Anaphylactic Release of a Prekallikrein Activator from Human Lung In Vitro

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ABSTRACT We have demonstrated the in vitro IgE-mediated release of a prekallikrein activator from human lung. The lung prekallikrein activator was partially purified by sequential chromatography on sulfopropyl-Sephadex, DEAE-Sephacel, and Sepharose 6B. Purified human prekallikrein was converted to its active form (kallikrein) by the lung protease. The generated kallikrein was shown to be biologically active; that is, it generates bradykinin from purified human high-molecular weight kininogen and also cleaves benzoyl-propyl-phenyl-arginyl-*p*-nitroanilide, a known synthetic substrate of kallikrein. The lung prekallikrein activator differs from the known physiologic activators of prekallikrein (the activated forms of Hageman factor) with respect to: (*a*) size (it has a mol wt of ~175,000); (*b*) synthetic substrate specificity (D-propyl/phenyl/arginyl-*p*-nitroanilide is a substrate for the activated forms of Hageman factor, but not the lung protease); (*c*) antigenic specificity (an anti-Hageman factor immunoadsorbent column did not remove significant amounts of the lung protease, while it removed most of the activity of activated Hageman factor fragments); and (*d*) inhibition profile (the lung proteases was not inhibited by corn trypsin inhibitor). This prekallikrein activator provides a physiologic mechanism by which prekallikrein can be directly activated during IgE-mediated reactions of the lung. While the role of this lung prekallikrein activator in immediate hypersensitivity reactions and in other inflammatory processes is not clear, it does represent a first and important interface between IgE-mediated reactions and the Hageman factor-dependent pathways of the inflammatory response.

INTRODUCTION

Human prekallikrein (PK) is a γ-globulin proenzyme that circulates in plasma (1). Once prekallikrein is converted to kallikrein, the latter may activate the Hageman factor (HF)[1]-dependent pathways via the proteolytic cleavage of zymogen HF (2–3). Alternatively, kallikrein may directly cleave kininogen to generate bradykinin (4), or cleave plasminogen to generate plasmin (5–6).

Activation of PK in human plasma was first shown to occur by a process of limited proteolytic digestion of the proenzyme by an active fragment derived from HF (HF_f) (1). Subsequently, a surface-bound activated form of HF (HF_a) was shown to cleave coagulation Factor XI as well as PK (7–8). These forms of HF (HF_f, HF_a) have heretofore presented the only known physiologic activators of PK.

The cross linking of human IgE antibody bound to receptors on mast cells and basophils, by an appropriate antigen, is known to initiate many events of allergic and inflammatory reactions. Until recently, this inflammatory reaction was thought to result primarily from mast cell- and basophil-derived small molecular weight mediators such as histamine, or from

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[1] *Abbreviations used in this paper:* AgE, ragweed antigen E; Bz-Pro-Phe-Arg-*p*NA, benzoyl-propyl-phenyl-arginyl-*p*-nitroanilide; D-Pro-Phe-Arg-pNA, *D*-propyl-phenyl-arginyl-*p*-nitroanilide; HF, Hageman factor; HF_a, activated HF; HF_f, HF fragment; HMW kininogen, high-molecular weight kininogen; LPKA, lung PK activator; PK, prekallikrein; SP, sulfopropyl.

effector cells brought to the lesion by chemotactic factors. However, neutral proteases have been isolated from human mast cells (9, 10) that have the potential to contribute to the inflammatory response by cleavage of protein substrates. For example, mast cell tryptase has been reported to cleave C3 to generate C3a anaphylatoxin (11). We have additionally described the IgE-mediated release of a group of high-molecular weight proteases from human lung, which interact with three proteins of the HF-dependent pathways (12–14). One of these proteases converts PK to its active form, kallikrein. The activation of PK by this lung PK activator (LPKA) could result in kinin generation (4) and fibrinolysis (5, 15), as well as the generation of chemotactic activity (3) and thereby participate in important aspects of inflammatory processes of human lung and other tissues. This manuscript describes the partial purification and characterization of the LPKA.

