RELEASE OF KALLIKREIN FROM GUINEA PIG LUNG DURING ANAPHYLAXIS*

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The kinins are a group of closely related polypeptides including bradykinin and methionyl bradykinin, which are potent agents in enhancing vascular permeability, contracting smooth muscle, and dilating small blood vessels (1). These polypeptides are formed in plasma and, probably, in extravascular tissues from a protein substrate, or substrates, kininogen, by the action of a number of proteolytic enzymes (2). Among these enzymes are the kallikreins, comprising a group of similar yet distinctly different enzymes present in plasma, various tissues such as the pancreas and salivary glands, and in urine (3). The kallikreins exist in an inactive precursor form, the prokallikreins or kallikreinogens and are activated by a step-wise, sequential process, which is incompletely understood (2).

In 1950, Beraldo demonstrated an increase in kinin in the blood of dogs following anaphylactic shock (4). More recently, Brocklehurst reported that kinin can be detected in the blood of guinea pigs, rats, and rabbits within 5 min after intravenous induction of anaphylaxis (5). Other workers have since studied the participation of kinin in anaphylactic shock of various species (6–13).

Brocklehurst and Lahiri have also demonstrated, in experiments with the isolated perfused guinea pig lung, that challenge with antigen led to the prompt appearance of kinin-forming activity (kallikrein) in the effluent. Kinin was absent, presumably because of the lack of kininogen in the perfusion preparation. The highly active kinin destroying enzyme, kininase, was present in the perfusate (14).

In these experiments, we have used a similar system of perfused sensitized guinea pig lung to study the nature of the kallikrein activity which is produced during anaphylaxis. We have compared its properties to those of plasma kallikrein, and have begun the study of the mechanism of activation of kallikrein in the lung by the antigen-antibody reaction.

Materials and Methods

All glassware used in the bioassay was siliconized.

Standard Solutions for Bioassay.—Bradykinin standards (Calbiochem Company, Los

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Angeles) were prepared in 0.25% casein, in dilutions of 100 nanograms per ml, and kept at −20°C for 6 months.

Human Urinary Kallikrein.—Human urinary kallikrein was supplied by Dr. Marion Webster, National Institutes of Health, Bethesda, Maryland.

Human Plasma Kallikrein and Pf/dil.—Human plasma kallikrein and human Pf/dil were supplied by Dr. David McConnell, Walter Reed Army Institute of Research.

Guinea Pig Plasma Kallikrein.—Guinea pig plasma kallikrein was prepared by elution of guinea pig serum from DEAE cellulose by 0.005 M sodium phosphate buffer, pH 8.10, exactly as described for the preparation of human plasma kallikrein (15).

Inhibitors.—Soybean trypsin inhibitor (SBTI), five times crystallized, was obtained from Mann Research Laboratories, New York City.

Diisopropylfluorophosphate (DFP) was obtained from Merck, Sharpe and Dohme Co., West Point, Pennsylvania.

Partially purified C'1 esterase inhibitor was supplied by Dr. N. Tamura, Howard Hughes Medical Research Institute, Miami.

Ellagic Acid.—Ellagic acid was obtained from K and K Laboratories, Jamaica, New York, lot 52062. It was made up in a 1×10^{-4} M solution in Tyrode's buffer.

Plasma Substrate.—Citrated fresh human plasma, collected in siliconized glassware, was heated to 61°C for 2 hr to destroy the plasma kallikrein system and prevent spontaneous formation of bradykinin. The heated plasma was dialyzed overnight against 0.15 M sodium chloride, acidified to pH 1.5 for 10 min at 37°C, and neutralized, to inactivate kininase. The bradykininogen of the final substrate (heated, dialyzed, and acidified plasma) was activated by the kallikreins but not by Pf/dil and did not spontaneously form bradykinin.

Preparation of Perfused Lungs.—The guinea pigs were anesthetized with intraperitoneal Nembutal® (200 mg) and the heart and lungs removed en bloc. The trachea was intubated and the pulmonary artery cannulated and perfused with Tyrode's solution at 37°C, at a rate of 2 ml per minute. The left atrium and ventricle were widely incised (16). Ventilation was achieved with Army Emergency Respirator (lent by Mr. Heinrich Straub, Harry Diamond Laboratories, Washington, D. C.) which produced an intermittent negative pressure of 10 to 20 cm of water within a closed chamber into which the heart-lung preparation was suspended, with the tracheal cannula open to the air. The lungs were perfused until free of gross blood (15 to 20 min) and bovine serum albumin, 2 mg, was injected into the pulmonary artery cannula. Perfusate was collected at 1 min intervals and immediately iced and centrifuged in the cold.

