Biochemical Pharmacology, Vol. 43, No. 6, pp. 1209–1217, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

NEW SPECIFIC ASSAYS FOR TONIN AND TISSUE KALLIKREIN ACTIVITIES IN RAT SUBMANDIBULAR GLANDS

ASSAYS REVEAL DIFFERENCES IN THE EFFECTS OF SYMPATHETIC AND PARASYMPATHETIC STIMULATION ON PROTEINASES IN SALIVA

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(*Received* 17 *September 1991; accepted* 16 *December* 1991)

Abstract—At least fourteen separate bands of proteinase activity, labelled A–N, were identified by an enzyme overlay membrane technique, using oligopeptide–7-amino-4-trifluoromethylcoumarin (AFC) substrates in rat submandibular gland extracts fractionated on pH 4–6.5 isoelectric focusing gels. The proteinases were eluted into an ammonium bicarbonate buffer pH 9.8 containing 0.1% Triton X-100 and the relative contribution of each band to total activity evaluated using D-Val-Leu-Arg-AFC (DVLR-AFC) and Z-Val-Lys-Lys-Arg-AFC (ZVKKR-AFC) as substrates. Immunoblotting of band eluants run on sodium dodecyl sulphate gels with antibodies showed that band A was identical with tonin and bands K–N contained tissue kallikrein. Tonin was found to hydrolyse ZVKKR-AFC but not DVLR-AFC. Estimates of the K_m values of tissue kallikrein for DVLR-AFC and tonin for ZVKKR-AFC were found to be similar (approx. 20 μM) yet the former enzyme hydrolysed its substrate five times faster. Tonin was inhibited by soybean trypsin inhibitor (SBTI) but not by aprotinin. Tissue kallikrein, on the other hand, was inhibited by aprotinin but was considerably more resistant to inhibition by SBTI. In tissue extracts 95% of the ZVKKR-AFC lytic activity in the presence of 1 μM aprotinin is due to tonin and a similar percentage of the DVLR-AFC hydrolysing activity in the presence of 10 μM SBTI is due to tissue kallikrein. These findings were used for the specific measurement of these two proteinases in submandibular gland extracts and in saliva without prior purification. Using these inhibitor based assays we revealed qualitative differences in the composition of proteinases secreted into saliva during parasympathetic versus sympathetic stimulation of the submandibular gland. The distribution of proteinases in sympathetic saliva is very similar to that found in submandibular extracts but on parasympathetic stimulation, although much less proteinase is released, the contributions of the more acidic isomers of tissue kallikrein are increased and that of tonin and other proteinases dramatically decreased. The data suggest that parasympathetic and sympathetic nerves induce proteinase secretion via different pathways.

Since the discovery of tissue kallikrein in urine by Frey in 1926 [1], a large family of closely related serine proteinases has been described. There are major differences in the number of kallikrein-like genes in the genome of different species; the mouse has approximately 24, the rat as many as 17 and human 3–5 [2–4]. In the rat submandibular salivary gland these enzymes are stored in high concentrations, packed into granules, in convoluted tubule cells and are released into saliva under autonomic control. While the function of these proteinases in saliva remains to be established their release into the vasculature may be of importance in the local regulation of blood flow and the processing of precursor proteins into pharmacologically active molecules. For example, tissue kallikrein is known to liberate the vasodilatory peptide, lys-bradykinin (kallidin), from kininogen and has been implicated in the processing of peptide hormones such as enkephalins [5–7]. Tonin is known to act on the tetradecapeptide renin substrate to release angiotensin I and then convert angiotensin I to the potent vasoconstrictor angiotensin II; it may also be involved in the activation of tissue pro-kallikrein [8, 9].

Specific assays for tissue kallikrein and tonin in saliva, urine or other body fluids could be of value as they can be used as indicators of changes in systemic physiology. Tissue kallikrein levels appear to be raised in the saliva of patients with connective tissue diseases such as rheumatoid arthritis [10] and in the urine in secondary hyperaldosteronism (Bartter's syndrome), immediately prior to the onset of menstruation and during dietary sodium restriction [11–13]. Dramatic reductions in the kallikrein content

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were seen in streptozotocin-induced diabetic rats [14] and kallikrein levels are known to be lower in the saliva of patients with sarcoidosis [15]. Although primates do not express tonin, for the proper assessment of the rat model of human hypertension, accurate measurements of tonin would be of value.

Earlier bioassays for tissue kallikrein based on the release of kinin from kininogen or by direct radioimmunoassay are relatively complicated, expensive, time consuming and may be difficult to reproduce between laboratories [16, 17]. Similar potential problems apply to the assay of tonin using angiotensin I [18] or by direct radioimmunoassay [19]. Assays based on the catalysis of low molecular weight synthetic oligopeptide substrates have become increasingly favoured because of their simplicity. An assay specific for tissue kallikrein using D-Val-Leu-Arg-*p*-nitroanilide in the presence of soybean trypsin inhibitor (SBTI‡) had been described by Jenzano *et al.* [20] without being fully characterized. Similarly a number of relatively insensitive synthetic substrates have been described for tonin [21]. This paper gives the first published account of a sensitive synthetic substrate for tonin.

