Biochemical Pharmacology, Vol. 21, pp. 3163–3169. Pergamon Press, 1972. Printed in Great Britain.

GLASS ACTIVATED KALLIKREIN FROM HUMAN PLASMA

OLGA B. HENRIQUES* and LINDA ALLAN
G.I. Unit, Western General Hospital, University of Edinburgh, Edinburgh, Scotland

(*Received* 21 *December* 1971; *accepted* 6 *July* 1972)

Abstract—It is shown that when preparing human plasma kallikrein by adsorption on glass surface a 30 sec period of shaking leads to more active preparations and a better yield than longer periods of shaking. A gradual decrease of the kininogenase specific activity of the eluates obtained after 1, 2, 5 and 10 min shaking of the plasma with glass beads was found. No parallelism between the kininogenase and esterolytic activities of the kallikrein preparations was observed, suggesting the presence of other esterase(s) in the eluates. Glass activation of prekallikrein was not prevented by previous incubation of the plasma with lima bean trypsin inhibitor which has been shown[6] to inhibit factor XII, generally considered to be the enzyme responsible for the activation of prekallikrein.

MARGOLIS[1] suggested that when factor XII is activated and adsobred by a surface it also activates another enzyme, compound A. Subsequently he thought that the enzyme was plasma prekallikrein,[2] which when activated, remains in solution and releases kinin from kininogen. It appears that this assumption is only partially correct. We found[3] that if enough glass powder is shaken with horse plasma, prekallikrein is in fact activated but it is also adsorbed on this surface and no kallikrein activity can be detected in the soluble phase (plasma supernatant). During repeated adsorptions on glass beads (200 mg/ml plasma) some active kallikrein remains in the first supernatant, while kininogen I (HMW kininogen) is rapidly consumed and only kininogen II (LMW) is left in the supernatant plasma. The presence of the latter can be seen by incubating the supernatant with trypsin, snake venom or pancreas kallikrein whilst the absence of the HMW-kininogen is shown by the impossibility of releasing kinin with plasma kallikrein.

Eisen and Vogt[4] suggest the existence of a double kinin-forming system, according to the hypothesis previously presented by Vogt.[5] They consider that two kinin-releasing enzymes exist in plasma: kininogenase I and II. All kininogenase I would be activated in a few minutes exposure to glass surface and then would be "destroyed" or "blocked". The evidence given is that a new glass contact does not give rise to any kinin formation. In fact we have shown[3] that by repeatedly exposing horse plasma to small amounts of glass ballotini (200 mg/ml) no detectable kallikrein could be found in the eluate from glass ballotini after the 6th adsorption. However the kallikrein is not "destroyed" or "blocked" in a few minutes but it is adsorbed to the glass surface from which it can be easily eluted by a buffer with high ionic strength and high pH. It is true that if the adsorbed kallikrein is left in contact with the supernatant plasma

* Present address: Centro Nacional de Investigaciones Cientificas, Havana, Cuba.

for longer periods of time it is gradually destroyed or blocked.[3] The experiments mentioned above were repeated with human plasma and the same results were obtained—therefore depletion of kinogen I and kallikrein were observed in the supernatant plasma. The first was consumed by the activated kallikrein and the second was gradually adsorbed on the glass ballotini.

Since various authors recommend a longer period of shaking of plasma with glass beads, in order to obtain a more active kallikrein in the soluble phase, we made a study of the influence of the time of shaking plasma with glass beads on the activity of the kallikrein adsorbed. In this paper we show the results of experiments in which samples of the same human plasma were repeatedly shaken with glass ballotini for 30 sec, 1, 2, 5 and 10 min under specific conditions and the kallikrein was determined in the eluates of the glass beads by measuring its kininogenase and esterase activities.

Some results are also given on the effect of lima bean trypsin inhibitor (LBTI), an inhibitor of factor XII,[6] on the activation and adsorption of human plasma kallikrein on the glass surface.

MATERIAL AND METHODS

Bradykinin, synthetic was kindly supplied by Sandoz, Ltd., Basle. Loss of bradykinin activity in the diluted solutions was prevented by the addition of oxalic acid in the final concentration of 10^{-3} M.