METHODS

Bradykinin triacetate (kindly provided by Sandoz Ltd., Basel) was used as the standard for native bradykinin. The following were purchased: hexadimethrine bromide, diisopropylfluorophosphate; Aldrich Chemical Co., Inc., Milwaukee, WI); DEAE-Sephacel, quaternary aminoethyl-Sephadex A-50, sulfopropyl (SP)-Sephadex C-25, Sephadex G-100 and G-200, and Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ); Tris base, potassium thiocyanate, EDTA, monobasic and dibasic sodium phosphate (Fisher Scientific Co., Pittsburgh, PA); benzoyl-phenyl-arginyl-*p*-nitroanilide (Bz-Pro-Phe-Arg-*p*NA; Vega-Fox Biochemicals Div., Tucson, AZ); D-propyl-phenyl-arginyl-*p*-nitroanilide (D-Pro-Phe-Arg-pNA; Pentapharm Ltd., Basel); and $Na^{125}I$ (New England Nuclear, Boston, MA). A Corning pH meter and a Yellowsprings conductivity meter were used to determine the pH and conductivity of buffers, respectively. Ragweed antigen E (AgE) was kindly provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Bethesda, MD.

PK-deficient plasma (0.38% citrate) was a gift from Dr. C. Albildgaard (University of California, Davis) and corn inhibitor was a gift from Dr. J. Pisano (NIH). Normal plasma used for assays of kinin generation was collected in EDTA (0.9 mg/ml of blood) and centrifuged at 900 *g* for 20 min at 4°C. Plasma used for the isolation of plasma proenzymes was collected in sodium citrate (0.38%) and hexadimethrine bromide (0.36 mg/ml).

Preparation of PK and high-molecular weight (HMW) kininogen. Prekallikrein was prepared, as previously reported (16), by successive chromatography using QAE-Sephadex, SP-Sephadex, Sephadex G-200 (phosphate-buffered normal saline, pH 7.4) and passage over an immunoadsorbent prepared with sheep antibody against human IgG and beta 2 glycoprotein I. HMW kininogen was prepared by the procedure of Thompson et al. (17) on QAE-Sephadex and SP-Sephadex.

Generation of human lung supernatants. Specimens of macroscopically normal human lung recovered from surgical specimens excised because of carcinoma were dissected free of pleura, cartilage, and large vessels, then cut into fragments (~100 mg) and washed with Tyrodes buffer until they were grossly free of blood (18). The lung fragments were then passively sensitized (18 h, 22°C) in a 1:40 dilution of a serum that contained 6.0 μg/ml of IgE, 1.5 μg/ml of which was specific for AgE. After passive sensitization, the tissue was extensively washed and then the fragments were challenged with AgE in Tyrodes buffer for 30 min at 37°C. The supernatant was recovered by gravity filtration of the mixture on a wire screen. Histamine content was measured in the supernatant spectrofluorometrically as previously described (19).

Amidolytic assays. To detect kallikrein activity, we used the synthetic substrate Bz-Pro-Phe-Arg-pNA (20). The assay was performed by incubating 5 μl of PK (70 μg/ml in 0.01 M phosphate buffer, pH 8.0) with 5 μl of PK activator (or column fractions to be tested for LPKA activity) for 30 min at 37°C. At the end of the first incubation, 50 μl of Bz-Pro-Phe-Arg-*p*NA (0.7 mg/ml in 0.01 M phosphate buffer, pH 8.0) was added to the mixture and the incubation continued for another 30 min. The reaction was terminated by the addition of 0.4 ml of 0.1 M acetic acid and the amount of free *p*-nitroaniline determined by its absorbance at 405 nm. Initial experiments demonstrated that the liberation of *p*-nitroaniline was linear with respect to the concentration of kallikrein in the range of 0.05 and 30.0 μg/ml and that significant cleavage of Bz-Pro-Phe-Arg-*p*NA was not obtained with the LPKA. Samples were diluted to levels of activity within the linear range of the assay.

To determine the presence of HF_a or HF_f, we used the synthetic substrate D-Pro-Phe-Arg-*p*NA (21). This assay was performed by incubating 5 μl of sample with 50 μl of D-Pro-Phe-Arg-*p*NA (0.7 mg/ml) for 20 min at 37°C. The reaction was terminated by the addition of 0.4 ml of 0.1 M acetic acid and the absorbance determined at 405 nm.

Bioassay. The ability of kallikrein to generate kinin from either heat-inactivated plasma or partially purified HMW kininogen was determined by bioassay using the isolated guinea pig ileum (22). The contractions were proportional to the concentration of bradykinin between 2 and 20 ng/ml. The sample containing the protease (LPKA) and PK was incubated for 30 min at 37°C and the mixture further incubated for 5 min with either 200 μl of EDTA treated heat-inactivated plasma (plasma heated to 61°C for 2 h) or 200 μl HMW kininogen (100 μg/ml). The control consisted of the same mixture that was added directly to the bioassay in the absence of any incubation.