MATERIALS AND METHODS

Chemicals. The oligopeptide fluorogenic substrates D-Val-Leu-Arg–7-amino-4-trifluoromethylcoumarin (DVLR-AFC) and *Z*-Val-Lys-Lys-Arg–AFC (ZVKKR-AFC) were from Enzyme Systems Products, U.S.A. Cellulose diacetate sheets were from Sartorious and 0.45 μm nitrocellulose was from Schleicher and Schuell. SBTI, affinity purified bovine lung aprotinin and angiotensin I were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Collection and preparation of submandibular fluids. Adult male Wistar rats, fasted overnight, were anaesthetized with pentabarbitone (35 mg/kg i.p.) followed by chloralose (75 mg/kg i.v.). A submandibular duct was cannulated and samples of saliva secreted during sympathetic or parasympathetic stimulation were collected. For parasympathetic saliva, the chorda lingual nerve was cut and the peripheral stump of the nerve was electrically stimulated on the submandibular duct at 10 Hz (4–6 V). Sympathetic saliva was evoked by stimulating the sympathetic nerve trunk in the neck, caudal to the superior cervical ganglion, at 50 Hz (4–6 V) in bursts of 1 sec every 10 sec. Four per cent homogenates were prepared at 4° from control (non-stimulated) glands by disrupting the tissue with an IKA Ultra-Turrax disperser, set at 20,000 rpm, in 100 mM phosphate buffer, pH 6.0, containing 2 mM EDTA and 0.02% Triton X-100. The homogenates were spun at 13,000 *g* for 20 min and the supernatants stored at −20°.

The enzyme overlay membrane (EOM) technique. Rat submandibular proteins were focused on 1 mm thick, pH 4.0–6.5, precast gels (Ampholine PAG, Pharmacia, Uppsala, Sweden) set up on a flat bed isoelectric focusing (IEF) unit (Multiphor II, Pharmacia) cooled to 10° (Multitemp, Pharmacia) and run according to the manufacturer's instructions. Fifty microlitres of test samples were applied to 50 mm^2 pieces of electrode wick placed 10 mm from the cathode. The proteinases were visualized under long wave UV light by overlaying the focused gels with cellulose diacetate membranes impregnated with 100 μM solutions of the fluorogenic oligopeptide proteinase substrates DVLR-AFC or ZVKKR-AFC as described previously [22]. Where permanent records were required the membrane was peeled off at this stage and dried; the gels were stained with Coomassie blue R-250. In experiments involving further characterization of individual proteinases, the fluorescent bands were carefully excised and the protein in the underlying gel eluted into 1 mL of 20 mM ammonium bicarbonate buffer, pH 9.8, containing 0.1% Triton X-100 (for biochemical studies) or 0.1% sodium dodecyl sulphate (SDS) (for electrophoresis on SDS gels) [23]. The proteinases were optimally eluted by agitating the gel pieces in buffer for 3 hr at 4°.

Immunoblotting. Major bands showing proteinase activity with the EOM technique were excised and eluted into 20 mM ammonium bicarbonate buffer, pH 9.8, containing 0.1% SDS, lyophilized and run on 10% SDS gels under reducing conditions in accordance to Laemmli [24]. The proteins were electroblotted onto 0.45 μm pore size nitrocellulose. The blots were quenched overnight in Blotto (5% low fat milk powder in 50 mM Tris buffer, pH 8.0, containing 0.15 M sodium chloride and 0.02% azide) before a 3 hr exposure to a 1:200 dilution, in Blotto, of a polyclonal antibody to rat urinary kallikrein or a monoclonal antibody to tonin. Thoroughly washed blots were incubated with a 1:100 dilution of the appropriate second antibody–gold conjugate (Biocell Research Laboratories, U.K.). After washing, blots were incubated in silver enhancing solution, fixed with Super Amfix and dried.

General assay for proteinase with AFC substrates. Initial reaction velocities of suitably diluted band eluants were measured against 10 μM AFC substrates (0.1–30 μM for K_m and V_{max} measurements) in 50 mM Tris buffer, pH 8.0, containing 100 μg/mL bovine serum albumin (BSA) and 0.02% azide. Extracts with and without inhibitors were pre-incubated for 5 min at 30° before addition of substrate. Following a 10 min incubation at 30° of the enzyme with substrate the fluorescence was read on an Ames Fluorostat (Miles Laboratories) fitted with a 405 nm excitation filter and a 500 nm emission filter and calibrated with 0.1–2 μM free AFC. The initial rate of hydrolysis of the substrate was maintained for at least 30 min of the reaction. BSA was included in the incubation medium because it was found to be very effective in preventing loss of enzymatic activity thought to be caused by the adsorption of the enzyme to the reaction vessels (manuscript in preparation); BSA does not itself appear to be a substrate for the proteinases under the conditions used in the experiments.

