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MECHANISM OF ACTIVATION OF INACTIVE RENIN IN HUMAN PLASMA BY PUFF ADDER VENOM

CHRISTOPHER H. LAWRENCE and BRIAN J. MORRIS *

Department of Physiology, The University of Sydney, Sydney, N.S.W. 2006 (Australia)

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Summary

Venom of the puff adder (*Bitis arietans*) contains a potent, basic, M_r 24 000 metalloproteinase activity that can destroy all detectable trypsin and chymotrypsin inhibitory activity, when venom is incubated with human plasma. We have found that during such incubation, concomitant activation of inactive renin occurs. In an examination of the mechanism involved we now report the activation, in addition, of plasma prekallikrein and serine proteinase activity, but not plasminogen, when human plasma is incubated with venom. Furthermore, venom was not able to release active trypsin from its complex with α_1-proteinase inhibitor and human renin was not inhibited by α_1-proteinase inhibitor. The activities in venom and venom/plasma mixtures were analysed using Sephacryl S-200 gel filtration and the effect of 10 mM EDTA and 5 mM phenylmethanesulphonyl fluoride on activities in column fractions was tested. The inactive-renin-activating, plasma prekallikrein-activating and serine proteinase-activating activities could be accounted for to a large extent by a venom metalloproteinase which was estimated to have a M_r of 24 000 by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. This enzyme activity appeared to complex with α_2-macroglobulin when venom was mixed with plasma. Since both EDTA and phenylmethanesulphonyl fluoride could inhibit the activation of inactive renin by this metalloproteinase, it is suggested that the enzyme activates serine proteinase(s), which then activate inactive renin. Plasma kallikrein may have a role in this process. Additional peaks of inactive-renin-activating activity eluted from Sephacryl S-200 at M_r 30 000 and 80 000 (minor) and an additional, minor peak of caseinolytic activity eluted at M_r 60 000. The M_r 24 000 metalloproteinase in venom may have considerable util-

* To whom requests for reprints should be addressed.

ity in activating inactive renin at physiological pH owing to its ability to destroy plasma proteinase inhibitors at the same time.

Introduction

The carboxyl proteinase renin (EC 3.4.99.19), which is secreted into the bloodstream by the kidney, is the rate-limiting enzyme in a reaction pathway that leads to formation of angiotensin II, an octapeptide with potent pressor, aldosterone-stimulating and dipsogenic activity [1]. An inactive form of renin that can be enzymatically activated by either carboxyl or serine proteinases [2], and which comprises 60—90% of the 'total' renin in biological fluids [1,3], has stimulated considerable speculation over the last ten years concerning the existence of a circulating renin precursor that has been termed, perhaps prematurely, 'prorenin'. Proof of this concept remains to be produced. Although Morris and co-workers have established activation by trypsin [1], glandular kallikrein [4], plasmin [5], pepsin [2,6—8], and cathepsin D [6], the significance of inactive renin in terms of a circulating pool of potential renin activity also remains hypothetical and must await the demonstration of an endogenous, physiologically-operational activating pathway in the bloodstream. Unfortunately, decidedly non-physiological conditions, viz. acidification to pH 3.3 or treatment to —4°C are needed to trigger endogenous activating mechanisms [1,3]. Furthermore, acid activation in human amniotic fluid involves an enzyme that is resistant to pH 1.5 [2] and inhibited by pepstatin (Shulkes, A.A. and Skinner, S.L., personal communication), but not by EDTA [2,9], phenylmethanesulphonyl fluoride or *N*-ethylmaleimide (Lawrence, C.H. and Morris, B.J., unpublished results), suggesting that the enzyme is pepsin which has been activated from pepsinogen at low pH [2], a process that would appear to have no physiological relevance. However, in plasma it has been shown recently that up to 70% of acid activation can be inhibited by trasylol, di-isopropylphosphorofluoridate or phenylmethanesulphonyl fluoride, which inhibit serine proteinases such as kallikrein (Refs. 10—12, Lawrence, C.H. and Morris, B.J., unpublished results). Furthermore, plasma kallikrein [13], as well as urinary and glandular kallikreins [4,13—16] can activate inactive renin. The ability of plasma kallikrein to activate inactive renin adds weight to hypotheses concerning an activating mechanism that may operate in vivo. It has been said that incubation of plasma at low pH or in the cold increases neutral proteinase activity by dissociating or denaturing plasma inhibitors of neutral proteinases [10]. In order to inactivate endogenous proteinase inhibitors at pH 7.4, we have utilized phenylmethanesulphonyl fluoride-treated, dialysed venom of the puff adder (*Bitis arietans*), since of all the venoms tested this is the most potent in preferentially destroying proteinase inhibitors in human plasma via an action of a metalloproteinase [17—21]. Inactive renin was activated and the activation could be inhibited by EDTA and dithiothreitol, which inactivate venom metalloproteinase activity [17], and also by phenylmethanesulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide, which inhibit neutral serine and thiol proteinases [21]. These results were consistent with the possibility of a role of plasma serine proteinase(s) in the activation of inactive renin,

the serine proteinase(s) having first been activated by venom metalloproteinase.

