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ACTIVATION OF A SPECIFIC HUMAN PLASMA KININOGENASE WITH TRYPSIN AND PLASMIN

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Abstract—In full human plasma, trypsin and plasmin attack prekininogenase I and form active kininogenase I, which is identical to serum kallikrein. Trypsin and plasmin do not attack prekininogenase II. Conversely, contact treatment of full human plasma with glass or quartz generates kininogenase II from its corresponding pre-enzyme, while under these conditions prekininogenase I remains unaltered.

DURING treatment of plasma with glass beads, a so far unknown activator might be consumed, interposing between the Hageman factor and prekininogenase I during kallikrein activation by acid or acetone.

Human plasma kininogenase can be activated *in vitro* in various ways; by treatment with acetone,[1–3] acid,[4–8] trypsin[9] and plasmin[10–13] and by exposure to surface active substances (glass, quartz).[14,15]

It was previously shown that acetone and acid activate kininogenase I,[3,16] while active kininogenase II originates from intensive surface contact.[17] In plasma, kininogenase I liberates kinin from its specific substrate kininogen I, while kininogenase II liberates kinin from kininogen II.[18]

Since also a small amount of kininogen I is consumed during glass contact, and, since by acid kininogenase I cannot be activated in previously contacted plasma[18] but only in fresh plasma, it was suggested that kininogenase I is activated during glass contact, too, but subsequently inactivated by endogenous plasma protease inhibitors so rapidly that cleavage of its specific substrate kininogen I is largely prevented.

Seidel *et al.*[13] demonstrated that plasmin can activate kininogenase I in fresh or contacted plasma. Therefore, activation and inactivation of kininogenase I during glass contact was assumed to be uncertain. In this paper, experiments are reported which indicate that trypsin and plasmin activate a specific kininogenase from the same pre-enzyme in fresh and also in previously contacted plasma.

MATERIALS AND METHODS

Substances. Bradykinin, synthetic product was obtained from Sandoz, Basel. Casein, biochemical grade, Merck, Darmstadt; further purified as previously described.[13] Flufenamic acid, Parke, Davis and Co., Munich; a 0·1 M solution of the

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sodium salt was prepared and stored at −20°. Heparin (Vetren®), Promonta, Hamburg. *p*-Iodobenzoic acid (PIBA), EGA-Chemie, Steinheim; purified by repeated crystallization and sublimation; a 0·74 M solution of the sodium salt was prepared and stored at −20°. *o*-Phenanthroline, 1,10-phenanthroline-HCl, Merck, Darmstadt; 1% solution in 0·9% NaCl. Plasmin, Forschungs-Plasmin Kabi, 3 U/mg protein, Deutsche Kabi, Munich. Sephazyme-TRY, insolubilized trypsin, Pharmacia, Sweden; the gel was equilibrated before use with 0·05 M phosphate buffer (pH 8·5); after each run it was restored according to the manufacturers. Streptokinase (Streptase®), Behringwerke, Marburg, was kept as a stock solution of 1000 U/ml in 0·9% NaCl at −20°. Trypsin, Boehringer, Mannheim.

Preparations. Plasma, free of kininogen II ("B-depleted plasma"), was prepared as previously described.[13] Plasma, free of kininogen II and kininogenase II ("A + B-depleted plasma"),[19] was prepared by stirring plasma four times with quartz powder (0·6 g/ml, surface 3·1 m^2/g) each time for 10 min at 37°. The quartz powder was separated by centrifugation and discarded. Kininogen I was prepared according to Vogt and Wawretschek.[16] A batch containing 7·5 μg bradykinin equivalents/ml was used. 61°-Plasma, i.e. substrate plasma, containing both kininogen I and kininogen II without any prekininogenase, was prepared according to Vogt *et al.*[18] A batch containing 4·5 μg bradykinin equivalents/ml was used. Crude kininogenase was prepared by precipitation of fresh human plasma with acetone according to Seidel and Vogt.[3]

Human venous blood was obtained by free flow through a polyethylene catheter into a plastic container. Clotting was prevented with sodium citrate (0·02 ml, 20% solution/ml). The blood was centrifuged (20 min at 1700 *g*) and the plasma separated. All experiments were performed in siliconized or plastic equipment.