Assay for tonin by angiotensin I cleavage. The

‡ Abbreviations: AFC, 7-amino-4-trifluoromethylcoumarin; DVLR-AFC, D-Val-Leu-Arg-AFC; ZVKKR-AFC, *Z*-Val-Lys-Lys-Arg-AFC; BSA, bovine serum albumin; EOM, enzyme overlay membrane; IEF, isoelectric focusing; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate; DFP, diisopropylfluorophosphate.

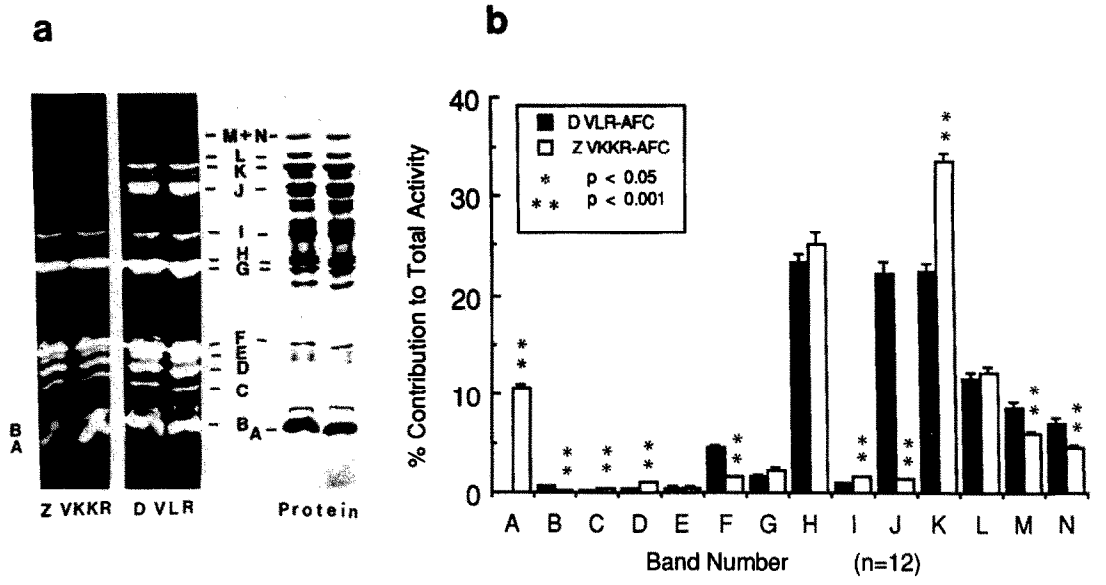

Fig. 1. (a) Proteinase bands A–N in rat submandibular gland homogenates run on pH 4–6.5 IEF gels were localized under UV light by overlaying focused gels with cellulose diacetate membranes impregnated with 100 μM ZVKKR-AFC and DVLR-AFC as substrates. The fluorograms were peeled off the gels and dried for permanent records. The IEF gel was stained for protein with Coomassie blue R250. (b) Proteinases in bands A–N were eluted into an ammonium bicarbonate buffer containing 0.1% Triton X-100 as described in Materials and Methods. Amydolytic activity against 10 μM DVLR-AFC and ZVKKR-AFC, respectively, was measured at pH 8.0 following a 10 min incubation at 30°. The results, based on an average of 12 experiments, are presented as percentages of total observed activity.

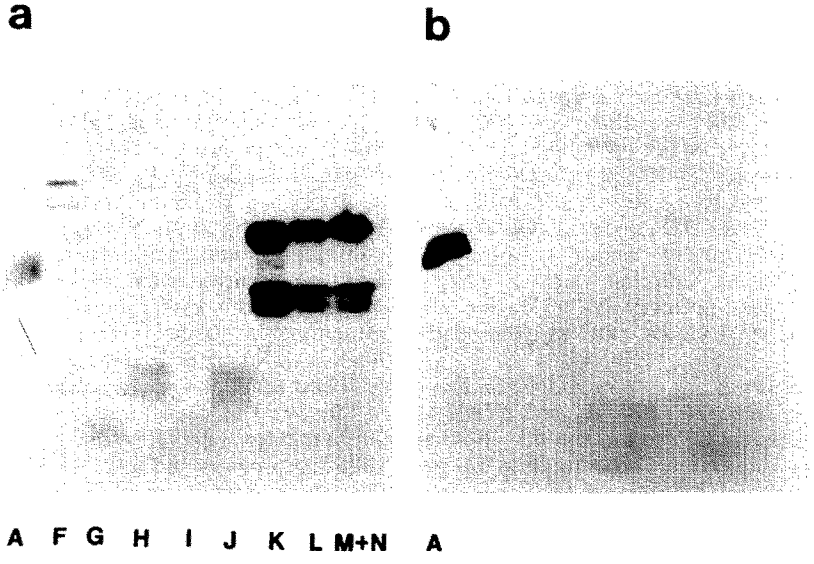

Fig. 2. Bands A and F–N showing proteinase activity with the EOM technique were eluted into an ammonium bicarbonate buffer, pH 9.8, containing 0.1% SDS, lyophilized and run on 10% SDS gels under reducing conditions in accordance to Laemmli [24]. The proteins were electroblotted onto 0.45 μM pore size nitrocellulose. The blots, quenched with Blotto, were probed with a polyclonal antibody to tissue kallikrein (a) and a monoclonal antibody to tonin (b). Immunopositive bands were detected by silver enhancement of blots incubated with gold-labelled second antibody.