Perfusions made in the presence of ethylenediaminetetraacetic acid (EDTA) were performed by addition of EDTA to the perfusion fluid (Tyrode's) in a final concentration of 0.005 M.

Perfusions made in the presence of ellagic acid were performed by rapid infusion of 10 ml of ellagic acid, 1×10^{-4} M in Tyrode's, into the pulmonary artery cannula after completion of a preliminary perfusion with Tyrode's buffer until the lungs were blood-free.

Sensitization of Guinea Pigs.—Hartley strain guinea pigs (250 to 400 g) were actively sensitized with subcutaneous bovine serum albumin (BSA) 3 wk previously and boosted with 1 to 2 intradermal injections of BSA at 4-day intervals until a marked Arthus reaction was observed; or, were passively sensitized intravenously 18 to 24 hr before use with rabbit anti-bovine serum albumin, 0.25 mg antibody nitrogen per animal. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Bioassay.—Bioassay of the perfusate was performed by incubation of 0.5 ml samples with 0.25 ml of plasma substrate, at 37°C for 10 min; the kinin released by this incubation was measured by contraction of the isolated estrus rat uterus, according to the method of Eisen (17), and compared to contractions obtained with synthetic bradykinin standards. Histamine and a slow reacting substance of anaphylaxis (SRS-A) were assayed by contraction of the

isolated guinea pig ileum, according to the method of Brocklehurst (18). Permeability activity was determined in "blued" guinea pigs according to the method of Miles and Wilhelm (19) as modified by McConnell et al. (20).

Inhibition Studies.—Inhibition of the activity of plasma and lung kallikrein was carried out by incubation of the enzyme with the inhibitor for 1 hr at room temperature. When diisopropylphosphofluoridate (DFP) was the inhibitor being tested, the DFP was removed prior to bioassay by passage of the sample through a column (1.0 × 20 cm) of Sephadex G-25 (medium) (Pharmacia Fine Chemicals, Piscataway, N. J.) immediately before testing.

Preparative Agar Electrophoresis.—Agar electrophoresis was performed as described by Ovary et al. (21), on 3¼ × 4 in. glass slides filled with 20 ml of 2% Ionagar (Consolidated Laboratories, Inc., Chicago Heights, Illinois) diluted with equal parts of 0.1 M glycine buffer at pH 8.6. A longitudinal well holding 0.3 ml of sample was cut in the center and reference sera were placed at either end for immunoelectrophoresis. Electrophoresis was done at 4°C in 0.05 M glycine buffer, using 40 v for 60 min. Nine longitudinal strips (6 × 0.5 cm) were cut out parallel to the center well, and eluted with 0.16 M sodium chloride after freezing and thawing one time to break up the agar.

Gel Filtration.—Fractionation of whole serum and of kallikrein preparations was carried out with gel filtration on columns (1.3 × 45 cm) of Sephadex G-200 (Pharmacia Fine Chemicals). Samples were made up in 10% sucrose with 10 mg of Blue Dextran 2000 (Pharmacia Fine Chemicals) as a marker; 1.0 ml samples were applied to the column and eluted with 0.05 M phosphate buffer at 4°C. Fractions of 1.0 ml were collected, and optical density was measured at 280 mμ.

Sucrose Gradient.—The positions of serum and lung kallikrein on a sucrose density gradient of 5 to 20%, were determined using the Spinco Model L ultracentrifuge at 36,000 RPM for 15 hr at 4°C. Twenty-five fractions of ten drops each were analyzed by bioassay and compared to the positions of marker proteins (BSA and human gamma globulin).

RESULTS

Release of Kallikrein

Time Sequence.—In vitro anaphylaxis in the actively or passively sensitized isolated guinea pig lung was induced by introduction of bovine serum albumin, 2 mg, into the pulmonary artery. Within 2 min, severe bronchospasm occurred, and the lungs remained in a hyperaerated condition.

As seen in Fig. 1, the perfusate collected immediately before antigen was injected contained little or no kinin-forming activity; perfusate collected each minute after antigen contained increasing amounts of kinin-forming enzyme as measured by kinin liberation from plasma substrate. Enzyme levels decreased after 6 min and were barely detectable 15 min after anaphylaxis.

