Effect of Plasma Kallikrein on Coagulation *in Vitro*[1] (36757)

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(Introduced by W. O. Weigle)

Activation of the intrinsic pathway of blood coagulation leads simultaneously to the generation of the oligopeptide, bradykinin (1). Both coagulation and kinin generation require the participation of Hageman factor, but only the former process requires calcium ions to proceed to completion. Analysis of highly purified presursor components of this system has shown recently that a low molecular weight moiety of Hageman factor activates prekallikrein directly by enzymatic cleavage into two fragments (2). The larger fragment releases kinin from kininogen. The studies described in this report were undertaken to determine whether kallikrein has any effect on the promotion of coagulation *in vitro* in rabbit or human plasma.

Materials and Methods. Highly purified Hageman factor, prekallikrein activator, and prekallikrein used in these studies were prepared as previously described (2, 3). Methods employed for anion and cation exchange chromatography, block electrophoresis in Pevikon (Pharmacia, Uppsala), kinin generation, and analytical disc-gel and sodium dodecyl sulfate (SDS)-gel electrophoresis have been published (2).

Rabbit plasma prekallikrein was purified from chelated plasma protected with hexadimethrine bromide. The final product was homogeneous by analytical disc-gel and SDS-gel electrophoresis. When activated, the enzyme hydrolyzed the synthetic amino acid substrate benzoyl-arginine ethyl ester (BAEe) with a specific activity of approximately 50 μM/min/mg at 25°. Prekallikrein was radiolabeled with ^{125}I by the chloramine-T method (4).

Human plasma prekallikrein was prepared by ammonium sulfate fractionation, DEAE-Sephadex and CM-Sephadex chromatography (2). The pooled fractions containing prekallikrein were subjected to electrophoresis in Pevikon at 10 V/cm for 22 hr in citrate–phosphate buffer (pH 6.2) (Wuepper, K. D., unpublished data). The final product was contaminated only by β-$_2$-glycoprotein, Type I, as determined by double diffusion in agarose against an antiserum to β_2GP(I) (Behring Diagnostics, Somerville, NJ).

Contamination of prekallikrein with coagulation factors XII or XI was excluded by (a) double diffusion in agarose against monospecific antisera to XII (5) or XI (6), (b) failure of prekallikrein to accelerate the kaolin-partial thromboplastin test with human XII or XI deficient plasmas as substrate, (c) the isolation of factors XII and XI separate from prekallikrein (3, 6), and (d) the different physical properties of XI from prekallikrein (6). Upon activation by the prekallikrein activator, kallikrein solutions could not be shown to contain thrombin. When 0.5 ml human fibrinogen 4 mg/ml (Nutritional Biochemical, Co.) in TBS which contained 0.05 M $CaCl_2$ was incubated with kallikrein for 30 min at 37°, a coagulum did not form. To exclude the presence of an inhibitor, 1 NIH unit of thrombin was added to the fibrinogen solution and it clotted promptly. Similarly, after the fibrinogen solution had been incubated with 2 μg kallikrein for 30 min, 1 NIH unit thrombin was added and a coagulum then formed.

Prekallikrein activator used in these

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studies was from rabbit or human sources of highly purified Hageman factor which had undergone spontaneous activation (3).

Alpha$_2$-macroglobulin (α_2M) was prepared from human plasma by precipitation with ammonium sulfate, chromatography on DEAE-Sephadex, gel filtration on Sephadex G-200 and electrophoresis in Pevikon. Soy bean trypsin inhibitor (5× recrystallized) (Nutritional Biochemical Co.) was dissolved in 0.15 *M* sodium chloride buffered with 0.01 *M* tris(hydroxymethyl)aminomethane (TBS).