Elimination of protease inhibitors. If not otherwise stated, the plasma samples were preincubated with PIBA (final concentration 0·074 M) or flufenamic acid (final concentration 0·01 M) for 60 min at 37°. By this treatment, the plasmatic protease inhibitors were largely destroyed.[20] To prevent factor XII-dependent activation of kininogenase, heparin was added to the plasma (final concentration 100 U/ml).[21]

Inhibition of kininases. To prevent spontaneous decay of kinins in plasma or related incubation mixtures, phenanthroline, an inhibitor of kininases, was added (0·1 ml/ml plasma).[22]

Activation of kininogenase by glass beads. PIBA-preincubated plasma was mixed with phenanthroline and rotated with glass beads (0·17 g/ml, 0·1 mm diameter) for 5 min at 37°. Ten min later the formed kinins were estimated.

Activation of kininogenase by trypsin. Sephazyme–TRY (gel volume 10 ml = 3200 U of trypsin) was poured into a siliconized tube and equilibrated with 0·05 M phosphate buffer (pH 8·5). Five ml of plasma were added to each equilibration. The plasma remained in contact with the fixed trypsin for about 45 min at room temperature. Only undiluted aliquots of plasma were in further use. During the passage through the column, all plasma kininogen was cleaved. Plasma treated with trypsin was used for further experiments only after decay of the formed kinins (60 min, room temperature).

Activation of kininogenase by plasmin. Plasma samples were incubated with streptokinase (final concentration 100 U/ml, 37°). The time-dependent formation of kinins was followed up for 2 hr.

Estimation of kininogenase. The presence of kininogenase was proved by the formation of kinins from endogenous plasma kininogen, from kininogen I-preparation or

from 61°-plasma. In two experiments, kininogenase was determined as arginine esterase by hydrolysis of BAEe.[23]

Estimation of kininogen. Kininogen was estimated according to Mouri *et al.*[24]

Estimation of kinins. Kinins were estimated by bioassay on the isolated guinea pig ileum with synthetic bradykinin as standard.

Estimation of plasmin. Plasmin was measured by caseinolysis according to Sgouris *et al.*[25] modified as previously described in detail.[13]

RESULTS

In the present experiments, all plasma samples were preincubated with PIBA or flufenamic acid for large elimination of plasmatic protease inhibitors. This pretreatment considerably reduced kininogen, which could be cleaved by trypsin (Fig. 1).

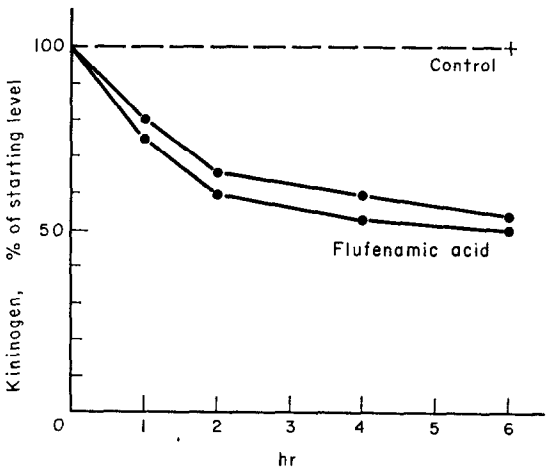

FIG. 1. Time-dependent decrease of trypsin-cleavable kininogen during incubation of plasma with flufenamic acid (final concentration 0·01 M) at 37°. Two experiments. Control with 0·9% NaCl instead of flufenamic acid.

Without any treatment at 37°, the plasma kininogen remained stable for at least 6 hr. This was in accord with findings of Armstrong and Dias da Silva.[26]

Activation of kininogenase by trypsin. Plasma, having passed the Sephazyme–TRY column, lost all its kininogen cleavable by trypsin. When left for 60 min at room temperature, the formed kinins had decayed during this time, activated kininogenase was still demonstrable by splitting kinin from newly added substrate (Table 1).

Experiments, performed under the same conditions with previously contact-activated (B-depleted) plasma, are listed in Table 2. Similar amounts of kinins were liberated out of 61°-plasma, as if fresh plasma had served as the enzyme source.