Histamine levels were determined in the perfusate by contraction of the atropinized guinea pig ileum. In Fig. 2, it is apparent that histamine was released in largest amounts within the first 5 min following anaphylaxis, and was not released in significant amounts after 10 min. SRS-A, as assayed by contraction of the atropinized guinea pig ileum in the presence of antihistamine, was released during the first 10 min after anaphylaxis and decreased in amount but was still present after 20 min.

Incubation of perfusate with bradykinin at 37°C resulted in progressive

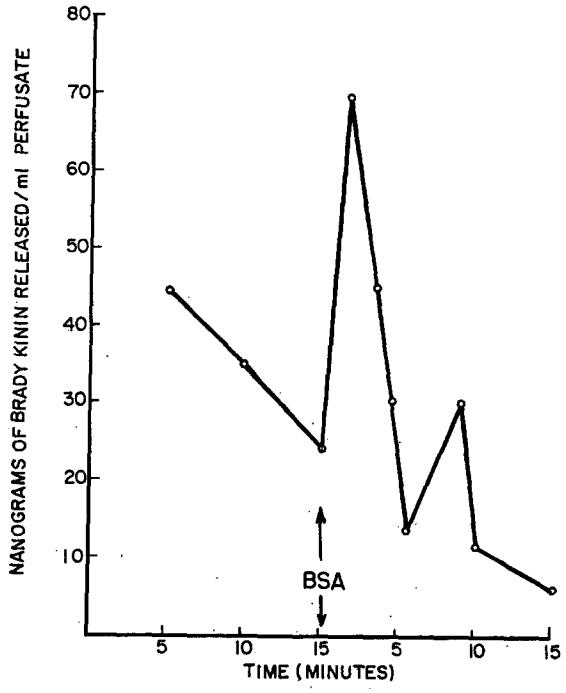

FIG. 1. Kallikrein activity in guinea pig lung perfusate during antigen administration.

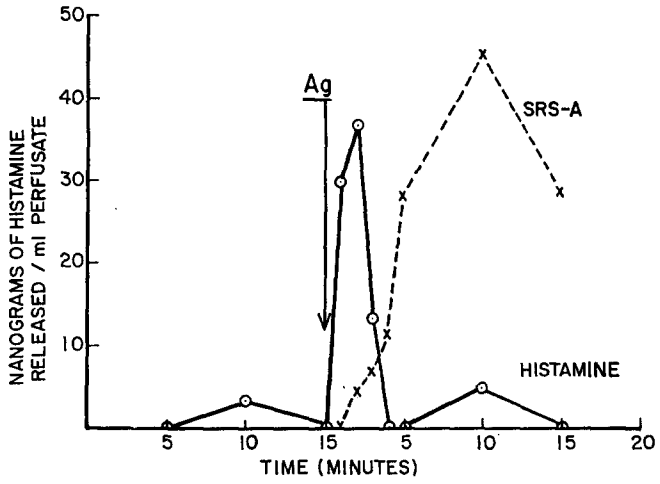

FIG. 2. Histamine and SRS-A in guinea pig lung perfusate during antigen administration.

decrease of kinin activity, due to the presence of kininase in the lung perfusate (14). This kininase activity was decreased significantly by dilution of the perfusate, and was inactivated by acidification of the perfusate.

TABLE I

*Effect of EDTA on Anaphylactic Release of Histamine, SRS-A, and Kallikrein by the Isolated Perfused Guinea Pig Lung**

Perfusate	Histamine		SRS-A		Kallikrein	
	No EDTA	EDTA	No EDTA	EDTA	No EDTA	EDTA
Control, before antigen	0	0	0	0	10	10
2 min, after antigen	20	0	20	0	50	64
4 min, " "	20	0	5	0		
6 min, " "	23	0	3		60	87
8 min, " "	20	0	5	0	30	70
10 min, " "	4	0	3		30	60
12 min, " "	3	0	8	0		

Height of contraction of guinea pig ileum or rat uterus, in millimeters.

* No histamine, SRS-A, or kallikrein was released with EDTA alone, in the absence of antigen.

TABLE II

Effect of Addition of 10^{-4} M Ellagic Acid to Fluid Perfusing the Unsensitized Guinea Pig Lung

Perfusate	Kinin production
Control perfusate, before ellagic acid	2*
2 min, after ellagic acid	2
4 min, " " "	4
6 min, " " "	6
8 min, " " "	35
10 min, " " "	30
Ellagic acid + control perfusate	2
Ellagic acid alone	0
Bradykinin, 10 nanograms	38

* Height of contraction of rat uterus, in millimeters.