Coagulation tests were performed in disposable 12 × 75 mm plastic tubes (Falcon Plastics, Oakland). The partial thromboplastin test (PTT) was performed by the serial addition of equal volumes of TBS or material to be tested, partial thromboplastin 1:10 (B & A cephalin, Sigma Chemicals), calcium chloride 0.05 *M*, and fresh platelet-poor plasma in acid citrate dextrose (ACD). All reagents were added at 37° and the time required for clot formation was recorded. In separate experiments, the test material was first preincubated with plasma for specified intervals before recalcification.

Platelet-poor plasma used in these tests was obtained from normal rabbits or from rabbits genetically deficient in complement component C6 (Rancho Conejo, Vista, CA). Tests were also performed with platelet-rich plasma in which case buffer was substituted for cephalin. Human plasma, obtained from laboratory personnel, was anticoagulated in ACD.

The kaolin-partial thromboplastin test (K-PTT) was performed by addition of equal volumes of TBS or test material, a mixture containing in final volume 5 mg/ml kaolin and 1:10 partial thromboplastin, and test plasma. After a 3 min incubation interval, the mixture was recalcified and the clotting time was determined.

Results. As shown in Figs. 1 and 2, the PTT of rabbit plasma was accelerated in fractions containing rabbit kallikrein (Fig. 1) or human kallikrein (Fig. 2). The peaks of clot-promoting and BAEe esterolytic activity and kinin generation were coincident. In controls, prekallikrein did not accelerate coagulation but required activation with the prekallikrein activator from Hageman factor (Fig. 1). Incubation was carried out for 10 min at 37°. A quantity of PKA was selected for activation of prekallikrein which did not cause a reduction in the PTT of the control. Plasma rich in platelets from normal rabbits or plasma from rabbits lacking C6 also showed clot promotion in the presence of kallikrein (Table I). Unlike rabbit plasma, the PTT of fresh human plasma was not appreciably accelerated by the addition of kallikrein.

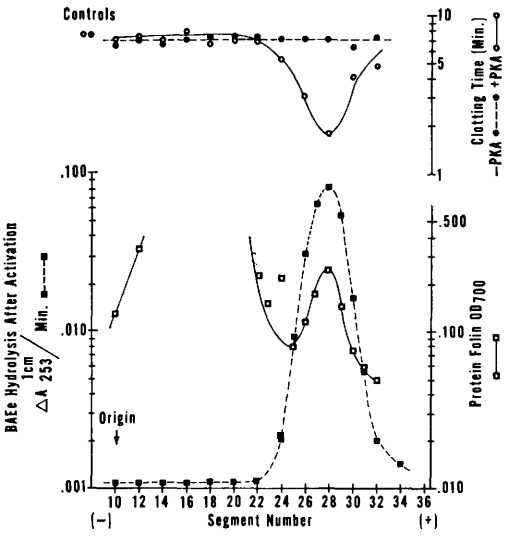

FIG. 1. Pevikon block electrophoresis of rabbit prekallikrein in the final stage of purification. Electrophoresis was in 0.05 *M* phosphate buffer (pH 8) at 10 V/cm for 20 hours. After activation, the enzyme hydrolyzed BAEe, given here as $\Delta A/1$ cm 253/min. Clotting was measured by the PTT prior to (—PKA) or following (+PKA) incubation with the prekallikrein activator.

Five micrograms of highly purified ^{125}I-rabbit prekallikrein was incubated with 0.55 μg PKA at 37° for 40 min and sedimented in linear gradients of sucrose 5 to 20%. Two labeled peaks corresponding to the 4.2 S and 0.9 S fragments were observed (2). Aliquots of 25 μl were brought to 0.1 ml in TBS and tested for clot promotion. The results are given in Fig. 3. Clot promotion corresponded to the larger labeled fragment which had es-