In a third series of experiments, plasma free from kininogen II and prekininogenase II (A + B-depleted) was passed through a Sephazyme–TRY column. As shown in Table 3, kininogenase I could still be activated in such plasma and formed kinins after addition of new substrate.

In further experiments, it was shown that trypsin did not activate kininogenase II. Fresh plasma, having passed the Sephazyme–TRY column and being left for 60 min at

TABLE 1. KININ FORMATION FROM 61°-PLASMA OR KININOGEN I-PREPARATION BY A KININOGENASE WHICH WAS ACTIVATED BY TRYPSIN IN PLASMA PREINCUBATED WITH PIBA

Exp. No.	Substrate	Bradykinin equivalents (μg/ml substrate)
1	61°-Plasma	0·5
2	61°-Plasma	0·6
3	61°-Plasma	1·0
4	Kininogen I	0·8
5	Kininogen I	1·3
6	Control (0·9% NaCl)	<0·06

Kininases were inhibited by phenanthroline.

TABLE 2. KININ FORMATION FROM 61°-PLASMA BY A KININOGENASE WHICH WAS ACTIVATED BY TRYPSIN IN PREVIOUSLY B-DEPLETED PLASMA PREINCUBATED WITH PIBA

Exp. No.	Substrate	Bradykinin equivalents (μg/ml substrate)
1	61°-Plasma	0·83
2	61°-Plasma	0·69
3	61°-Plasma	0·61
4	61°-Plasma	0·74
5	Control (0·9% NaCl)	<0·06

Kininases were inhibited by phenanthroline.

room temperature for decay of trypsin-formed kinins, was subsequently incubated with 61°-plasma (120 min, 37°). In parallel incubates it was found that within this time the kinin formation had terminated and the liberated kinins had already decayed. Thus, the kininogen portion in 61°-plasma cleavable by trypsin-activated kininogenase was consumed. After addition of *o*-phenanthroline, the mixture was stirred with glass beads. About 0·21 μg of bradykinin equivalents/millilitre of 61°-plasma were formed by this procedure (three experiments), while controls without additional glass contact yielded no measurable kinin (<0·06 μg bradykinin equivalents/millilitre of 61°-plasma).

Reactivation of kininogenase from an enzyme–inhibitor complex by trypsin. In two experiments, crude acetone-activated kininogenase was exposed to fresh plasma for 2 hr at 37°. At the end of this period, the arginine esterase activity of the mixture was estimated. During the 2 hr of incubation, the activity of the added kininogenase decreased to about one-third of its initial amount. Subsequently, the mixture was treated with PIBA for 1 hr at 37° and then passed through the Sephazyme–TRY column in the usual way. Almost none of the added esterolytic activity could be regained by the passage through the insolubilized trypsin (Table 4). This indicates that the added and obviously inhibited kininogenase in fresh plasma could not be re-activated by trypsin.

Activation of kininogenase with plasmin. In order to study the kinin formation via

TABLE 3. KININ FORMATION FROM 61°-PLASMA OR KININOGEN I-PREPARATION BY A KININOGENASE WHICH WAS ACTIVATED BY TRYPSIN IN PREVIOUSLY A + B-DEPLETED PLASMA PREINCUBATED WITH PIBA

Exp. No.	Substrate	Bradykinin equivalents (μg/ml substrate)
1	61°-Plasma	1·2
2	61°-Plasma	0·7
3	61°-Plasma	0·9
4	Kininogen I	1·3
5	Kininogen I	0·9
6	Kininogen I	1·2
7	Control (0·9% NaCl)	<0·06

Kininases were inhibited by phenanthroline.

plasmin in connection with different conditions of the kininogenase II–kininogen II-system, three samples of plasma, each from the same donor, were mixed with *o*-phenanthroline and streptokinase (final concentration 100 U/ml) and incubated for 60 min at 37°: fresh, noncontacted plasma, plasma free from kininogen II (B-depleted), or plasma free from kininogen II and prekininogenase II (A + B-depleted). Practically identical formation of kinins was found within the three different trials (Fig. 2.).