Effect of EDTA.—Release of kallikrein from the lung tissue was found to be independent of the release of histamine and SRS-A, it not being affected by the presence of ethylenediaminetetraacetic acid (EDTA) in the fluid used to perfuse the lungs. Histamine and SRS-A were not present in the recalcified perfusate after anaphylaxis, when 0.005 M EDTA in Tyrode's solution was used to perfuse the lungs (Table I). Kallikrein was present in these perfusates,

in concentrations equal to those of control perfusions using Tyrode's solution alone. Bronchospasm was reduced in the lungs receiving EDTA perfusion.

Effect of Ellagic Acid.—Table II shows the results of an experiment in which ellagic acid was perfused through unsensitized guinea pig lung. No kallikrein

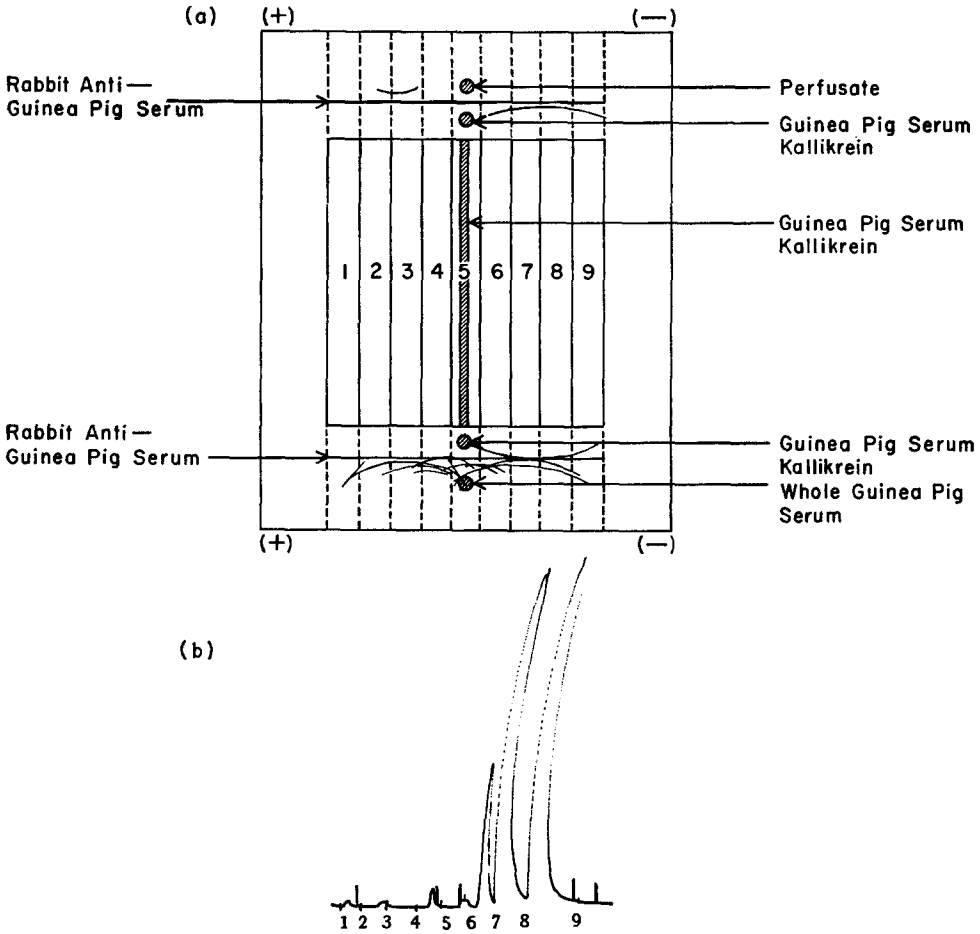

FIG. 3. (*a*) Preparative agar electrophoresis of guinea pig serum kallikrein. (*b*) Bioassay of eluates from strips 1 to 9 of agar electrophoresis. Rat uterus.

activity was present in the perfusate of unsensitized lungs prior to the infusion of ellagic acid into the pulmonary artery cannula. Following addition of ellagic acid, kallikrein activity appeared in the perfusate, reaching maximal levels at 8 min, and declining by 10 min. Addition of ellagic acid in equal amounts to the inactive preliminary perfusate failed to produce kallikrein activity. Ellagic acid alone added to the plasma substrate, did not produce kinin.

In a second experiment, not shown here, the same results were obtained with infusion of ellagic acid, with the exception that, when ellagic acid 10^{-4} M was incubated with the control perfusate, kinin was produced when plasma substrate was added. Again, neither the control perfusate nor ellagic acid alone had kallikrein activity.