The aim of the present investigation was to identify the enzyme activities in *B. arietans* venom involved in the activation of inactive renin in plasma and to attempt to delineate the reaction pathway involved.

Materials and Methods

Materials. Fresh citrated plasma was obtained from the Sydney Blood Bank and pooled samples, each from ten subjects, were stored at −20°C. Frozen samples of third trimester human amniotic fluid were obtained from the Department of Biochemistry, Royal Prince Alfred Hospital. The lysate of granules from polymorphonuclear leucocytes was generously provided by Dr. R. Stephens, Royal North Shore Hospital, Sydney. Lyophilized *B. arietans* venom was from Sigma (Lot No. 53C-2080), as were phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, soybean trypsin inhibitor, α_1-proteinase inhibitor, trypsin, streptokinase, ribonuclease A, α-chymotrypsinogen and ovalbumin. Bovine serum albumin (Cohn fraction V) was from the Commonwealth Serum Laboratories, Sydney, catalase was from Pharmacia, and casein was from Hammerstein. Fletcher factor-deficient plasma (George King Co., TX) was a kind gift from Professor P. Castaldi, Westmead Hospital, Sydney.

Activation in inactive renin by venom. A solution of 6 mg lyophilized venom/ml was treated with 2.5 mM phenylmethanesulphonyl fluoride, extensively dialysed to remove unbound phenylmethanesulphonyl fluoride and then incubated with 4 vol. plasma at 25°C, pH 7.4, as described previously [17,21].

Assay of renin. Renin was measured as the initial velocity of formation of angiotensin I during incubation of a 50 μl sample at 37°C with 200 μl plasma collected from a sheep 6 days after nephrectomy. The latter contained 20 × K_m angiotensinogen (2500 pmol/ml) and negligible renin. Also present in the incubation mixture were 15 mM EDTA, 1.6 mM 2,3-dimercapto-1-propanol and 3 mM 8-quinolinol (which inhibit angiotensinases) and 50 mM sodium phosphate buffer, pH 7.4. The reaction was stopped by adding 1 ml cold, distilled water and placing tubes in a boiling water bath for 3 min. Angiotensin I was then quantified by radioimmunoassay [22] and activity was expressed as pmol angiotensin I/h per ml.

Assay of neutral proteinase. Hydrolysis of heat-denatured casein at pH 7.6 was measured spectrophotometrically by the method of Kunitz [23]. Samples of 0.5 ml were mixed with 0.5 ml 100 mM sodium, potassium phosphate buffer, pH 7.6, and 1.0 ml of a 1% solution of casein in buffer (this solution having been previously boiled for 10 min and cooled). The mixtures were incubated at 37°C for 7, 15 or 26 h. Controls, in which casein was not added, were included to allow for background effects of protein in the samples. The reaction was stopped by addition of 3 ml 5% (w/v) trichloroacetic acid. The samples were cooled for 30 min at 4°C, then centrifuged at 10 000 × *g* for 30 min and absorbance at 280 nm was measured. In each assay caseinolytic activity in samples was compared with the caseinolytic activity of trypsin, 20—500 ng/ml and activity was expressed according to Kunitz as TU^{cas}/ml [23].

Assay of inactive-renin-activating activity. Portions of 0.05 ml column fractions were incubated for 15 h at 25°C with 0.2 ml of either plasma or amniotic

fluid, and after addition of EDTA to 10 mM and phenylmethanesulphonyl fluoride to 5 mM, renin was measured as described above.

Assay of plasma kallikrein. Plasma kallikrein activity was measured spectrophotometrically at 405 nm as the rate of release of *p*-nitroaniline, at 37°C, from the peptide amide chromozym PK (Boehringer Mannheim, Lot No. 1257102), using the method of Mattler and Bang [24]. Samples of 0.3 ml were mixed with 0.3 ml 0.2 M ϵ-caproic acid, 0.6 ml 40 mM Tris, pH 7.9, containing 150 mM NaCl and 10 mM EDTA and 0.6 ml water. A portion of 0.6 ml was mixed with 1.65 ml of the Tris buffer and after addition of 0.25 ml 0.8 mM chromozym PK, absorbance at 405 nm was measured at intervals of 15 s, for 3 min. Arginine amidase activity in venom treated with 5 mM phenylmethanesulphonyl fluoride and 10 mM EDTA was taken into account in venom/plasma mixtures and plasma kallikrein activity was expressed as μU/ml [24].