Kinin formation in plasma produced by streptokinase-activated plasmin had terminated 60 min after the beginning of incubation. Another 60 min later, the formed kinins had decayed, as was observed in incubates without *o*-phenanthroline. Addition of *o*-phenanthroline and glass beads led to a corresponding kinin liberation in plasma samples with and without streptokinase pretreatment (Fig. 3).

TABLE 4. ATTEMPT TO REACTIVATE THE ARGININE ESTER SPLITTING ACTIVITY OF CRUDE KININOGENASE WHICH WAS REDUCED DURING THE EXPOSURE TO FRESH HUMAN PLASMA

Procedure	Exp. No. 1	Exp. No. 2
Total esterolytic activity in a mixture of plasma and crude kininogenase		
(a) At 0 min (calculated value)	184	103
(b) After 120 min 37° incubation	60	36
Plasma control after 120 min 37° incubation	4	2
Esterolytic activity after treatment with PIBA and insolubilized trypsin	86	71
Esterolytic activity in control plasma after treatment with PIBA and insolubilized trypsin	27	33
Reactivated activity	3	4

All activities in mU/ml of incubation mixtures.

6·6 ml of kininogenase preparation were added to 5 ml of original plasma (control: 6·6 ml of 0·025 M $NaHCO_3$ instead of kininogenase preparation) and incubated for 120 min at 37°. Subsequently, the samples were treated with PIBA (1 hr, 37°) and passed through the Sephazyme-TRY column.

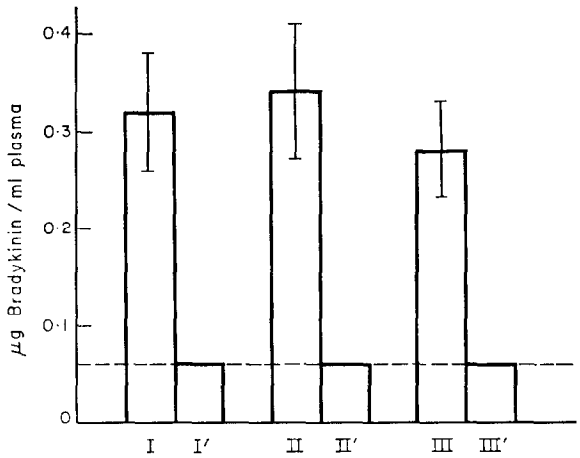

FIG. 2. Kinin formation via plasmin in plasma incubated with streptokinase (final concentration 100 U/ml). Kininases were inhibited by phenanthroline. I: Noncontact plasma. II: B-depleted plasma. III: A + B-depleted plasma. I′, II′, III′ = controls without streptokinase. Means ± S. D., N = 3, – – –: limit for kinin measurement.

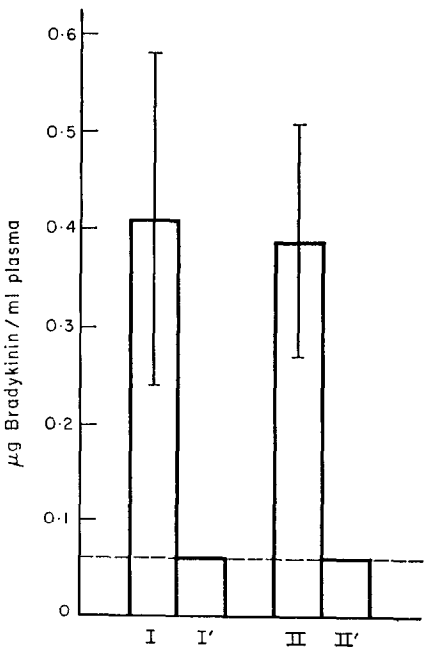

FIG. 3. Kinin formation by contact activation in plasma with (I) and without (II) previous streptokinase incubation. I′, II′ = controls without contact activation. Means ± S. D., N = 3, – – –: limit for kinin measurement.

Streptokinase generated plasmin from endogenous plasminogen only in amounts causing no direct cleavage of kininogen.[13] In two experiments, only 0·14 and 0·15 U/ml plasma were found 60 min after the addition of streptokinase.