Trypsin, 2 × crystallized, Sigma. The solution of trypsin was prepared in 10^{-3} M hydrochloric acid, immediately before use.

LBTI, was supplied by Sigma, chromatographically prepared.

Plasmas, for the preparation of kallikrein, outdated, citrated human plasma was obtained from the Blood Bank of the Western General Hospital.

Heat-treated plasma. Three-hundred and fifty ml of human blood was collected in plastic bags containing 75 ml of acid citrate dextrose. One-hundred ml of this solution contain 0·8 g citric acid, 2·2 g sodium citrate and 2·24 g dextrose in water for injection, B.P. The blood was collected in the evening at the Blood Transfusion, Royal Infirmary, kept in the cold (4°), centrifuged next morning in a refrigerated centrifuge and the plasma immediately heated at 60° for 1 hr in a siliconized flask. The temperature was brought rapidly to 60° by heating the plasma in a boiling water-bath and then transferred to a water-bath at 61°, a thermometer being constantly maintained inside the plasma. This plasma was used as such, small samples being kept frozen in plastic containers. Once thawed, the remaining plasma was discarded. Dialysis of this plasma against 5×10^{-3} M phosphate buffer, pH 7·4, leads to considerable loss of kininogen I. The plasma was always pipetted with a siliconized pipette. Heating the same human plasma at 56° for 3 hr caused a very marked loss of kininogen I, which meant that the volume then needed for use as substrate for plasma kallikrein was so large that it made it impossible for the measurement of kallikrein activity.

Kinin-releasing determination. The activity of the dialysed kallikrein eluates was tested on the isolated guinea-pig ileum, using Tyrode solution containing diphenhydramine and atropine,[3] by incubation with heat-treated human plasma, its activity being expressed as bradykinin. The incubation was conducted either directly in the organ bath or in polythene tubes.[3] Instead of 1,10-phenantroline which was shown to decrease the response of the ileum to bradykinin,[7] EDTA was used to inhibit any traces of kininase present in the heat-treated plasma. A kallikrein preparation was

used as standard, its activity having been determined by a four point assay. We used the "bracketing" assay employing this kallikrein for comparison. We observed that the ileum reacts more constantly to this incubation mixture than to bradykinin, the response of the latter varying very much after each addition of enzyme or incubate, several additions of bradykinin to the bath being needed to obtain a constant response. Using an excess of kallikrein, we were able to release an equivalent to 1 μg/ml of bradykinin from the heated human plasma (61°).

In order to demonstrate the specificity of the substance released by glass activated plasma kallikrein from heated plasma, parallel assays on cat jejunum,[8] rat colon,[9] rat duodenum[10] and guinea pig ileum were superfused in cascade[11] with Krebs solution, containing a mixture of antagonists[12] to acetylcholine, histamine and 5-hydroxytryptamine, at a rate of 5 ml/min. To the superfusion fluid, plasma kallikrein, heated plasma or heated plasma plus glass activated kallikrein were infused. The response of the assay tissues to these infused solutions were compared to standards of angiotensin, prostaglandin E_2 and bradykinin.

Esterase activity was determined by the method of Brown[13] on *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and on benzoyl-L-arginine ethyl ester (BAEE). The activities are expressed in μmoles of ester hydrolysed per mg of protein/min.

Protein was determined according to Lowry, Rosebrough, Farr and Randall.[14]

Glass beads (ballotini), 75–105 micra, Sigma, were activated with 4 M HCl.

Glycine–saline buffer, pH 10, 0·1 M, was prepared according to Sörensen,[15] containing NaCl in the final concentration of 0·1 M.

Plasma kallikrein was prepared from outdated citrated human plasma by batch adsorption on glass ballotini, as previously described for horse plasma kallikrein,[3] 500 mg/ml being used. Two adsorptions were made with each sample of plasma and each batch of ballotini was eluted twice with glycinate buffer after being thoroughly washed with 0·9% sodium chloride. The proportion of buffer to ballotini was kept the same: 1 ml for each 2·5 g ballotini.