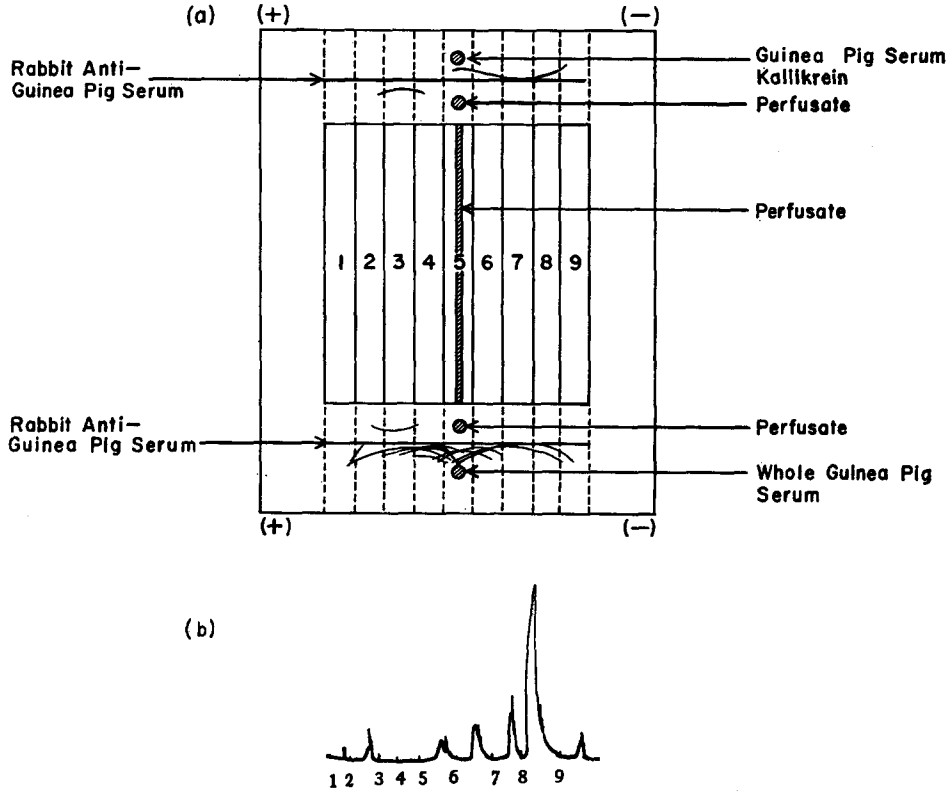

FIG. 4. (*a*) Preparative agar electrophoresis of guinea pig lung perfusate. (*b*) Bioassay of eluates from strips 1 to 9 of agar electrophoresis.

Properties of Lung Kallikrein

Certain properties of guinea pig lung kallikrein have been found identical to those of guinea pig serum kallikrein.

Electrophoretic Mobility.—Guinea pig serum kallikrein and lung perfusate were subjected to preparative agar electrophoresis (Figs. 3 and 4). Eluates from the areas of slow gamma globulin mobility only, were active in each instance. This area corresponded to a weak precipitin band in the adjacent immunoelectrophoresis patterns.

Size.—Guinea pig serum kallikrein and lung perfusate were subjected to gel filtration on columns of Sephadex G-200. Activity came off (with a tenfold dilution) in two areas, identical in each sample. The first area, tubes 45 to 55, corresponded to the dip between macroglobulins and 7S globulins. The second area of activity, tubes 70 to 80, was unexpected and appeared in each sample in the dip immediately prior to the albumin peak (Fig. 5). Further characterization of this peak was not carried out.