DISCUSSION

In the present experiments, the amount of kinins formed by kininogenases from endogenous plasma kininogen or kininogen preparations was relatively small. Obviously, the use of flufenamic acid or PIBA necessary to destroy plasmatic protease inhibitors[20] causes an unspecific loss of kinin precursors. A similar suggestion was made by Heimburger for other plasma proteins.[27]

Activated kininogenase in whole plasma remains stable only if the endogenous inhibitors are eliminated.[13] Therefore, the negative effect of the inhibitor destroying substances on kininogen had to be accepted. This seemed to be permissible because qualitative rather than quantitative aspects were of interest.

Treatment of human plasma with acid or acetone[3,16] as well as with plasmin, endogenously activated by streptokinase,[13] and trypsin generates a specific kininogenase, which utilizes kininogen I. Nevertheless, the activation mechanism with acid or acetone seems to differ from that with plasmin and trypsin. In the first two cases, activation of the kininogenase is impossible after previous contact of the plasma with certain surfaces, a procedure which is known for activation of kininogenase II.

The assumption that in the course of glass contact kininogenase I is activated and subsequently inactivated by plasma inhibitors[18] is no longer tenable. For trypsin (Sephazyme–TRY) forms kininogenase in fresh human plasma as well as in plasma pretreated once with glass beads (B-depleted = free from kininogen II) or repeatedly treated with quartz powder (A + B-depleted = free from prekininogenase II and kininogen II). This was found when new substrate (61°-plasma or kininogen I-preparation) was added to the Sephazyme–TRY-passed plasma samples. Obviously, plasma prekininogenase I remains untouched during surface contact and can be activated further by trypsin. This kininogenolytic activity did not originate from a kininogenase I inhibitor complex, because kininogenase I inhibited by adding fresh plasma could not be regenerated by passage of the mixture through the Sephazyme–TRY column.

In contrast to prekininogenase I, prekininogenase II seemed not to be activated by trypsin. When plasma, having passed the Sephazyme–TRY column, was added to 61°-plasma containing both kininogen I and II, kinins were formed. But kininogen II remained present in the incubate and was transposed to kinin not before the sample was rotated with glass beads, whereby kininogenase II in the Sephazyme–TRY-passed plasma was activated. PIBA-pretreated plasma of the same batch, which had not passed the insolubilized trypsin, did not form more kinin from 61°-plasma than previously trypsin-exposed plasma, when it was activated with glass beads.

Similar results were obtained for plasmin instead of trypsin. The kinin formation via plasmin kininogenase was also independent from the presence of kininogen II and prekininogenase II. Conversely, kinin formation by contact activation was not influenced by previous incubation of the plasma with streptokinase.

Activation of kininogenase I with acid or acetone is only possible in the presence of Hageman factor.[7] According to our results, this activation can be interpreted with a

special factor interposing between factor XII and the prekininogenase I. This activator must be consumed during a single glass contact, because acetone and acid do not generate kininogenolytic activity in glass-treated plasma. Prekininogenase I in plasma remains untouched by contact treatment and is further available.

Recently, Kaplan and Austen[28,29] and Movat *et al.*[30–32] described a prekallikrein activator (PKA) that was formed from purified active Hageman factor (factor XIIa) by plasmin or trypsin or during contact exposure. PKA certainly differs from the newly postulated factor, because in plasma once treated with glass, only a small portion of Hageman factor disappears, and kininogenase II can be induced repeatedly by contact activation.

It seems that in whole plasma, factor XII and PKA are not of equal importance for plasmin-induced kinin formation as in purified systems. Higher concentrations of plasmin, as are possibly involved in the activation of kininogenase II during contact of plasma with certain surfaces and as they are formed by streptokinase, seem to activate kininogenase I directly. It is an open question whether plasmin does not activate kininogenase II in the presence of Hageman factor. Possibly, the Hageman factor has to change into factor XIIa before plasmin can split it into PKA.

At this point, it is impossible to reconcile the findings about the two kinin forming systems in whole human plasma with the findings of kinin formation in purified systems. In the latter experiments, there might have been artificial conditions without any relevance for kinin liberation under plasmatic conditions. On the other hand, the two kinin forming systems in whole plasma are possibly only simulated because of interactions between inhibitors and enzymes occurring when kinin formation is induced in whole plasma in different ways.