RESULTS

The addition of glass activated plasma kallikrein (ranging from 0·50 μg protein of the most active preparation to 14·5 μg of the less active one) or up to 0·3 ml of heated plasma to the organ bath did not contract the isolated guinea pig ileum preparations while the subsequent addition of both caused a slow contraction. The substance released by glass activated kallikrein from heated plasma substrate contracted the superfused cat jejunum and guinea-pig ileum, relaxed the rat duodenum and had no effect on the rat colon. This pattern is characteristic of bradykinin-like substances, excluding the release of angiotensin, prostaglandin, acetylcholine, histamine and 5-hydroxytryptamine.

Influence of time of shaking of human plasma with glass powder on the kallikrein activity of the eluates. Outdated samples of the same human plasma (50 ml) were shaken with ballotini, respectively for 30 sec, 1, 2, 5 and 10 min. The shaking was performed in 250 ml conical flasks by smooth circular movements to prevent foaming, at an average rate of 120 shakings per min. The supernatant plasmas were immediately decanted and the ballotini was immediately and thoroughly washed with 0·9% NaCl eluted with glycine buffer and the eluates dialysed against 5×10^{-3} M phos-

phate, pH 7·4, in the cold. A fresh portion of 500 mg ballotini/ml was added to the decanted supernatant plasma and the whole procedure repeated again. The proportion of washing solution, eluting buffer, etc. were kept as described before.[3] The supernatant plasmas were tested for the presence of kininogen I and II. No kininogen I could be detected by incubating the supernatant with glass activated kallikrein, while trypsin released considerable amounts of kinin from all the supernatants. Two eluates were made after each adsorption on glass beads. The dialysed eluates of each preparation (30 sec, 1, 2, 5 and 10 min shaking) were separately tested for their esterolytic activity on BAEE and TAME as well as for kininogenase activity by bioassay (Table 1). For the kininogenase determination on the ileum, amounts as little as 0·5 μg protein was used of the 30 sec shaking preparations whereas for the preparations in which plasma has been shaken for longer periods, the amount of protein in the test had to be gradually increased.

TABLE 1. ACTIVITY OF HUMAN PLASMA KALLIKREIN ELUTED FROM GLASS BALLOTINI

Eluates	Time of shaking with ballotini	Protein (μg/ml)	μmoles BAEE/min/ mg prot.	μmoles TAME/min/ mg prot.	μg Br/mg prot.
GK_1El_1	30 sec	58	5·1	6·2	67
El_2	30 sec	50	1·6	2·6	27
GK_2El_1	30 sec	50	4·5	5·3	79
El_2	30 sec	32	2·2	2·7	62
GK_1El_1	1 min	54	6·6	6·3	49
El_2	1 min	47	1·2	2·4	21
GK_2El_1	1 min	48	4·5	3·0	16·7
El_2	1 min	29	0·70	2·0	9·0
GK_1El_1	2 min	76	4·0	4·8	31
El_2	2 min	44	1·6	3·0	27
GK_2El_1	2 min	49	1·1	1·6	4·0
El_2	2 min	27	*	1·5	5·8
GK_1El_1	5 min	62	1·6	2·5	11
El_2	5 min	34	0·90	1·6	4·4
GK_2El_1	5 min	31	0·43	1·3	5·1
El_2	5 min	12	*	*	1·3
GK_1El_1	10 min	56	1·2	1·9	5·7
El_2	10 min	26	*	1·5	4·5
GK_2El_1	10 min	27	*	*	2·9
El_2	10 min	12·5	*	*	1·3

Human plasma was shaken twice with fresh portions of ballotini (500 mg/ml) for the times indicated above, the ballotini were then immediately washed with saline and eluted twice with pH 10 glycinate buffer (see details in text). The eluates were dialysed against pH 7·4 phosphate buffer and the kinin released was measured directly by adding the kallikrein samples and heat-treated human plasma to the isolated guinea pig ileum in the bath. The kinin activity is expressed as bradykinin (Br).

