25 °C in 0.1 M Tris/HCl, 0.1 M NaCl, 0.1 mM EDTA, pH 8.0, in a volume of 0.44 ml. The reaction was started by the addition of enzyme (0.8 D-ValLeuArg-pNA-mU of gpK1 or gpK1a or 8 mU of gpK2a). 100 μl samples were withdrawn at 5, 20, 60 and 200 min, stopped with 20 μl 2 N HCl and analysed for kinins in the isocratic pH 3 HPLC system (Fiedler and Geiger, 1988). Enzyme activity was determined in parallel with the assay with D-ValLeuArg-pNA in the presence of 0.1 M NaCl.

The hypotensive action of TKs was measured on adult (750 g) guinea pigs anaesthetised with intraperitoneal urethane. Carotid arterial blood pressure was recorded with a Statham pressure transducer linked to a Beckman R511A multichannel recorder. 100 μl-samples were injected in 10 min-intervals through the cannulated external juglar vein and washed immediately into circulation with 200 μl 0.9% NaCl containing 3.5 units of heparin. Activities were match assayed against bradykinin standards.

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