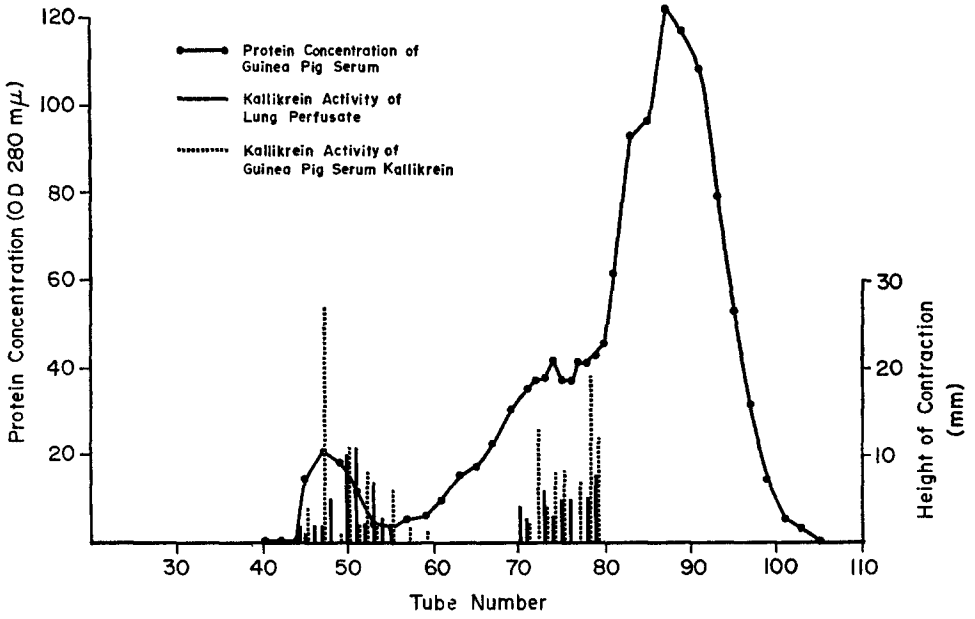

FIG. 5. Gel filtration on Sephadex G-200 (45 × 1.3 cm). Note two separate peaks of kallikrein activity; tubes 45 to 55, and tubes 70 to 80. Sample size, 1 ml.

Sucrose density gradient ultracentrifugation of serum kallikrein and of lung kallikrein each yielded activity over a broad area without distinguishing two peaks of activity as had been noted with gel filtration. The broad area of activity began in samples before the human gamma globulin marker protein, and extended to but did not include the samples containing the bovine serum albumin marker protein. This broad distribution of kallikrein activity was compatible with the molecular size heterogeneity of kallikrein found by gel filtration.

Inhibition of Activity.—

Soybean trypsin inhibitor (SBTI): Samples of lung perfusate were incubated with SBTI, 50 μg per ml of perfusate, for 1 hr at room temperature, and were

then added to plasma substrate. Bioassay with the rat uterus (Fig. 6), demonstrated complete inhibition of activity of the lung perfusate by SBTI.

These results were confirmed with assay of permeability activity in "blued" guinea pigs. As seen in Table III, permeability activity of bradykinin was not

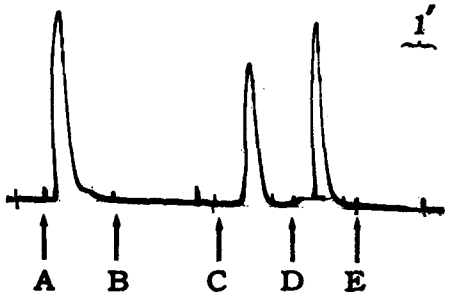

FIG. 6. Inhibition of lung perfusate with soybean trypsin inhibitor (SBTI). Substrate: human plasma (heated, dialyzed, and acidified). Rat uterus. (A) Bradykinin, 5 nanograms; (B) perfusate, 1 min, 0.5 ml, + SBTI, 50 nanograms; (C) perfusate, 1 min, 0.5 ml; (D) perfusate, 4 min, 0.5 ml; and (E) perfusate, 4 min, 0.5 ml, + SBTI, 50 nanograms.

TABLE III

Permeability Activity

Dilutions	Bradykinin 1.0 μg/ml		Perfusate	
		SBTI 50 μg		SBTI 50 μg
1:2	11*	11	10	0
1:4	10	9.5	8.5	0
1:8	10	8	0	0
1:16	9.5	8.5	0	0
1:32	9	7	0	0
1:64	6	6.5	0	0
1:128	3	0	0	0
Tyrode's	4	3	3	3

* Diameter of blue spot on skin of blued guinea pig in millimeters; 0.2 ml volumes.

affected by SBTI, while permeability activity of lung perfusate kallikrein was abolished by SBTI.

Diisopropylphosphofluoridate (DFP): Samples of lung perfusate were incubated for 1 hr at room temperature with DFP at concentrations of 1.0×10^{-3} and 5.0×10^{-4} M. After removal of DFP, bioassay using the rat uterus (Fig. 7) demonstrated incomplete inhibition by the lower concentration of DFP, and complete inhibition by the higher concentration of DFP.