assay is a modification of the procedure first described by Boucher *et al.* [9]. Suitably diluted homogenates were incubated with 50 μg of angiotensin I in 1.5 mL of 50 mM phosphate buffer, pH 7.0, containing 1.33 mM EDTA and 10 μL of 20% dipyridyl in methanol. After a 1 hr incubation at 37° the enzymatic reaction was terminated by addition of 250 μL of 2 M sodium hydroxide. For the detection of the released His-Leu dipeptide 50 μL of fresh 0.1% *O*-phthaldialdehyde in 25% methanol were added and the mixture left for 5 min. Two hundred microlitres of 34% orthophosphoric acid was added and the developed fluorescence read on an Ames Fluorostat after 5 min. The instrument was fitted with 360 nm excitation and 440 nm emission filters and calibrated with 0–60 nM His-Leu dipeptide.

RESULTS

Submandibular gland homogenates were focused on pH 4–6.5 IEF gels, and following this the positions of the proteinases were located by the EOM technique using DVLR-AFC and ZVKKR-AFC as substrates. Using DVLR-AFC at least 13 proteinase bands of varying intensities (labelled B–N), were identified as shown in Fig. 1a. It failed to reveal the presence of a major proteinase demonstrated by ZVKKR-AFC at position A. With the exception of band A the latter substrate produced bands of lower intensity for similar incubation times. Direct comparison of fluorescent bands seen on EOMs with stained IEF gels showed that proteinase activity is associated with major protein bands. Figure 1b compares the distribution of activities associated with each band, eluted into a Triton X-100 buffer, using DVLR-AFC and ZVKKR-AFC as substrates. The results are expressed as percentages of total activities observed for each substrate. Although the overall activities are at least four times higher when DVLR-AFC is used as a substrate (data not shown), within the confines of its total activity ZVKKR-AFC is the preferred substrate for bands A and K but was found to be much less reactive towards band J.

The positions of tissue kallikrein and tonin on focused gels were identified by immunoblotting, following SDS–polyacrylamide gel electrophoresis (Fig. 2). Band A was found to run at a molecular mass of approximately 33 kDa and crossreacted with a monoclonal antibody to tonin. A polyclonal antibody to rat urinary kallikrein reacted strongly with a molecular mass species of 38–39 kDa and less strongly with one at 31 kDa in extracts from bands K–N.

Analysis of the preceding data on individual band eluants indicates that both DVLR-AFC and ZVKKR-AFC could potentially be used in the detection of tissue kallikrein isomers. In order to develop an assay selective for tissue kallikrein in crude mixtures a number of potential inhibitors were tested to determine whether it is possible selectively to inhibit interfering proteinases while still retaining tissue kallikrein activity. Figure 3a shows the effect of a range of concentrations of SBTI on an extract of tissue kallikrein. DVLR-AFC lytic activity appears to be more resistant than ZVKKR-AFC hydrolysing activity to inhibition by this inhibitor. The multiphasic

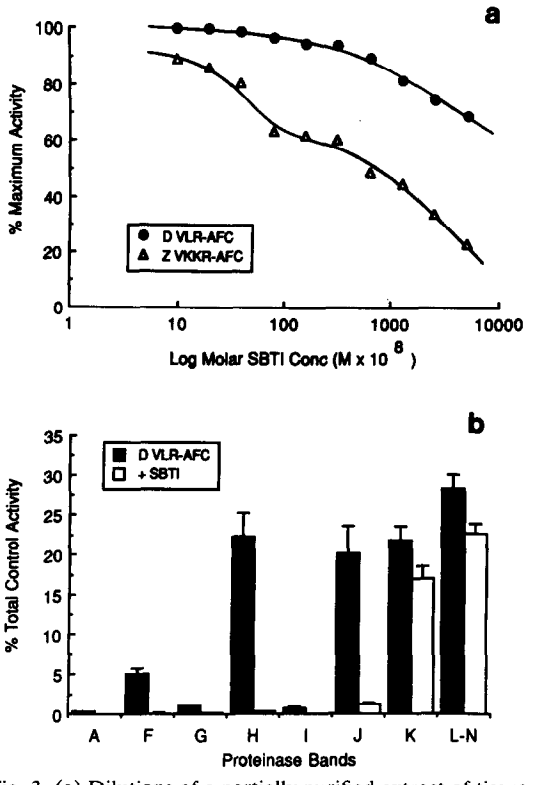

Fig. 3. (a) Dilutions of a partially purified extract of tissue kallikrein (bands K–N) were preincubated for 5 min at 30° with a range of concentrations of SBTI. Residual amidolytic activity was measured using DVLR-AFC and ZVKKR-AFC as substrates. (b) The inhibitory effect of 10 μM SBTI on the amidolytic activity of some of the major proteinase bands using DVLR-AFC as a substrate is shown. The data is based on four experiments.