Assay of plasmin. This was performed by a method analogous to that above, but using the chromogenic substrate S-2251 (Ortho Diagnostics, Raritan, NJ; Lot No. C318). Samples of 0.03 ml were mixed with 0.12 ml 50 mM Tris buffer, pH 7.4, containing 10 mM EDTA, and 0.05 ml of the mixture was added to 2.15 ml buffer, followed by 0.3 ml 0.3 mM chromogenic substrate S-2251. Absorbance at 405 nm was measured as above and activity was expressed as ΔA_{405}/min. This activity was compared with activity in a range of concentrations of plasma (pooled from 60 subjects) that had been incubated with HCl (83 mM) at 25°C for 15 min to activate plasminogen.

Gel filtration. Samples of 2 ml were applied to a 1.5 × 90 cm column of Sephacryl S-200 (Pharmacia) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 200 mM NaCl and 3 mM NaN_3 at 4°C and fractions of approx. 2.8 ml were collected at a flow rate of 0.2 ml/min. Elution volumes were determined after weighing each fraction. The column was calibrated using Blue Dextran (M_r 2 000 000), catalase (M_r 210 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), α-chymotrypsinogen (M_r 25 000), soybean trypsin inhibitor (M_r 20 000) and ribonuclease A (M_r 14 000).

Gel electrophoresis. Electrophoresis was performed with a 10 μl sample on 12.5 and 14% acrylamide, 0.1% SDS-polyacrylamide slab gels (10 cm long) in Tris-HCl buffer (0.375 M, pH 8.8) at 20 mA and 20°C [25]. Gels were stained with 0.1% Coomassie brilliant blue G-250 in 50% (w/v) trichloroacetic acid and destained in 7% (v/v) acetic acid. Relative mobility (R_m) was determined in relation to Bromophenol blue. Each run included protein standards and M_r was determined from a plot of R_m vs. M_r of standards.

Results

Gel filtration of venom. Gel filtration of 25 mg venom on Sephacryl S-200 revealed two peaks of caseinolytic activity (M_r 60 000 and less than 14 000) and three peaks of inactive-renin-activating activity (M_r 80 000, 30 000 and less than 14 000) Fig. 1). Similar results were obtained in a second experiment. Both peaks of caseinolytic activity were inhibited by 10 mM EDTA, but 5 mM phenylmethanesulphonyl fluoride did not inhibit the M_r less than 14 000 peak (Table I). The major peaks of inactive-renin-activating activity at M_r 30 000 and less than 14 000 were inhibited by EDTA and by a combination of phenyl-

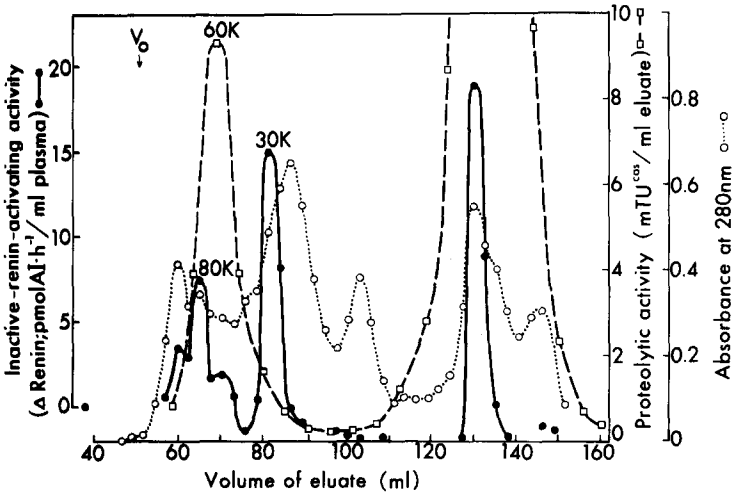

Fig. 1. Gel filtration of puff adder venom on Sephacryl S-200. Shown are inactive-renin-activating activity, caseinolytic activity and protein. The basal level of active renin (6 pmolAI/h per ml plasma) has been subtracted from the total renin measured in each fraction to give the Δ renin values shown. (AI = angiotensin I, V_0 = void volume, TU^{cas}/ml, activity expressed according to Kunitz [23].)

methanesulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide, whereas only EDTA inhibited the minor peak of activity at M_r 80 000 (Table I). The major peaks caused full activation of plasma-inactive renin at a similar rate of 0.094 pmol angiotensin I/h renin/ml per min, compared with 0.064 for the M_r 80 000, minor peak, which activated only 48% of the inactive renin present by 5 h at 25°C. Since there was a coincident peak of caseinolytic activity, inactive-renin-activating activity and protein at M_r less than 14 000, which, as will be discussed later, was probably a basic proteinase likely to be retarded on Sephacryl, protein in the M_r less than 14 000 peak was examined by SDS-polyacrylamide gel electrophoresis. With 12.5 and 14% gels a predominant band of protein was found with a M_r of 24 000 (Fig. 2).