C'1 esterase inhibitor: Varying volumes of lung perfusate and of guinea pig

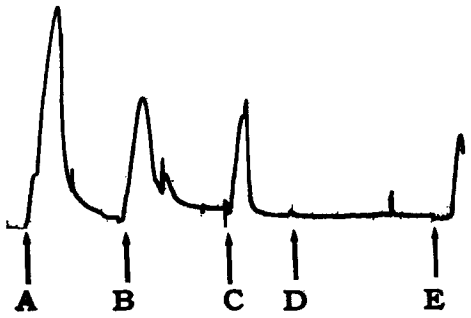

FIG. 7. Inhibition of lung perfusate with diisopropylphosphofluoridate (DFP). Substrate: human plasma (heated, dialyzed, and acidified). Rat uterus. (A) Bradykinin, 2 nanograms; (B) bradykinin, 0.5 nanograms; (C) perfusate, 2 min, 0.5 ml, incubated at 25°C for 1 hr, then passed through Sephadex G-25; (D) perfusate, 2 min, 0.5 ml, incubated with DFP (1.0×10^{-3} M) at 25°C for 1 hr, then passed through Sephadex G-25; (E) perfusate, 2 min, 0.5 ml, incubated with DFP (5.0×10^{-4} M) at 25°C for 1 hr, then passed through Sephadex G-25.

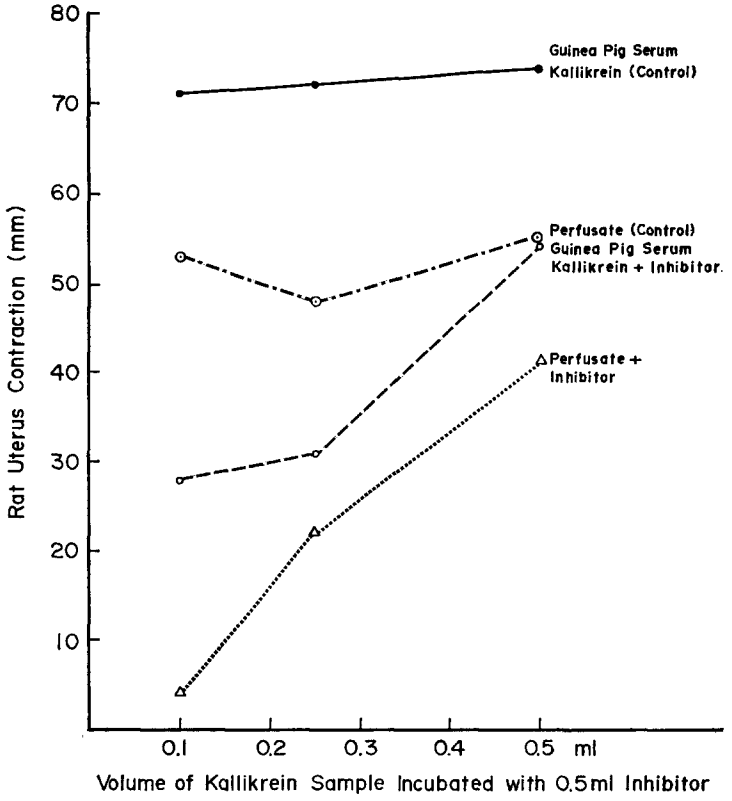

FIG. 8. Inhibition of guinea pig serum kallikrein and lung perfusate by C'1 esterase inhibitor. Volume of inhibitor constant (0.5 ml); volume of kallikrein sample varied from 0.1 to 0.5 ml. Incubation for 1 hr at 25°C.

serum kallikrein were incubated at room temperature for 1 hr with 0.5 ml of guinea pig C'1 esterase inhibitor. Bioassay with the rat uterus (Fig. 8) revealed progressive inhibition of enzyme activity of serum and lung kallikrein with higher concentrations of inhibitor. Within the broad limits of this experiment, it was also apparent that the degree of increase of inhibition of serum and lung kallikrein with each decreasing concentration of enzyme, was similar.

DISCUSSION

These experiments confirm the reports of Brocklehurst and Lahiri, that the antigen-antibody reaction is associated with the release of kallikrein into the effluent of the perfused guinea pig lung (14). In all properties we have studied, the lung kallikrein is indistinguishable from plasma kallikrein. Soybean trypsin inhibitor and DFP in the same concentrations inhibit the lung kallikrein to apparently the same degree as guinea pig plasma kallikrein. The inhibition of guinea pig lung and plasma kallikrein by a preparation containing guinea pig C'1 esterase inhibitor, extends to this species the findings of Kagan that human C'1 esterase inhibitor inactivates human plasma kallikrein (22). C'1 esterase inhibitor is, so far as it is known, rather specific, inhibiting only C'1 esterase, kallikrein, and Pf/dil; the rather closely related enzymes, plasmin and thrombin, are not affected.