Immunologic procedures. To obtain antibody to HF, 0.5 mg of purified HF was emulsified in complete Freund's adjuvant and injected into the sheep on day 1 and day 21.

The sheep were bled a month after the last injection. The IgG immunoglobulin fraction was obtained by octanoic acid precipitation of the serum followed by dialysis against 0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. The immunoglobulin fraction was coupled to Sepharose 4B by cyanogen bromide coupling (23) in order to prepare the immunoadsorbent. In the soluble inhibition studies the immunoglobulin fraction resulting from octanoic acid precipitation was adjusted to a concentration of 1 mg/ml protein.

RESULTS

Purification of LPKA. The lung supernatant was diluted 1:6 with distilled water and the pH adjusted to 5.0 with 1 M HCl. The supernatant was then fractionated by chromatography on a column of SP-Sephadex C-25 that was equilibrated with 0.01 M phos-

phate buffer pH 5.0 (Fig. 1). The size of the SP-Sephadex column was determined by the quantity of lung tissue used to generate the supernatant (3 ml of gel/g of tissue). Fractions were neutralized to pH 7.0 by collection of the eluent in 0.1 M phosphate buffer pH 7.0. The LPKA activity was superimposed on the protein peak that was found in the column wash (1 mM phosphate buffer pH 5.0). The fractions containing LPKA activity were adjusted to pH 8.0 with 0.1 M NaOH and further fractionated on a DEAE-Sephacel column equilibrated with 0.01 M phosphate buffer pH 8.0 (0.1 ml of gel/g of tissue). After the sample was applied, the gel was washed with 0.01 M phosphate buffer pH 8.0 and eluted with a NaCl gradient consisting of the wash buffer as the initial buffer and the wash buffer with 1.0 M NaCl as the terminal buffer (Fig. 2). The gradient volume was ten times the bed volume of the DEAE-Sephacel column. Two major absorbance peaks were eluted from the DEAE-Sephacel during the NaCl gradient (Fig. 2), with the LPKA activity in the first absorbance peak. At this point, the yield of activity was 50% and the purification factor was ~100-fold.

The fractions containing LPKA activity were concentrated by vacuum dialysis to 3 ml in a B12 collodion bag with a mol wt cutoff of 25,000 (Arthur H. Thomas Co., Philadelphia, PA). The concentrate was further fractionated on a 1.6 × 95-cm Sepharose 6B column equilibrated and eluted (10 ml/h) with a 0.01 M phosphate buffer pH 7.0 (Fig. 3). The LPKA eluted on the Sepharose 6B column in the region where proteins of ~175,000 mol wt fractionate. These three chromatographic steps were used to fractionate supernatants from 16 lungs, and we obtained essentially the same elution pattern in each instance. After the third column, the protease had been purified ~500-fold.

LPKA released by IgE-mediated mechanisms. To demonstrate that LPKA was released by an IgE-mediated mechanism, supernatants from both antigen- and buffer-challenged lung fragments were fractionated on SP-Sephadex and assayed for the relative amounts of LPKA released. 40 g of lung was cut into 400 fragments, passively sensitized, and randomly divided into two, 200-fragment pools. One was incubated at 37°C for 30 min in Tyrodes buffer and AgE (0.5 μg/ml), while the second served as a control and was incubated with buffer alone under the same conditions. Both pools generated LPKA and histamine activity but the amount of LPKA and histamine released by the control tissue (challenged with buffer) was only 16 and 5%, respectively, of that released from the antigen challenged tissue. AgE alone was fractionated on SP-Sephadex and no LPKA activity was detected. Three similar experiments were carried out, and the LPKA activity in the control preparation ranged from 9 to 16% of that obtained from the antigen-challenged tissue.