nature of the observed inhibition curve with ZVKKR-AFC suggests differences in the susceptibility of different isomers of tissue kallikrein to inhibition by SBTI. Indeed with ZVKKR-AFC as a substrate 10 μM SBTI produced a 62% inhibition of band K and only a 37% inhibition of a pooled extract of bands L–N ($P < 0.01$, $N = 6$). On the other hand, only a 20% inhibition of the various isomers of tissue kallikrein occurred when DVLR-AFC was used as a substrate. Tissue kallikrein is in fact the only major proteinase in submandibular extracts that shows relative resistance to inhibition by SBTI. The effect of 10 μM SBTI on individual band eluants is shown in Fig. 3b. This concentration of SBTI produces a 20% inhibition of DVLR-AFC lytic activity of tissue kallikrein isomers and a 95% inhibition of band J, the only other major band showing resistance to the inhibitor.

As opposed to the proteinases detected by DVLR-AFC, ZVKKR-AFC has an advantage in that unlike DVLR-AFC it also forms a good substrate for tonin. The selective inhibition, by aprotinin, of ZVKKR-AFC hydrolysing activity of all submandibular proteinases other than tonin has been used to develop a new fluorometric assay specific for tonin.

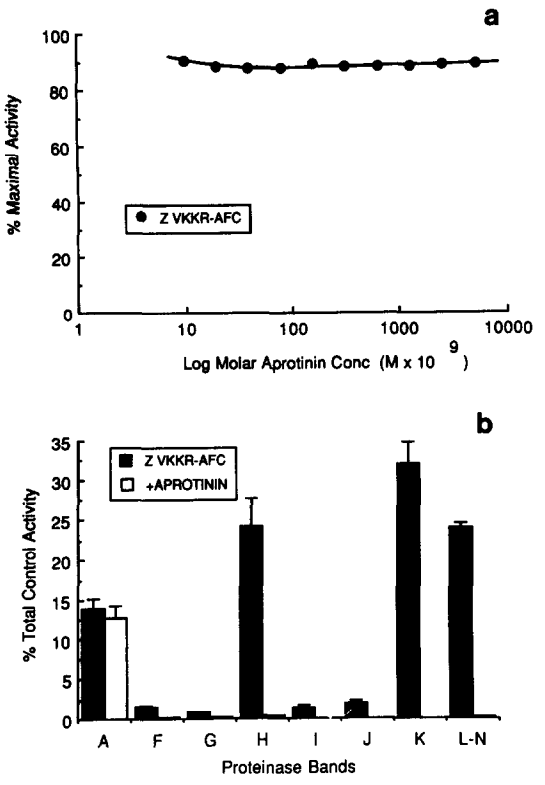

Fig. 4. (a) The inhibitory effect of a serial dilution of aprotinin on ZVKKR-AFC lytic activity in band A extracts is shown. (b) The inhibitory effect of 1 μM aprotinin on amidolytic activity in various band eluants using 10 μM ZVKKR-AFC as a substrate is presented. The results are based on four experiments.

Figure 4a shows that the ability of band A eluants to hydrolyse ZVKKR-AFC is resistant to inhibition by aprotinin. The small amount of inhibition seen with very low concentrations of aprotinin probably represents contamination of these extracts by band B. Further analysis of the effect of aprotinin on individual band eluants showed that in homogenates nearly 95% of the activity resistant to 1 μM aprotinin is due to tonin (Fig. 4b).

Comparison of tonin recovered from the gel, measured by the two tonin assays, with that present in the original homogenates showed that a large percentage of the activity never entered the gel and remained in the fluid at the application site (Table 1). Expanding the pH gradient was found to increase the quantity of tonin moving into the IEF gel although a substantial percentage of this proteinase still remained at the application site. This contrasts dramatically with the situation found with tissue kallikrein where only a small percentage of the activity was retained within the applicator.

Interestingly, although no loss of tonin activity was incurred as a result of IEF, it was possible to account for only 76% of the original kallikrein content in homogenates after IEF. Retention of a large percentage of tonin activity at the application site together with the observed losses of some tissue kallikrein activity as a result of electrophoresis raised the question whether further isomers of these proteinases may be present in crude homogenates.

Having established that 95% of the SBTI resistant DVLR-AFC lytic activity is due to tissue kallikrein and a similar percentage of aprotinin resistant ZVKKR-AFC lysing activity is due to tonin, checks were made on the effect of a second inhibitor in a direct comparison between homogenates and purified band eluants. The near identity of the inhibition curves for the proteinases in their crude and purified states, as shown by Fig. 5a and b probably exclude the possibility of any unaccounted for subspecies of these proteinases.