* When less than 10 per cent of hydrolysis of the substrate was observed no data are given due to the error involved in the determination.

GK_1 means first adsorbate; El_1 means first eluate; El_2 means second eluate; GK_2 means second adsorbate.

Table 2 gives the total protein, total esterase and kininogenase activities obtained from 50 ml of plasma.

Effect of lima-bean trypsin inhibitor on the activation and adsorption of plasma kallikrein on glass ballotini. Five ml of citrated human plasma were incubated with

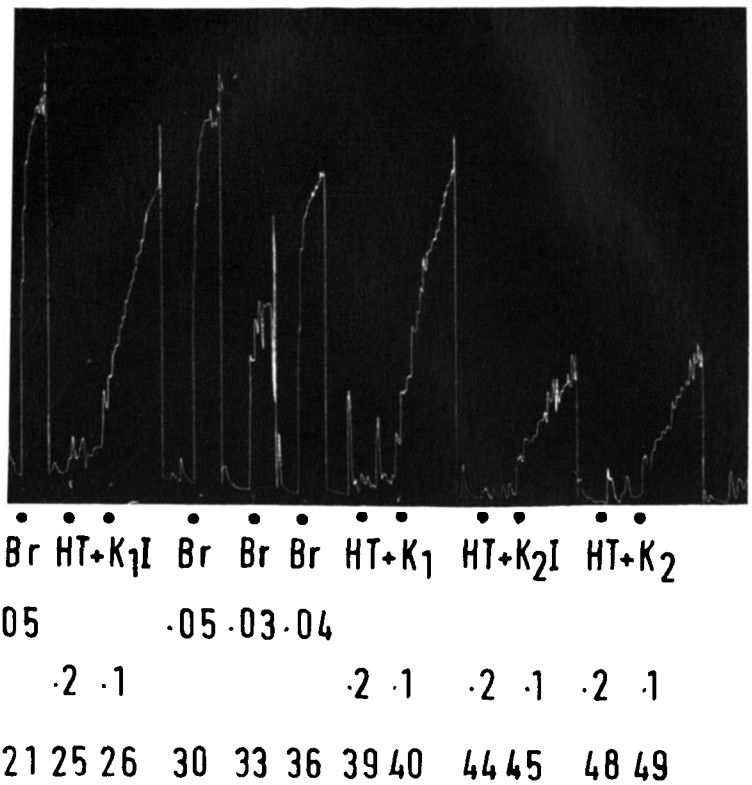

FIG. 1. The activity of kallikrein obtained from human plasma by activation and adsorption on glass surface in presence and absence of LBTI was measured on the guinea pig ileum using heat treated human plasma (HT) as substrate. Br: bradykinin; K_1: kallikrein eluate after first adsorption; I: LBTI; K_2: second eluate after first adsorption on glass.

B.P.—*facing page* 3166

TABLE 2. TOTAL PROTEIN AND TOTAL SPECIFIC ACTIVITIES OF THE FOUR ELUATES AFTER DIFFERENT PERIODS OF SHAKING 50 ml OF HUMAN PLASMA WITH GLASS BALLOTINI (500 mg/ml PLASMA)

Time of shaking	mg prot.	μg Br	Yield* (%)	μmoles BAEE/ min	Yield* (%)	μmoles TAME/ min	Yield* (%)
30 sec	1·90	112	100	6·7	100	8·4	100
1 min	1·78	47	42	6·5	97	6·6	79
2 min	1·96	39	35	4·3	64	6·2	74
5 min	1·39	10	8·9	1·4	21	2·5	30
10 min	1·22	5·3	4·7	0·7	10	1·5	18

* Considering the eluates after 30 sec shaking as representing 100 per cent of the activity.

1 ml of LBTI solution in saline (7·5 mg) for 10 min at 37°.[6] A control was made incubating a sample of the same plasma with saline. From both incubates kallikrein was prepared by adsorption on glass beads, as described for the experiments above, shaking the plasma samples for 2 min with ballotini. Figure 1 shows that LBTI did not interfere in the activation or adsorption of plasma kallikrein. The protein concentration of the parallel eluates in the two preparations was the same.