Gel filtration of venom/plasma mixture. Gel filtration was also used to examine the venom activities after the phenylmethanesulphonyl fluoride-treated, dialysed venom had been incubated with 4 vol. human plasma for 5 h at 25°C. Two peaks of caseinolytic activity (M_r approx. 800 000 and 55 000)

TABLE I

EFFECT OF ENZYME INHIBITORS ON ACTIVITIES IN FIG. 1

	Percentage inhibition of activity		
Caseinolytic activity		60 000 peak	low M_r peak
10 mM EDTA		97	100
5 mM phenylmethanesulphonyl fluoride		92	20
Inactive-renin-activating activity	80 000 peak	30 000 peak	low M_r peak
10 mM EDTA	86	100	92
5 mM phenylmethanesulphonyl fluoride +0.32 mg/ml soybean trypsin inhibitor +5 mM *N*-ethylmaleimide	10	88	95

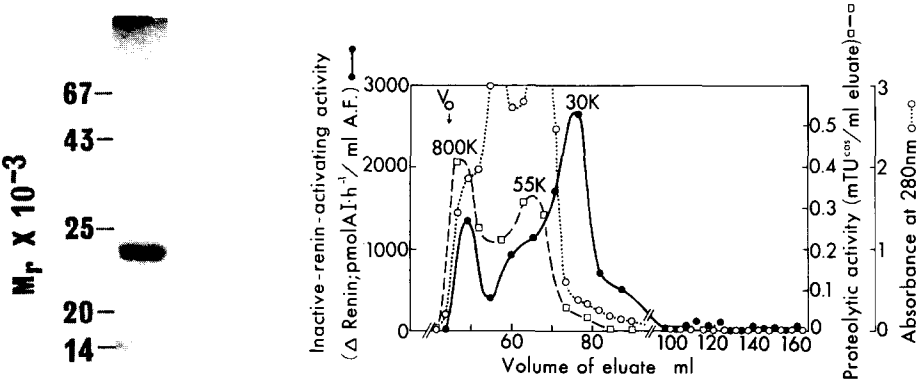

Fig. 2. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of the low M_r venom peak from Sephacryl S-200. The gel was 14% in acrylamide and in adjacent lanes bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), α-chymotrypsinogen (M_r 25 000), soybean trypsin inhibitor (M_r 20 000) and ribonuclease A (M_r 14 000) were run as calibration standards. From a plot of R_m vs. M_r of standards (r = 0.89) the M_r of the major band was found to be 24 000. A value of 24 000 was also obtained using a gel 12.5% in acrylamide (r = 0.93 for R_m vs. M_r of standards).

Fig. 3. Gel filtration of venom/human plasma mixture on Sephacryl S-200. A 2 ml sample containing human plasma that had been incubated with 1.2 mg venom for 5 h at 25°C, was chromatographed. Proteolytic activity of odd-numbered fractions and inactive-renin-activating activity of even-numbered fractions was determined. The basal level of active renin (300 pmolAI/h per ml amniotic fluid) was subtracted from the total renin measured in each fraction to give the Δ renin values shown. (AI = angiotensin I, V_0 = void volume, TU^{cas}/ml, activity expressed according to Kunitz [23].)

and three peaks on inactive-renin-activating activity (M_r approx. 800 000, 80 000 and 30 000) were observed (Fig. 3). The latter activity was measured using human amniotic fluid, which contains high concentrations of inactive renin. Similar results were obtained in a second column run. EDTA caused complete inhibition of the M_r 800 000 peak of caseinolytic activity and substantial inhibition of the M_r 55 000 peak, whereas phenylmethanesulphonyl fluoride had little effect on the M_r 800 000 peak, but caused partial inhibition of the M_r 55 000 peak (Table II). The peaks of inactive-renin-activating activity were substantially inhibited by EDTA and by phenylmethanesulphonyl fluoride (Table II). The activities in the M_r 800 000 peak thus resembled those of the low M_r metalloproteinase in whole venom.