Biochemical parameters for the two proteinases are shown in Table 2. Although tissue kallikrein and tonin react with their respective substrates with similar affinities the former can hydrolyse its substrate approximately five times faster. Measurements of the protein content of IEF band eluates necessary for V_{max} measurements were as described previously [25]. Data on inhibitors show that although tonin is some 300 times less resistant than tissue kallikrein to inhibition by SBTI it is more resistant to inhibition by aprotinin and by DFP.

By using SBTI resistance and aprotinin resistance as indices we have found that there is a large compositional difference in the tissue kallikrein and tonin content of parasympathetic saliva when compared to their respective percentages in homogenates and in sympathetic saliva (Table 3).

Although parasympathetic stimulation produced a nearly 10-fold faster mobilization of fluid, the proteinase content of the evoked saliva is some 2–4000 times lower, depending on the substrate. The ZVKKR-AFC to DVLR-AFC ratio of parasympathetic saliva is only half that observed in homogenates or sympathetic saliva ($P < 0.01$), further indicating a change in proteinase composition. Measurements of tissue kallikrein and tonin content by the specific inhibitor-based assays showed that in parasympathetic saliva the net contribution of tissue kallikrein is doubled and that of tonin is only a quarter that found in homogenate or in sympathetic saliva.

Further confirmation of an altered composition of proteinase content of parasympathetic saliva came from analysis of extracts from IEF gels. Highly concentrated parasympathetic saliva and suitably diluted sympathetic saliva were matched for proteinase content with homogenate (Fig. 6a). DVLR-AFC was used as a substrate on the overlay membranes. The EOMs indicated that the percentage contribution of bands L, M and N was increased and that of B–K reduced in parasympathetic saliva. Decreased Coomassie blue staining in the band A region further indicated that the contribution of tonin too was considerably reduced.

Biochemical measurements with DVLR-AFC on band eluants from IEF gels run with sympathetic and parasympathetic saliva are presented in Fig. 6b. The distribution of DVLR-AFC hydrolysing activity, in the various band eluants, in sympathetic saliva was found to be very similar to that in homogenates (Fig. 1b). Apart from the minor band G, the

Table 1. Distribution of tissue kallikrein and tonin between gel and applicator following electrophoresis of homogenates on IEF gels

Proteinase	pH range of IEF gel	% of activity before IEF		
		In applicator	In gel	% accounted for
Tissue kallikrein	4–6.5	4.23 ± 0.46	71.86 ± 8.66	76.1 (N = 4)
Tonin	4–6.5	52.99 ± 5.95	42.09 ± 4.75	95.1 (N = 6)
Tonin	3.5–9.5	37.80 ± 1.59	65.19 ± 1.34	103.0 (N = 3)

Homogenates were focused on IEF gels. The proteinases in the sample applicator strips and in the appropriate regions of gel were eluted. Extracts were assayed for tonin and tissue kallikrein content by inhibitor-linked assays, described in Results, except for the 3.5–9.5 gel where the older angiotensin I based assay was used.

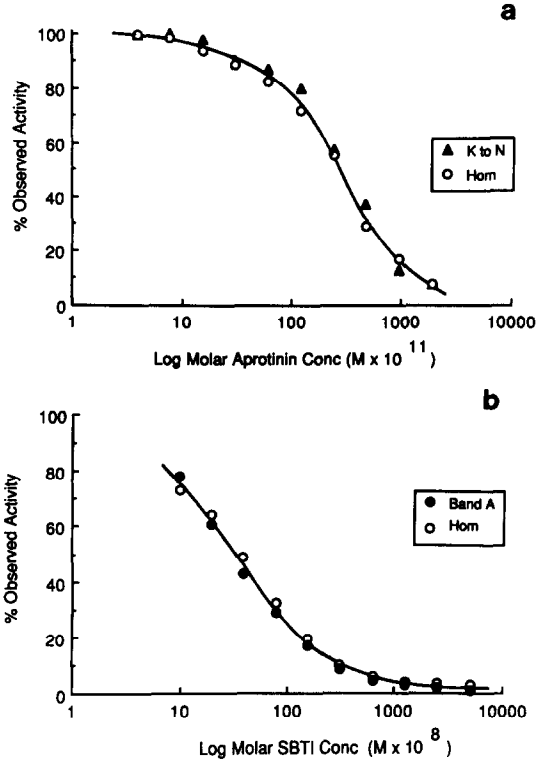

Fig. 5. (a) The inhibitory effects of a serial dilution of aprotinin on 10 μM SBTI resistant DVLR-AFC lytic activities in homogenate (Hom) and purified tissue kallikrein extracts were investigated. (b) The inhibitory effects of a range of concentrations of SBTI on the 1 μM aprotinin resistant ZVKKR-AFC amidolytic activities in a homogenate (Hom) and a purified band A extract are shown.

distribution of proteinases in parasympathetic saliva differed radically from that in sympathetic saliva. The estimates of the net contribution of tissue kallikrein to the proteinase contents of parasympathetic and sympathetic saliva, as determined on IEF gel extracts (85% and 50%, respectively), were remarkably close to the values obtained by the *in vitro* assay of the salivas in the presence of SBTI (88% and 45%, respectively). Different extracts were used in the two measurements.