Not only are the kallikreins of guinea pig lung and plasma similar in their susceptibility to inhibitors, but they are also similar in those physical properties which we have studied. On electrophoresis, both have mobility in the slow gamma region. When subjected to gel filtration on a column of Sephadex G-200, the plasma and lung preparations each gave two peaks of kallikrein activity in identical areas; one corresponding to a molecular weight less than the macroglobulins but greater than 7S gamma globulin, and the other corresponding to a molecular weight less than the 7S gamma globulin but somewhat greater than albumin.

Both peaks contained activity of the kallikrein type as defined by kinin production from heated plasma (which is not capable of activation by Pf/dil or enzymes other than kallikrein in the activation sequence of kinin). These results further emphasize the similarity of lung and plasma kallikrein, and also reveal a completely unexpected heterogeneity in molecular size. An investigation of this heterogeneity is under way in our laboratory.

An unequivocal conclusion as to the relationship of lung and plasma kallikrein would be possible only after a much more extensive and detailed comparison than was attempted here. Nevertheless, the similarities in the various properties of the two kallikreins disclosed by the present study suggests that the kinin-forming activity from the two sources is due to the same enzyme.

The acceptance of the hypothesis that the kallikreins of lung and plasma are the same immediately raises the question of the source of these enzymes. It is possible that the lung kallikrein is derived from residual plasma remaining in

the perfused lung system, although it seems unlikely that the enzyme could have been present in measurable quantities in the small amounts of plasma present after the extensive perfusion. It is more likely that the kallikreinogen has been adsorbed from the plasma on to the endothelial cells or other pulmonary tissues, and is activated and released during the antigen-antibody reaction. Alternatively, the lung may be a source of kallikrein, and the kallikrein found in the perfusate as well as that found in plasma might have come directly from this tissue. Exploration of these possibilities must await purification of the kallikreins beyond what has been presently attempted.

EDTA has no effect on the activation of lung kallikrein by an antigen-antibody reaction, although it completely blocks the release of histamine and SRS-A. The mechanisms of immune activation of kallikrein and of histamine and SRS-A are obviously different; the mechanism of the first apparently does not invlove a cation-dependent step, whereas, release of the two latter factors does. The insensitivity to EDTA of the activation of lung kallikrein is, however, in accord with the findings of Margolis and Bishop (23) that in human plasma, kinin formation by glass activation of the kallikrein system occurs in the presence of 0.01 M EDTA. Also, Armstrong et al. (24) have observed kinin formation in human plasma in the presence of citrate. Thus, to the other similarities between lung and plasma kallikrein already described, one can add that activation of either kallikrein does not involve a cation-dependent step.

Ratnoff has reported that derivatives of gallic acid, especially ellagic acid, will activate Hageman factor in plasma, causing an acceleration of clotting (25), and the appearance of permeability activity (26). There is good evidence that in plasma the formation of kallikrein from prokallikrein involves the initial activation of Hageman factor (2). Therefore, from several viewpoints it is significant that active kallikrein appears in the perfusate of unsensitized lungs following the infusion of ellagic acid into the pulmonary artery. Another point of similarity between lung and plasma kallikrein is brought out, and it is suggested that a system of kallikrein activation similar to that present in plasma is also present in perfused lung. This in turn suggests, although it does not demand, the working hypothesis that the initiating event in the activation of kallikrein by an antigen-antibody reaction is the activation of Hageman factor.

SUMMARY

An antigen-antibody reaction occurring in the perfused sensitized guinea pig lung, has been demonstrated to release kallikrein, a proteolytic enzyme related to the formation of kinins. This lung kallikrein is similar to plasma kallikrein in all properties studied, including susceptibility to the same inhibitors, electrophoretic mobility, and heterogeneity in molecular size.

The release of kallikrein during anaphylaxis in the guinea pig lung occurs in the presence of ethylenediaminetetraacetate. Perfusion of ellagic acid into

nonsensitized lungs will also release kallikrein, presumably through activation of Hageman factor. On the basis of these findings the hypothesis is suggested that the kallikrein in perfused lung activated by the antigen-antibody reaction is, in fact, plasma kallikrein. It is further suggested that activation of such kallikrein by the antigen-antibody reaction proceeds through Hageman factor.

Guinea pig C'1 esterase was kindly supplied by Dr. N. Tamura of the Howard Hughes Medical Research Institute, Miami, Florida.

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