Activation of plasma serine proteinase activity by venom. Most of the caseinolytic activity in column fractions was due to venom metalloproteinases. In order to determine whether an increase in plasma proteinase activity occurred during treatment with venom, phenylmethanesulphonyl fluoride-treated, dialysed venom was incubated with 4 vol. of human plasma at 25°C, pH 7.4, for 0—5 h and, after addition of EDTA to 10 mM to inhibit venom metalloproteinases, caseinolytic activity was measured. Untreated plasma had no caseinolytic activity, but addition of venom resulted in the generation of caseinolytic activity which reached a maximum by 2 h (Fig. 4). Basal activity at the 0 h point was probably due to residual venom activity. In the presence of 5 mM phenylmethanesulphonyl fluoride 97% inhibition of the initial rate of activation of caseinolytic activity was observed (Fig. 4), indicating that the new activity was probably due to serine proteinases.

TABLE II

EFFECT OF ENZYME INHIBITORS ON ACTIVITIES IN FIG. 3

	Percentage inhibition of activity	
Caseinolytic activity	approx. 800 000 peak	55 000 peak
10 mM EDTA	100	72
5 mM phenylmethanesulphonyl fluoride	6	45
Inactive-renin-activating activity	approx. 800 000 peak	30 000 peak
10 mM EDTA	79	88
5 mM phenylmethanesulphonyl fluoride	94	71

Effect of venom on trypsin-α_1-proteinase inhibitor complex. The release of serine proteinases in plasma may have been a consequence of either (i) liberation from complexes with proteinase inhibitors in plasma, or (ii) activation of precursors of serine proteinases in plasma. Alternative (i) was tested by incubating 30 μl phenylmethanesulphonyl fluoride-treated, dialysed venom (equivalent to 0.18 mg powder) for 5 h at 25°C with a 1 ml mixture (in 50 mM sodium phosphate, pH 7.4/200 mM NaCl), which contained 100 μg α_1-proteinase inhibitor and 0—20 μg trypsin that had been pre-incubated overnight at 4°C. α_1-Proteinase inhibitor accounts for 90% of the trypsin-inhibitory capacity of human plasma [26]. EDTA was added to give 10 mM and trypsin activity was measured by incubation with heat-denatured casein at 37°C for 7 h, as described above for assay of neutral proteinase activity. Fig. 5 shows that α_1-proteinase inhibitor markedly inhibited trypsin activity and that after addition of phenylmethanesulphonyl fluoride-treated, dialysed venom to the trypsin-α_1-proteinase inhibitor complex, trypsin activity remained low. However, pretreatment of α_1-proteinase inhibitor with phenylmethanesulphonyl fluoride-treated, dialysed venom markedly impaired its ability to inhibit trypsin (Fig. 5), as has been shown previously [17]. Thus, in the case of the α_1-proteinase inhibitor complex active proteinase is not released when the complex is incubated with venom.

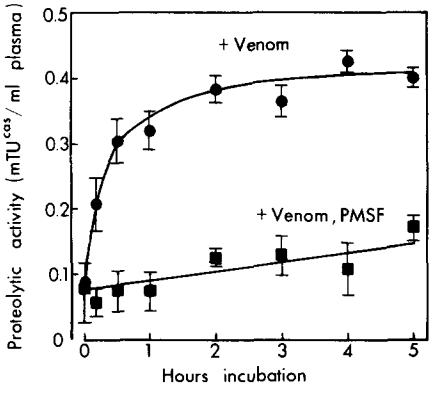

Fig. 4. Activation of caseinolytic activity in human plasma by venom and inhibition of activation by 5 mM phenylmethanesulphonyl fluoride (PMSF) (mean ± S.E., n = 6, TU^{cas}/ml, activity expressed according to Kunitz [23]).

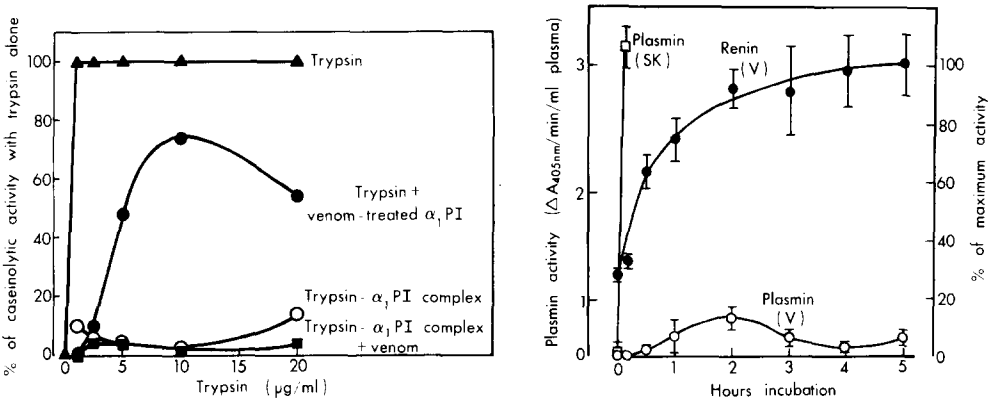

Fig. 5. Effect of venom on trypsin-α_1-proteinase inhibitor complex. Trypsin activity was measured using heat-denatured casein as substrate. For comparison, the caseinolytic activity of the trypsin-α_1-proteinase inhibitor complex itself, as well as trypsin that had been incubated with 100 μg venom-treated α_1-proteinase inhibitor, and trypsin alone was also determined. Results are expressed as a percentage of the latter activity at each concentration of trypsin. (α_1PI = α_1-proteinase inhibitor.)