DISCUSSION

In this study, we have developed simple sensitive assays for tissue kallikrein and tonin using DVLR-AFC and ZVKKR-AFC, respectively, as substrates. When used in conjunction with SBTI the DVLR-AFC assay will accurately measure tissue kallikrein against a background of other closely related serine proteinases. Similarly, ZVKKR-AFC assay in the presence of aprotinin is selective for tonin. Of central importance in the development of these assays was the use of the EOM technique. Although this method was first described by Smith and Grabske nearly a decade ago [26] its true potential as a powerful analytical tool has until now remained unrealized. The technique was found to be particularly useful, when coupled with an extraction protocol in the rapid identification of tissue kallikrein and tonin following electrophoresis of rat submandibular gland homogenates on IEF gels and in the characterization of individual proteinases.

There is a growing volume of literature describing a large number of closely related serine proteinases in the rat submandibular gland and recently attempts have been made to link specific enzymes with the products of specific genes [27]. This has become increasingly important as careful analysis of published data reveals that the same enzyme may have been given more than one name by different authors [28–31]. The situation is made even more complex where several isomers of the same protein exist [32]. Undoubtedly, the EOM technique can play a central role in helping to resolve many of the current duplications of enzymes in the literature, and should provide a sound biochemical basis for the reclassification of duplicated enzymes.

ZVKKR-AFC is an unusual substrate for the serine proteinases, having been previously used for the measurement of cysteine proteinases such as cathepsin B [33] which operate optimally at low pH values. Interestingly the pH optimum (9.0) with the AFC derivative was higher than that found when angiotensin I is used as a substrate (pH 7.0, data not shown).

Table 2. Estimates of biochemical parameters of tissue kallikrein and tonin: effect of inhibitors

Enzyme	Tissue kallikrein	Tonin
Substrate	DVLR-AFC	ZVKKR-AFC
K_m (M × 10^6)	22.33 ± 0.67 (N = 7)	20.25 ± 1.30 (N = 12)
V_{max}/min/mg	24.99 ± 2.08 (N = 7)	4.40 ± 0.33 (N = 12)
IC_{50} (M × 10^9)		
Aprotinin	1.5	NI
SBTI	103,000	309
Resistance to DFP (%)		
1 mM	20.27 ± 2.15 (N = 5)	89.31 ± 2.36 (N = 8)
10 mM	3.36 ± 0.28 (N = 5)	63.24 ± 1.47 (N = 4)

K_m and V_{max} measurements were on purified band eluants in the absence of inhibitor. Effect of inhibitors was measured on homogenates using the inhibitor-linked assays. IC_{50} values are based on the concentration of inhibitor producing 50% inhibition of activity. To evaluate the effects of DFP, homogenates were preincubated overnight at room temperature with the inhibitor or with water before estimation of tissue kallikrein or tonin content.

NI, not inhibited.

Table 3. Altered percentage contributions of tonin and tissue kallikrein to the proteinase content of parasympathetic saliva compared to homogenate and sympathetic saliva

	Flow rate (μL/min)	Total proteinase activity* DVLR-AFC	Total proteinase activity* ZVKKR-AFC	Tissue kallikrein (DVLR-AFC: %)	Tonin (ZVKKR-AFC: %)
Homogenate (N = 12)	—	—	—	41.31 ± 1.96	11.10 ± 0.44
Parasympathetic saliva (N = 12)	40.04 ± 6.75†‡	0.047 ± 0.007†	0.005 ± 0.0001†	88.32 ± 1.42†	3.20 ± 0.34†
Sympathetic saliva (N = 7)	4.12 ± 0.55	99.36 ± 20.44	18.59 ± 4.18	45.11 ± 2.15	12.59 ± 0.27

Sympathetic and parasympathetic salivas were collected from anaesthetized rats as described in Materials and Methods. Tissue kallikrein and tonin content was measured by inhibitor-linked assays.

Figures assume 20% inhibition of tissue kallikrein by SBTI (DVLR-AFC) assay.

* μmol/min/mL with 10 μM substrate.

† $P < 0.01$.

‡ N = 6.

In many ways tonin does not appear to behave like a typical serine proteinase in the classification scheme proposed by Hartley [34]. For although it is inhibited by SBTI it is not inhibited by purified aprotinin and only partially inhibited by high concentrations of DFP. This situation is very different from the tissue kallikrein isomers which are considerably more resistant to inhibition by SBTI but very susceptible to inhibition by low concentrations of DFP and aprotinin. Reports, using synthetic peptide derivatives, claiming inhibition of tonin by DFP or aprotinin have to be treated with caution [8, 21]. The results may be explained in terms of contamination of tonin preparations by band B which could represent salivain [35]. The proteinase in this band has a pI and molecular mass close to that of tonin (data not shown), but has a different substrate specificity (DVLR-AFC vs ZVKKR-AFC). It may thus have been difficult to separate the protein from tonin in some experimental protocols that have used ion-exchange chromatography, chromatofocusing or gel filtration [31].