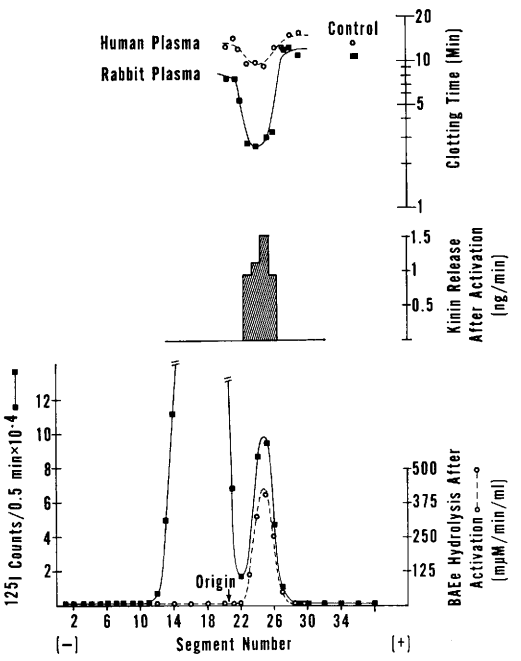

FIG. 2. Block electrophoresis of human kallikrein in Pevikon with citrate-phosphate buffer, μ M 0.04, (pH 6.2) for 20 hr at 10 V/cm. The major peak of protein was IgG; the minor, more anodal peak of protein contained prekallikrein. BAEe hydrolysis represents the contribution of human kallikrein subsequent to activation. Aliquots of 0.1 ml were used in the PTT with either human or rabbit plasmas as substrate.

ter-hydrolyzing activity and kallikrein activity.

Finally, prekallikrein was electrophoresed in 7% acrylamide gels at a running pH of 9.4 in Tris–glycine buffer. The gel was cut in segments and eluted in TBS. An aliquot from each segment was activated and tested for acceleration of the PTT. Clot promotion occurred in segments with a relative mobility which corresponded to the band of protein in a gel run in parallel and stained with Coomassie brillant blue.

The quantity of kallikrein employed was varied to determine the dose–response. With rabbit plasma substrate, the effect was linear between 0.1 and 1 μg when the log clotting time was plotted as a function of the log enzyme added (Fig. 4). A sharp acceleration of the dose response occurred at approximately 1 μg, which suggested that an inhibitor or regulatory factor might have been overcome.

Evidence for a plasma inhibitor of the clot-promoting effect of kallikrein was obtained. The enzyme was first preincubated with plasma for specified intervals prior to recalcification (Fig. 4, insert). Definite inhibition to control values occurred during the first 2 min

TABLE I. Effect of Rabbit Plasma Kallikrein on Normal and C6 Deficient Plasmas.

	PTT (min)[a]		
		Kallikrein[b]	
Plasmas substrate	Buffer	0.35 μg	1.4 μg
Platelet-poor, rabbit	9.6	4.6	1.75
Platelet-poor, rabbit	7.8	3.6	1.8
C-6 Deficient platelet-poor, rabbit	9.0	4.05	1.9
Platelet-poor, human	7.2	5.7	4.0

[a] Mean of duplicate determinations.
[b] Highly purified rabbit kallikrein.

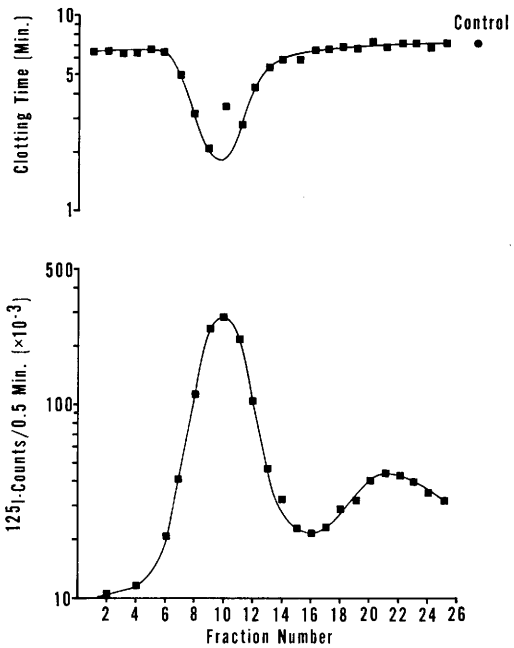

FIG. 3. ^{125}I-Rabbit prokallikrein was incubated with prekallikrein activator for 40 min (pH 7.5) and sedimented in a gradient of sucrose. Aliquots of the recovered fractions were tested by PTT with platelet-poor rabbit plasma as substrate.