Fig. 6. Plasmin activity in human plasma during treatment with venom (V) (mean ± S.E., $n = 6$). The increase in plasmin activity with 2000 U/ml streptokinase (SK) and the activation of inactive renin by venom, designated renin (V), in the same samples in these experiments is shown for comparison.

Effect of venom on plasminogen activation. Alternative (ii) was examined by measuring the activity of the specific serine proteinases, plasmin and plasma kallikrein, since these exist in the form of inactive precursors in plasma, have caseinolytic activity [27—29], and can activate inactive renin [5,13,30]. Incubation of human plasma with phenylmethanesulphonyl fluoride-treated, dialysed venom at 25°C resulted in only a small increase in plasmin activity compared with the effect of 2000 U/ml of streptokinase, a specific activator of plasminogen (Fig. 6). By 5 h, renin had increased by 270% ± 41 ($n = 6$) with phenylmethanesulphonyl fluoride-treated, dialysed venom, and by 73% ± 10 with streptokinase. Incubation of plasma with porcine plasmin, 400 μg/ml at 25°C for 5 h, did not lead to an increase in renin, presumably due to inhibition of plasmin by α_2-plasmin inhibitor [31]. The latter may also explain the lower level of activation of inactive renin after activation of endogenous plasmin by streptokinase. As with venom, activation of inactive renin by pH 3.3 treatment [9] was also not accompanied by an increase in plasmin, although subsequent addition of streptokinase, in each case, caused plasmin to rise to the level seen in normal streptokinase-treated plasma. In addition, 1—100 mM ϵ-caproic acid, a plasmin inhibitor, did not inhibit the activation of inactive renin by phenylmethanesulphonyl fluoride-treated, dialysed venom.

Effect of venom on plasma prekallikrein activation. In contrast to the results with plasmin, addition of phenylmethanesulphonyl fluoride-treated, dialysed venom to plasma at 25°C caused a rapid increase in plasma kallikrein activity (Fig. 7). The values shown were obtained after subtraction of venom blank (11.1 ± 1.1 μU/ml). Kaolin at 0°C [24] also increased plasma kallikrein activity. The activation of plasma prekallikrein preceded the activation of inactive renin. Renin increased 400% ± 90 ($n = 5$) with venom, but very little with

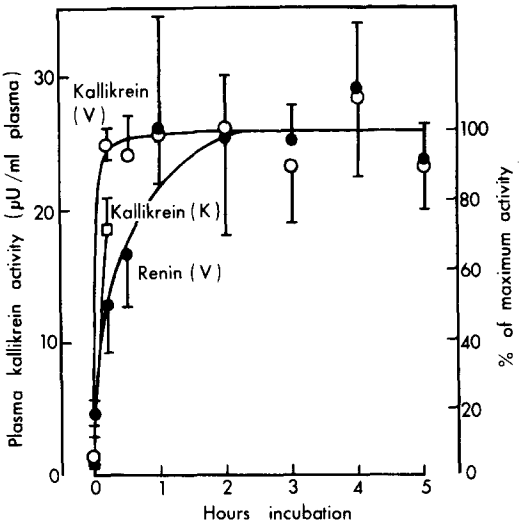

Fig. 7. Plasma kallikrein activity during treatment of plasma with venom (V) (mean ± S.E., $n = 6$). Values have been corrected for arginine amidase activity in controls consisting of phenylmethanesulphonyl fluoride/EDTA-treated venom. The increase in plasma kallikrein with kaolin (K) and the activation of inactive renin by venom, designated renin (V), are also shown, for comparison.

kaolin, possibly due to inhibition of plasma kallikrein by its inhibitors: $C\bar{1}$-inactivator, antithrombin III and α_2-macroglobulin [32]. Renin in prekallikrein-deficient samples increased by 322% ± 69 ($n = 6$) with venom and by 315% ± 36 with conventional pH 3.3 treatment [9,33] which represents incomplete (10—20%) activation of inactive renin, since treatment with trypsin has been shown to increase renin from 5—10 to 200—600 μU/ml in 4 Fletcher factor-deficient plasmas (Ref. 15, Derkx, F.H.M. and Schalekamp, M.A.D.H., personal communication). Incubation of column fractions with 4 vol. plasma at 25°C for 30 min, resulted in an increase in plasma kallikrein activity from 1.4 to 19.4 μU/ml in the case of the low M_r activity shown in Fig. 1, but no change with the M_r 30 000 activity. In controls, chromozym PK-hydrolysing activity of venom was confined to the M_r 30 000 venom enzyme (23.5 μU/ml) and this was inhibited completely by 5 mM phenylmethanesulphonyl fluoride and 56% by 10 mM EDTA.