LPKA is a unique PK activator. Since the activated forms of Hageman factor (HF_f, HF_a) are known prekallikrein activators, it was necessary to demonstrate

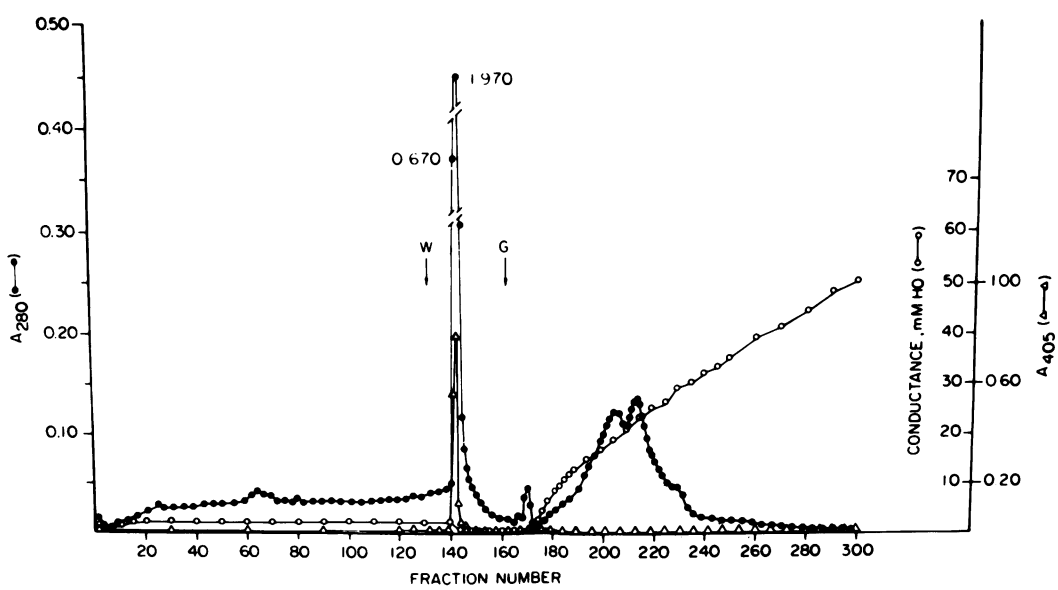

FIGURE 1 SP-Sephadex chromatography of LPKA. The sample was applied during the first 130 10-ml fractions. The LPKA eluted during the wash (*W*) with 1 mM phosphate buffer pH 5.0. No further LPKA was eluted with a salt gradient (*G*) (1.5 liters of wash buffer vs. 1.5 liters of wash buffer containing 1.0 M NaCl).

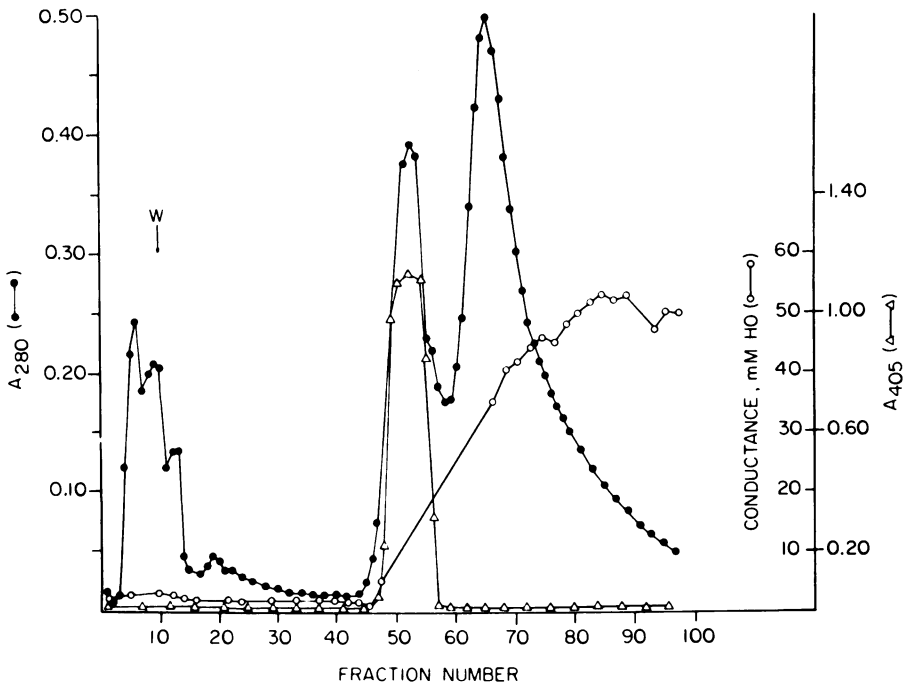

FIGURE 2 DEAE-Sephacel chromatography of LPKA. The 40-ml pool of LPKA from the SP-Sephadex column wash was further fractionated on DEAE-Sephacel. The LPKA activity eluted in the first absorbance peak. Fractions 49 to 56 were concentrated to 3 ml for fractionation on Sepharose 6B.