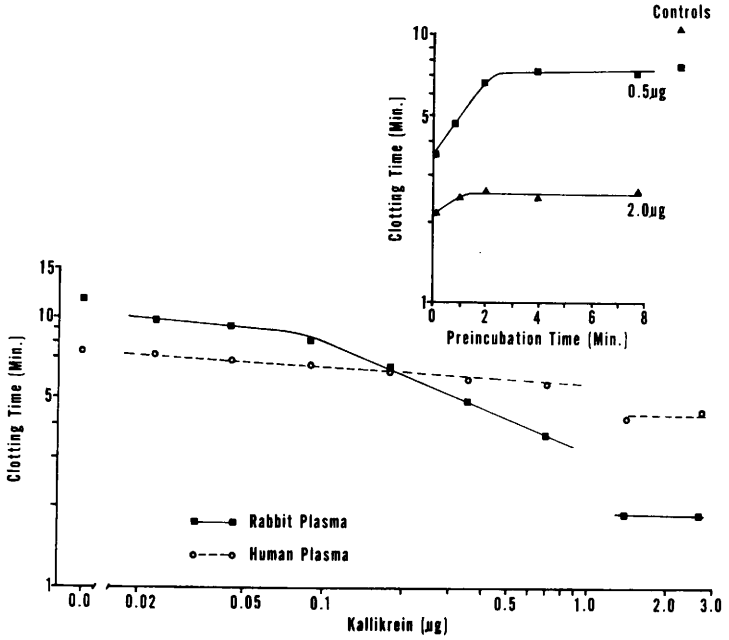

FIG. 4. Dose response of rabbit kallikrein with rabbit or human plasma as substrate. Initial studies were performed without preincubation. The insert shows the effect of preincubation of 0.5 or 2 μg kallikrein with rabbit plasma for variable periods prior to the addition of $CaCl_2$.

of preincubation when 0.5 μg kallikrein was added; no inhibition of 2 μg kallikrein occurred during 8 min of preincubation with plasma.

Inhibition of the clot-promoting activity of kallikrein was investigated with two inhibitors of the enzyme, soy bean trypsin inhibitor (SBI) and plasma alpha$_2$-macroglobulin (α_2M). When the quantity of SBI was varied, inhibition of the clot-promoting effect of kallikrein corresponded to a decrease in esterolytic activity (Table II). By contrast,

TABLE II. Inhibition of the Clot-Promoting Effect of Rabbit Plasma Kallikrein by Soy Bean Inhibitor.

Rabbit kallikrein[a]	SBI[b]	PTT clot time (min)	Hydrolysis of BAEe[c] (mμM/min)	Inhibition (%) of hydrolysis
—	—	7.5	—	—
+	—	1.9	75.0	0
+	5×10^{-6}	5.7	17.9	76
+	2.5×10^{-6}	2.8	21.4	71
+	1.25×10^{-6}	2.0	46.4	38
+	6.2×10^{-7}	1.8	64.3	14
—	5×10^{-6}	8.9	0.0	—
—	2.5×10^{-6}	7.7		
—	1.25×10^{-6}	7.5		
—	6.2×10^{-7}	6.7		

[a] +, present in the test; —, not present, TBS substituted.

[b] Final concentration (moles/liter).

[c] Following incubation with inhibitor, the sample was divided for tests of coagulation (PTT) or determination of BAEe hydrolysis.

α_2M protects approximately 50% of the esterolytic activity while completely inhibiting the *in vitro* coagulation effect (Table III).

TABLE III. Inhibition of Clot Promotion of Rabbit Kallikrein by Alpha$_2$ Macroglobulin.