Effect of polymorphonuclear leucocyte granule lysate. The ability of the serine proteinases, polymorphonuclear leucocyte elastase and cathepsin G to active inactivate renin was tested by incubating (for 5 h at 25°C) 100 μl lysate from the granules of polymorphonuclear leucocytes which are rich in these enzymes, with 200 μl human amniotic fluid. However, only a negligible increase in renin was observed.

Discussion

The present study has identified in *B. arietans* venom an enzyme activity that can hydrolyse casein and cause activation of plasma prekallikrein and inactive renin. The M_r of the protein (24 000) was similar to the very basic *B. arietans* 'protease A' [34] and to *Crotalus adamanteus* 'proteinases I and II'.

The latter are responsible for inactivation of α_1-proteinase inhibitor [18]. The activities emerged at approx. $3 \times V_0$ (M_r less than 14 000) on gel filtration (Ref. 18 and present results), possibly because Sephacryl retards basic proteins [35]. Gel filtration of *B. arietans* venom by others has also revealed two peaks of caseinolytic activity [36]. Fractions of M_r less than 100 000 are not toxic in mice [37]. Moreover, *B. arietans* venom is only a relatively weak inhibitor of trypsin, plasma kallikrein and plasmin [38], and its inhibitors do not interfere in experiments with plasma inhibitors [17,18].

The inactivation of α_1-proteinase inhibitor involves hydrolysis of a X-Met bond 8 residues inside the terminal M_r 7000 fragment, that is removed by trypsin during formation of the trypsin-inhibitor complex [19]. Therefore, our inability to release active trypsin from this complex may be because the site of attack by the venom proteinase was no longer present.

Our work and preliminary reports with *C. adamanteus* venom show that α_2-macroglobulin (M_r 725 000) [39] complexes with the M_r 24 000 proteinase, as it does with all proteinases [40]. Moreover, caseinolytic and inactive-renin-activating activities were retained in the complex. Such residual caseinolytic activity has been reported for trypsin in its complex with α_2-macroglobulin [41]. Proteinase II inactivates α_2-macroglobulin without enzymatic digestion and is released by a high M_r venom proteinase [20]. Proteinases generally cleave the M_r 185 000 subunits of α_2-macroglobulin, during their inhibition, to yield -S-S-bonded M_r 85 000 components [42] and in addition, heating to 90°C yields M_r 62 000 and 125 000 fragments [43]. In preliminary experiments treatment of venom/plasma mixtures with $(NH_4)_2SO_4$ (1.5—3.5 M), which destroys the ability of α_2-macroglobulin to bind trypsin [39], or lyophilization, resulted in an additional peak of EDTA-sensitive caseinolytic activity of M_r 130 000—170 000. It is thus possible that the M_r 24 000 venom proteinase may affect the internal structural integrity of α_2-macroglobulin while remaining attached to fragments of the molecule, as occurs when an excess of trypsin is incubated with α_2-macroglobulin [44].

It has been reported that α_1-proteinase inhibitor can competitively inhibit semi-purified hog renin [45]. However, we found no evidence of inhibition of renin in normal or acidified human plasma or amniotic fluid by 0.01—100 μM α_1-proteinase inhibitor and similar observations have been made by Derkx and Schalekamp (personal communication).

The activation of plasma prekallikrein by the M_r 24 000 venom proteinase may be direct since, at least for bovine prekallikrein, this involves hydrolysis at an Arg residue [46] and proteinase II cleaves bonds with Arg [18]. In contrast, the activation of inactive renin is probably indirect because both metallo- and serine-proteinase inhibitors impaired activation. Moreover, since plasma kallikrein can activate inactive renin [13] and is inhibited by phenylmethanesulphonyl fluoride [32], it may be involved in the activation by venom.

Although papain can also activate plasma prekallikrein [47] and destroy purified α_1-proteinase inhibitor [48], we have found that it does not cause activation of inactive renin in amniotic fluid [5] or plasma. This may be due to inhibition of papain by α_2-thiol proteinase inhibitor [49].