REFERENCES

1. H. KRAUT, E. K. FREY and E. WERLE, *Hoppe-Seyler's Z. Physiol. Chem.* **222,** 73 (1933).
2. M. E. WEBSTER and J. V. PIERCE, *Proc. Soc. exp. Biol.* **107,** 186 (1961).
3. G. SEIDEL and W. VOGT, *Naunyn-Schmiedeberg's Archs Pharmak. exp. Path.* **262,** 135 (1969).
4. E. WERLE, *Biochem. Z.* **287,** 235 (1936).
5. M. ROCHA E SILVA and E. L. HOLZHACKER, *Archs int. Pharmacodyn. Thér.* **122,** 168 (1959).
6. E. HABERMANN and M. E. OKON, *Hoppe-Seyler's Z. Physiol. Chem.* **326,** 171 (1961).
7. V. EISEN, *J. Physiol, Lond.* **166,** 496 (1963).
8. D. F. ELLIOT and G. P. LEWIS, *Biochem. J.* **95,** 437 (1965).
9. E. WERLE, M. M. FORELL and L. MAIER, *Naunyn-Schmiedeberg's Archs exp. Path. Pharmak.* **225,** 369 (1955).
10. M. E. WEBSTER and J. V. PIERCE, *J. Pharmac. exp. Ther.* **130,** 484 (1960).
11. V. EISEN, *J. Physiol., Lond.* **166,** 514 (1963).
12. W. VOGT, *J. Physiol., Lond.* **170,** 153 (1964).
13. G. SEIDEL, H.-U. STÜCKER and W. VOGT, *Biochem. Pharmac.* **20,** 1859 (1971).
14. J. MARGOLIS, *J. Physiol., Lond.* **144,** 1 (1958).
15. J. MARGOLIS, *J. Physiol., Lond.* **151,** 238 (1960).
16. W. VOGT and W. WAWRETSCHEK, *Nauyn-Schmiedeberg's Archs exp. Path. Pharmak.* **260,** 223 (1968).
17. G. SEIDEL, *Naunyn-Schmiedeberg's Archs Pharmak.* **264,** 18 (1969).
18. W. VOGT, G. GARBE and G. SCHMIDT, *Naunyn-Schmiedeberg's Archs exp. Path. Pharmak.* **256,** 127 (1967).
19. J. MARGOLIS, *Ann. N.Y. Acad. Sci.* **104,** 133 (1963).
20. K. N. v. KAULLA, J. EISELE and M. EINS, *Thromb. Diath. haemorrh.* **10,** 151 (1963).
21. D. J. MCCONNELL and B. MASON, *Br. J. Pharmac.* **38,** 490 (1970).
22. E. G. ERDÖS, A. G. RENFREW, E. M. SLOANE and J. R. WOHLER, *Ann. N.Y. Acad. Sci.* **104,** 222 (1963).
23. I. TRAUTSCHOLD and E. WERLE, *Methoden der enzymatischen Analyse*, p. 880. Verlag Chemie, Weinheim (1962).

24. T. MOURI, *Tohoku J. exp. Med.* **98,** 147 (1969).
25. J. T. SGOURIS, J. K. INMAN, K. B. MCCALL, L. A. HYNDMAN and H. D. ANDERSON, *Vox Sang.* **5,** 357 (1960).
26. D. ARMSTRONG and W. DIAS DA SILVA, *Bradykinin and Related Kinins,* p. 31. Plenum Press, London (1970).
27. N. HEIMBURGER, *Thromb. Diath. haemorrh.* Suppl. **XXXII,** 93 (1969).
28. A. P. KAPLAN and K. F. AUSTEN, *J. Immunol.* **105,** 802 (1970).
29. A. P. KAPLAN and K. F. AUSTEN, *J. exp. Med.* **133,** 696 (1971).
30. H. Z. MOVAT, M.-C. POON and Y. TAKEUCHI, *Int. Arch. Allergy* **40,** 89 (1971).
31. M. J. SOLTAY, H. Z. MOVAT and A. H. ÖZGE-ANWAR, *Proc. Soc. exp. Biol. Med.* **138,** 952 (1971).
32. A. H. ÖZGE-ANWAR, H. Z. MOVAT and J. G. SCOTT, *Thromb. Diath. haemorrh.* **27,** 141 (1972).