Influence of LBTI on the esterolytic and kininogenase activities of kallikrein eluates. We then looked at the possibility that the dialyzed eluates contained an esterase inhibited by LBTI (Factor XII?). Trypsin incubates were simultaneously run to control the activity of the inhibitor. The eluates were preincubated with LBTI (1·25 mg/ml incubate) for 10 min at 37° and its esterolytic activity on TAME was determined. Parallel controls were run incubating the same eluates with saline. The esterase activity of all the eluates was slightly inhibited, the inhibition varying between 8 and 10 per cent. No inhibition was observed when the same kallikrein–LBTI incubates were tested in comparison to the corresponding kallikreins, on the guinea pig ileum.

Influence of SBTI on the kininogenase and esterase activities of the kallikrein eluates. Samples of the eluates (30 sec–10 min shaking), containing 20–25 μg of protein were incubated for 10 min at 37° with 100 μg of SBTI dissolved in saline. The kininogenase activity was measured directly by adding the kallikrein sample and heat-treated human plasma to the isolated guinea pig ileum bath. No kininogenase activity could be detected in any of the eluates incubated with SBTI. To obtain the esterase activity on TAME similar samples were incubated with 500 μg of SBTI in the same conditions as above. A strong inhibition was observed, around 25 per cent of the activity being left in the 30 sec, 1 and 2 min-shaking samples and around 40 per cent in the 5 and 10 min shaking samples.

DISCUSSION

The results show that a 30 sec period of shaking plasma with glass beads leads to more active preparations with a better yield of kininogenase activity than longer periods of shaking. Table 1 shows that there is a gradual decrease of the kininogenase specific activity of the eluates after more prolonged periods of shaking. Comparing the total kininogenase activity of the eluates, it can be seen that the ones obtained after shaking the plasma with ballotini for 1, 2, 5 and 10 min, had respectively 42,

35, 8·9 and 4·7 per cent of the activity of the corresponding eluates obtained after 30 sec shaking period (Table 2). The longer the contact of the activated kallikrein with the plasma from which it is adsorbed, the lower its activity. It is possible that human plasma contains a kallikrein degrading enzyme similar to the one found by Werle and Schmal[16] in the rat serum. When working with horse plasma in the same conditions as in the present experiments, we also observed inactivation of the kallikrein.[3]

The inhibition experiments with SBTI as well as the absence of parallelism between the kininogenase and esterase activities of the kallikrein preparations indicate the presence of other esterase(s) in the eluates. However the very low inhibition by LBTI would exclude factor XII.

It is generally accepted that factor XII is the activator of plasma kallikrein and in fact many authors use serum or plasma treated ballotini to activate plasma prekallikrein.[2,17] In doing so they are also adding active kallikrein to the serum, plasma or γ-globulin solution containing prekallikrein. The possibility therefore exists that part of the esterolytic or kininogenase activity measured after adding treated ballotini to a solution of prekallikrein is due to kallikrein previously adsorbed on the ballotini. The possibility that plasma kallikrein may activate prekallikrein is also not excluded.

Nagasawa *et al.*[18] claim to have activated purified prekallikrein by Hageman factor purified according to Schoenmakers.[6] They also claim to be able to stop this activation reaction by adding LBTI to the incubate as well as to prevent the activation of prekallikrein by Hageman factor preincubating the Hageman Factor with lima trypsin inhibitor. Our results for the inhibition with LBTI do not agree with the results of Nagasawa *et al.*[18] We were not able to prevent the glass activation of prekallikrein when plasma was preincubated with 1·25 mg of LBTI per millilitre of incubate. The absence of details in the paper of Nagasawa *et al.* make it difficult to analyse their

TABLE 3.