that the LPKA activity was not derived from contamination with HF_f and/or HF_a. Four criteria were used to distinguish LPKA from the active forms of HF: molecular weight, synthetic substrate specificity, antigenic specificity, and inhibitor specificity. LPKA was shown to have a molecular weight of ~175,000 by chromatography on Sepharose 6B (Fig. 3), while the active forms of Hageman factor, HF_a and HF_f, have mol wt of ~100,000 and 40,000, respectively, on gel filtration (24). Thus, the molecular weights of the free forms of HF_a and HF_f differ from that of LPKA on gel filtration. D-Pro-Phe-Arg-*p*NA (a known substrate of HF_a and HF_f) was exposed to aliquots of LPKA and HF_f of similar functional activity. As shown in Table I, D-Pro-Phe-Arg-*p*NA was not a substrate for LPKA while, as anticipated, it was cleaved by the activated form of HF, HF_f (25). This difference in substrate specificity further suggests that the LPKA activity is not attributable to the activated forms of HF. Additional

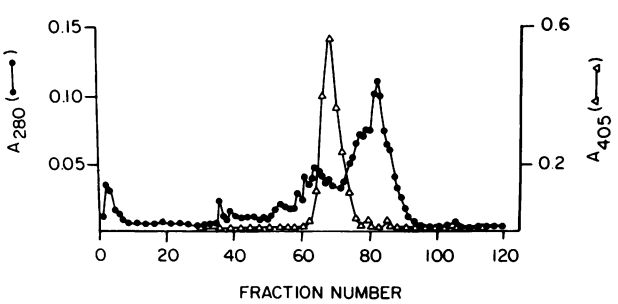

FIGURE 3 Sepharose 6B chromatography of LPKA. A 3-ml concentrate of LPKA activity from a DEAE-Sephacel column was fractionated on Sepharose 6B and the LPKA eluted in fractions 66 to 74 (2 ml/fraction), which correspond to the fractionation of a protein with a mol wt of ~175,000.

TABLE I

D-Pro-Phe-Arg-pNA Is a Substrate for HF_f but Not LPKA

	Amidolytic assay for LPKA*		Amidolytic assay for HF_f‡
	+PK	−PK	
LPKA	0.450	0.028	0.031
HF_f	0.482	0.005	0.268

* Aliquots of LPKA and HF_f of similar PK activator activity (A_{405} of 0.450 vs. 0.482 with Bz-Pro-Phe-Arg-*p*NA as substrate [see Methods]) showed dissimilar effects on the substrate D-Pro-Phe-Arg-*p*NA (0.031 vs. 0.268).

‡ This assay was performed by incubating 5 μl of either LPKA or HF_f with 50 μl of D-Pro-Phe-Arg-*p*NA for 20 min at 37°C. The reaction was terminated by the addition of 400 μl of 0.1 M acetic acid, and the absorbance was determined at 405 nm.

data distinguishing LPKA from the activated forms of HF were obtained by using an anti-HF immunoadsorbent. An immunoadsorbent was prepared by cyanogen bromide coupling of monospecific anti-HF antibody to Sepharose 4B (26). This antibody recognized both HF (or HF_a) and HF_f. The coupled antibody was used to prepare two 10-ml immunoadsorbent columns. A preparation of LPKA was fractionated on one column, while a preparation of HF_f of equal PK activating activity was fractionated on the second column. The columns were washed with 0.15 M NaCl in 1 mM phosphate buffer pH 7.4. When the wash fractions were assayed (Fig. 4) for their ability to activate PK by the amidolytic assay, the LPKA activity was recovered in fractions 15 to 35, while nearly all of the PK activating activity of the sample containing HF_f was removed by the anti-HF immunoadsorbent. These data indicate that LPKA does not share antigenic determinants with HF.

To further establish that LPKA is immunologically unrelated to HF, soluble antibody inhibition experiments were performed with fluid-phase antibody. The lower panel of Fig. 5 shows that the immunoglobulin fraction of anti-HF did not inhibit the PK activating activity of LPKA. The upper panel shows that this antibody totally inhibited HF_f of comparable activity to LPKA, from converting PK to kallikrein, while the