Activation of prekallikrein by acidification of plasma was reported in 1933 [50] and coupled with findings in 1971 of activation of inactive renin during

treatment of plasma to pH 3.3 [9], together with the demonstration of partial inhibition of such activation with inhibitors of serine proteinases [10—12] has led to the proposal of a role of plasma kallikrein in the activation of inactive renin in plasma. The present results support such a role. Nevertheless, they do not exclude the possibility or primary activation by venom of Hageman factor (Factor XII), which activates plasma prekallikrein, or of a role of other proteinases in the intrinsic coagulation pathway activated by plasma kallikrein, with the exception of plasmin. However, it has been shown that addition of Hageman factor to prekallikrein-deficient plasma does not result in the activation of inactive renin [51], whereas addition of plasma kallikrein to semipurified inactive renin does [13], suggesting that plasma kallikrein may indeed activate inactive renin directly.

Although venom was shown to increase caseinolytic (serine proteinase) activity in plasma, such activity was negligible in column fractions obtained after gel filtration of venom/plasma mixtures. This may have been due to low concentrations or instability of the activity when diluted or incubated for extensive periods with casein. The caseinolytic activity generated in plasma was equivalent to that of 250 ng/ml trypsin, a value similar to the concentration of trypsinogen in plasma: 273 ± 66 ng/ml [52]. However, trypsin hydrolyses the plasmin substrate S-2251 and yet little hydrolysis of S-2251 was observed in venom-treated plasma.

A second peak of inactive-renin-activating activity after gel filtration of *B. arietans* venom was found at an apparent M_r of 30 000. This fraction could hydrolyse chromozym PK, indicating arginine amidase activity. However, it did not activate plasma prekallikrein and did not have caseinolytic activity. Furthermore, it eluted in a similar position to a reported L-*p*-toluenesulphonyl-arginine methyl ester-hydrolysing activity observed after gel filtration of *B. arietans* venom on Sephadex G-75 [36]. The inhibition of inactive-renin-activating activity of this fraction by EDTA and phenylmethanesulphonyl fluoride may reflect a dependence for divalent metal ions, as has been reported for trypsin [53]. The mechanism of activation of inactive renin by the M_r 30 000 proteinase is more obscure and a direct effect, at least in part, cannot be excluded.

A third, minor peak of inactive-renin-activating activity eluted from Sephacryl S-200 at an apparent M_r of 80 000. This had no caseinolytic activity and its inhibition by EDTA, but not by phenylmethanesulphonyl fluoride, suggests that it may play a minor overall role in the activation of inactive renin by *B. arietans* venom by a direct effect. There was no evidence of binding of the M_r 80 000 and M_r 30 000 activities by α_2-macroglobulin, possibly indicating a greater affinity of the M_r 24 000 venom metalloproteinase.

The present results provide evidence for activation of plasma prekallikrein and thence inactive renin by a M_r 24 000 metalloproteinase in puff adder venom (Fig. 8). This proteinase also appears to complex with α_2-macroglobulin and is most likely identical with the M_r 24 000 venom metalloproteinase reported to inactivate plasma proteinase inhibitors. This enzyme would therefore appear to be useful in examination of the mechanism of activation of inactive renin in human plasma at physiological pH. The possibility of in vivo activation of inactive renin by kallikreins remains to be demonstrated. However, such an event would increase angiotensin II, the vasoconstrictor effect of which

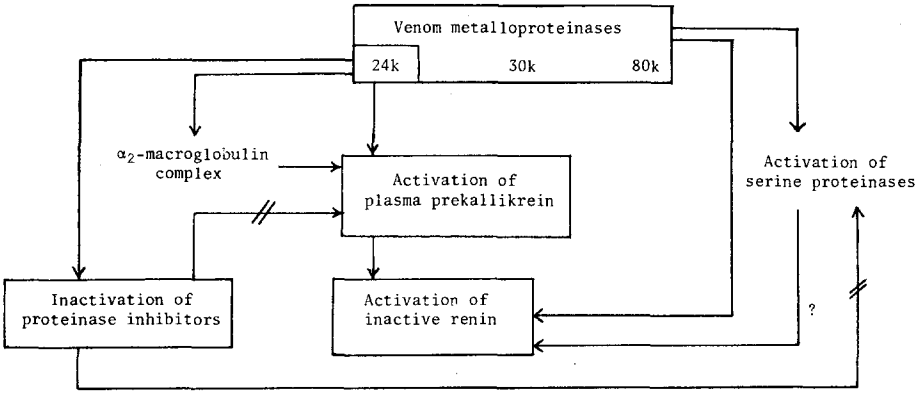

Fig. 8. Reaction scheme for the activation of inactive renin in human plasma by metalloproteinases in puff adder venom.

would be opposed by newly-formed kinins at vascular sites, but not at the non-vascular sites of action of angiotensin II, such as in the adrenal and brain, and may thus provide a mechanism for the selective channelling of the physiological effects of the renin-angiotensin system.

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