Rabbit plasma kallikrein	Dilution α_2 macroglobulin	Coagulation time (min)[a]
—	—	9.2
—	1:1	9.3
+	—	3.0
+	1:1	9.7
+	1:2	9.7
+	1:4	7.5
+	1:8	6.1
+	1:16	4.7
+	1:32	3.8

[a] Mean of duplicate determinations.

This esterolytic activity cannot be further reduced with SBI. This observation suggested that the enzymatic site remains available for small molecules (BAEe, DFP) but not larger proteins (SBI, plasma substrate) (Table IV).

The ability of prekallikrein to correct the coagulation abnormalities of human clotting factor-deficient plasmas was next tested. The effect of prekallikrein on these substrates was tested by the kaolin-PTT. The results are given in Table V.

Discussion. The enzyme of plasma which, in its active form, releases bradykinin from kininogen has been shown to accelerate the PTT of rabbit plasma. This phenomenon was an unexpected observation which could only come from analysis of the enzyme in highly purified form. The substrate acted upon by kallikrein and the difference between rabbit and human plasmas to be affected remain to be explained.

Prekallikrein did not substitute for the coagulation factor abnormalities of human plasmas abnormal in Hageman factor (XII), plasma thromboplastin antecedent (XI), Christmas factor (IX), or antihemophilic globulin (VIII). Kallikrein does not insolubilize fibrinogen or lyse fibrin clots. Studies of a preliminary nature have shown, however, that highly purified precursor Hageman factor from rabbit or human sources is activated rapidly by 0.1 to 0.5 μg of kallikrein (C. G. Cochrane *et al.*, unpublished data). Prekallikrein or DFP-treated kallikrein lacked this property. Plasmin, which is also able to activate Hageman factor (5, 7), could be

TABLE V. Failure of Prekallikrein to Correct the Coagulation Abnormalities of Defined Plasmas.

Human plasma substrates	Kaolin-PTT (min)	
	Buffer	Prekallikrein[a]
Normal plasma	1.1	1.1
XII deficiency[b]	11.7	11.4
XI deficiency[c]	2.4	2.2
IX deficiency[c]	2.8	2.7
VIII deficiency[c]	>15	>15

[a] 4 μg rabbit prekallikrein added.

[b] Obtained through the courtesy of Mrs. Gunda Hiatt.

[c] Kindly supplied by Dr. S. I. Rapaport.

TABLE IV. The Protective Effect of Alpha$_2$ Macroglobulin on the Esterolytic Activity of Rabbit Kallikrein.

Order of addition[a]			BAEe hydrolysis		PTT clot time (min)[b]
Kallikrein	α_2 M	SBI	ΔA 1 cm/253/min	(mμM/min)	
+	—	—	0.0245	87.3	2.9
—	+	—	0.001	3.6	9.3
—	—	+	0.000	0.0	—
+	—	+	0.000	0.0	—
+	+	—	0.0125	44.4	9.7
+	+	+	0.0115	41.1	—

[a] +, present in the test; —, substituted with TBS.

[b] —, not determined.

shown not to contaminate the preparations.

Human and rabbit plasmas are clearly different in their susceptibility to the clot-promoting effects of kallikrein. These differences might be explained by the rate of inhibition of the enzyme or the quantity of inhibitory substances in whole plasma. The susceptibility of the substrate to enzymatic activation by kallikrein provides an alternate possibility. Such differences are amenable to investigation.

Summary. Human or rabbit plasma kallikrein in highly purified form accelerated the partial thromboplastin test of rabbit, but not human, plasma in a dose-dependent fashion. The clot-promoting effect of kallikrein was inhibited by preincubation with plasma or by treatment of the enzyme with plasma α_2-macroglobulin or soybean inhibitors. Prekallikrein did not substitute for the coagulation abnormalities of human plasma deficient in Hageman factor, plasma thromboplastin antecedent, Christmas factor or antihemophilic globulin.

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