ZVKKR-AFC forms an excellent substrate for tonin as our estimates of the Michaelis–Menten constant using ZVKKR-AFC as a substrate (K_m approx. 20 μM) are lower than published by Boucher *et al.* [9] (53 μM) using angiotensin I as a substrate and considerably lower than those found by Thibault *et al.* [21] with some other synthetic substrates.

Although IEF followed by elution gives an accurate measure of the composition, before electrophoresis, of most proteinases there was some retention of tonin on applicators. This could have arisen from preferential interactions of the proteinase with other proteins such as the large 350,000 kDa "inhibitor" plasma protein or the 720,000 kDa α_2-macroglobulin [36, 37]. Further, the observed loss of some tissue kallikrein activity on IEF gels could

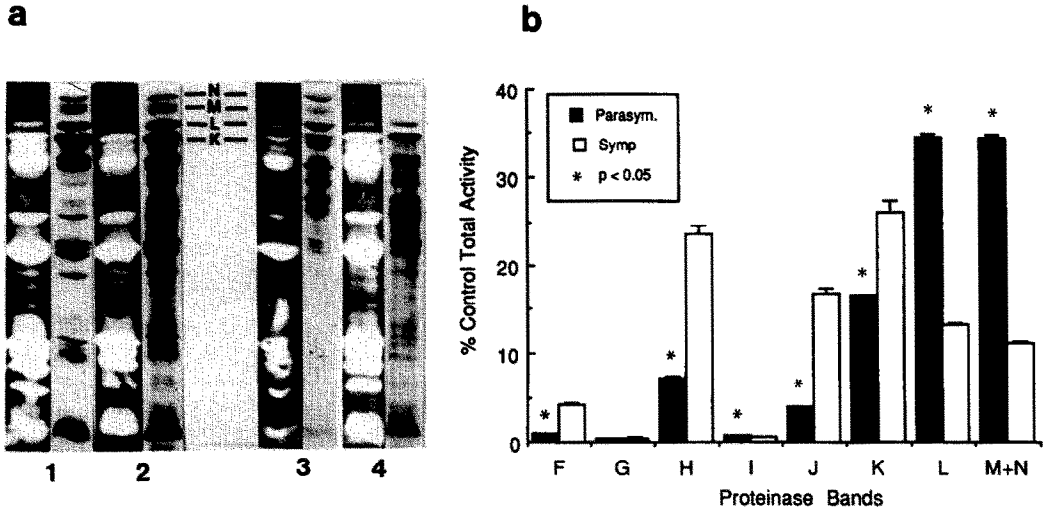

Fig. 6. (a) A comparison was made of the DVLR-AFC lytic activity in sympathetic saliva and homogenate (1,2) with parasympathetic saliva and homogenate (3,4) using the EOM technique. The appropriate strips of IEF gels after staining with Coomassie blue R250 are also shown. The kallikrein content of 5% sympathetic saliva (1) was found to match with 4% homogenate (2) and 17 times concentrated parasympathetic saliva (3) with 1.3% homogenate (4). Parasympathetic saliva had been concentrated by centrifugation in a Centricon-10 microconcentrator (Amicon) spun overnight at 2000 rpm, at 4°. The concentrate was washed through with homogenization buffer before application to IEF gels. (b) The distribution of DVLR-AFC lytic activities, with band, in extracts of IEF gels run with parasympathetic saliva and sympathetic saliva. Parasympathetic saliva was concentrated before application to IEF gels as described in (a). The data is based on an average of four observations.

be due to partial dissociation of an autolysed form of this proteinase into its component chains during electrophoresis [38]. The 31 kDa form of tissue kallikrein seen on immunoblots represents a fragment of the complete chain of the proteinase (unpublished data).

Morphometric studies by Garrett *et al.* [39] on the rat submandibular gland showed that sympathetic stimulation is responsible for exocytosis from both granular tubule cells and acinar cells into small volumes of fluid. This contrasts with parasympathetic stimulation which induces flows of large volumes of saliva of low protein content without any obvious degranulation.

The techniques described in this paper have been used to show a dramatic difference in the composition of proteinases secreted under sympathetic stimulation compared with those secreted under parasympathetic stimulation. We have not at this stage totally excluded the remote possibility of selectively stimulating a very small sub-population of highly specialized cells. However, the more likely explanation is that during parasympathetic stimulation newly synthesized proteins are being secreted from convoluted tubule cells through a constitutive pathway without prior pre-packaging [40]. Sympathetic stimulation on the other hand causes protein secretion through degranulation via the regulated pathway.

Acknowledgements—The study was supported by a project grant from the Wellcome Trust (U.K.). We also thank Dr R. E. Smith, of Enzyme Systems Products, U.S.A. for help with the AFC substrates.

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