Method of activation	Source of plasma	μmoles TAME/min/mg prot.*	Reference
Casein precipitation and ammonium sulphate fractionation	Hog	0·325	Habermann and Klett, *Biochem. Z.* **346**, 133–158 (1966)
Acetone activation	Human	0·009/ml plasma (mg prot.?)	Pierce and Webster, *Hypotensive peptides*, p. 130 (Eds. Erdos, Back and Sicuteri) (1966)
Kaolin activation	Human	3·48/ml plasma (mg prot.?)	Colman, *Biochem. biophys. Res. Commun.* **35**, 273–279 (1969)
Kaolin activation	Human	1·29/ml plasma (mg prot.?)	Colman, Mattler and Sherry, *J. clin. Invest.* **49**, 23–32 (1969)
Pseudoglobulin after DEAE–Sephadex A-50 chromatography	Bovine	$0·09/A_{280}$	Nagasawa, Koida, Takahashi, Suzuki and Schoenmakers, *Biochem. biophys. Res. Commun.* **32**, 644–649 (1968)
Glass adsorption followed by elution	Human	6·2	Henriques and Allan, **21**, 3163 (1972).

* Where there is only a figure given: units as top of page.

results. We have always found it extremely difficult to prevent activation of prekallikrein dialysis, chromatography or any contact with glass surface. On the other hand no results on the kinin release experiments by the activated kallikrein and controls are given in the mentioned paper.

From the point of view of plasma kallikrein preparation the adsorption and elution steps using glass beads by the batch procedure is very convenient (see Table 3) as a starting phase for further purification of the enzyme or for the use of the eluates to identify kininogen I during its isolation and purification from plasma. So far the only plasma kallikrein described in the literature with a higher TAMEase and kinin-releasing activity than that obtained by our one-step method is the highly purified preparation obtained by Habermann and Klett[19] after a 7-step laborious procedure. The specific TAMEase activity of our best eluate is about $\frac{1}{4}$ as high as that of their preparation.

The kallikrein described here is very stable, it can be kept frozen without loss of activity at least for 3 months. It can also be frozen and thawed several times during a week's work without losing its activity. It does not contain kininase and the presence of any other interfering substance has not been observed.

Acknowledgements—We would like to thank Dr. S. H. Ferreira for helping us with the superfusion technique. This work was supported by a grant from the Medical Research Council.

REFERENCES

1. J. Margolis, *J. Physiol., Lond.* **144,** 1 (1958).
2. J. Margolis, *Hypotensive Peptides*, p. 198. Springer, New York (1966).
3. O. B. Henriques, V. Stolpnik, V. Kusnetsova and M. Astrakan, *Biochem. Pharmac.* **19,** 2915 (1970).
4. V. Eisen and W. Vogt, *Handbook of Experimental Pharmacology—XXV*, p. 82. Springer, New York (1970).
5. W. Vogt, *Hypotensive Peptides*, p. 185. Springer, New York (1966).
6. J. G. Schoenmakers, R. Matze, C. Haanen and F. Ailliken, *Biochim. biophys. Acta.* **101,** 166 (1965).
7. O. B. Henriques, *Biochem. Pharmac.* **20,** 2759 (1971).
8. S. H. Ferreira and J. R. Vane, *Br. J. Pharmac. Chemother.* **29,** 367 (1967).
9. D. Regoli and J. R. Vane, *J. Physiol., Lond.* **183,** 513 (1966).
10. E. W. Horton, *Br. J. Pharmac. Chemother.* **14,** 125 (1959).
11. J. R. Vane, *Br. J. Pharmac. Chemother.* **23,** 360 (1964).
12. N. Gilmore, J. R. Vane and J. H. Wyllie, *Nature* **218,** 1135 (1968).
13. M. E. Brown, *J. Lab. clin. Med.* **55,** 616 (1960).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
15. G. Gomori, *Methods of Enzymol.* p. 138. Academic Press, New York (1955).
16. E. Werle and A. Schmal, *Z. physiol. Chem.* **349,** 521 (1968).
17. G. E. Davies, G. Holman and J. S. Lowe, *Br. J. Pharmac. Chemother.* **29,** 55 (1967).
18. S. Nagasawa, H. Takahashi, M. Koida, T. Susuki and J. G. Schoenmakers, *Biochem. biophys. Res. Commun.* **32,** 644 (1968).
19. E. Habermann and W. Klett, *Biochem. Z.* **346,** 133 (1966).