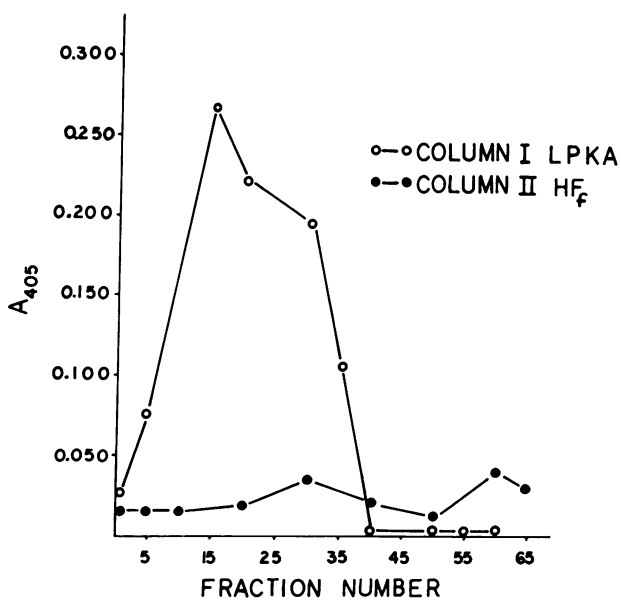

FIGURE 4 Anti-HF immunoadsorbent columns. Column I (open circles) was used to fractionate the LPKA, which eluted with the wash. Column II (closed circles) was used to fractionate the HF_f, which was not eluted with the wash. The columns were eluted with PBS, pH 7.4, and the fractions were 0.25 ml.

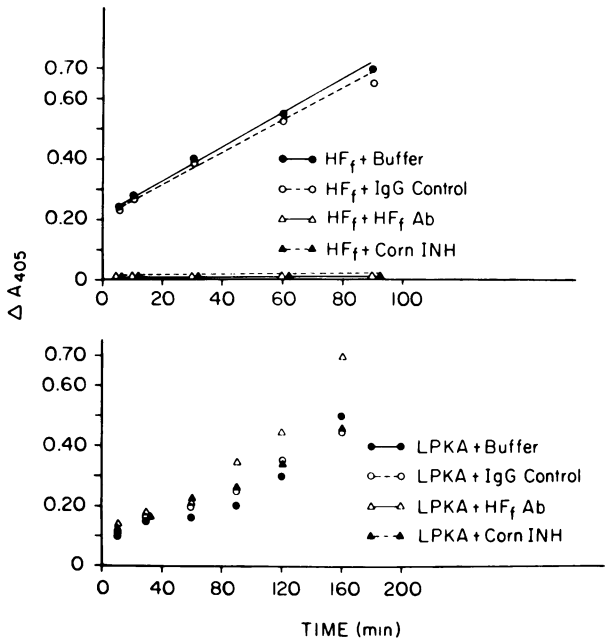

FIGURE 5 This figure demonstrates that neither antibody to HF nor corn trypsin inhibitor inhibit LPKA (lower panel), while both inhibit HF_f activity (upper panel). 10 μl of HF_f (10 ng/ml) were placed in tubes containing 10 μl of either PBS, the IgG fraction from nonimmune sheep, ("control"; 1 mg/ml), the IgG fraction (1 mg/ml) from a sheep immunized with HF, or the trypsin inhibitor from corn (0.1 mg/ml) and incubated for 10 min at 37°C. A separate set of tubes contained 10 μl of LPKA (with ~½ the prekallikrein activating activity of the HF_f) instead of HF_f. After the incubation, both sets of tubes were brought up to 400 μl with PBS containing both prekallikrein and Bz-Pro-Phe-Arg-*p*NA so that the final concentrations were 10 μg/ml and 125 μM, respectively. The change in absorbance over time was measured for the above incubation mixtures.

IgG fraction from normal sheep serum had no effect on either LPKA or HF_f. To determine the inhibition specificity of LPKA vs. HF_f, the enzymes were interacted with the trypsin inhibitor from corn (27). As shown in Fig. 5, the activity of LPKA was unaffected by the corn inhibitor (lower panel), while the activity of HF_f was completely inhibited by the corn inhibitor (upper panel).

Proteolytic cleavage of PK. The cleavage of PK by activated HF occurs within a disulfide bridge so that the resulting active kallikrein consists of two polypeptide chains that are disulfide linked (28). To compare the cleavage of PK by HF_f and LPKA, 2 μl of ^{125}I-PK (175 μg/ml, 0.5 μCi/μg) was incubated with either PBS, HF_f (2 μg/ml), or partially purified LPKA (the LPKA and HF_f had equal PK activating activity as determined by the amidolytic assay) for 30 min at 22°C. The resulting incubation mixtures were boiled

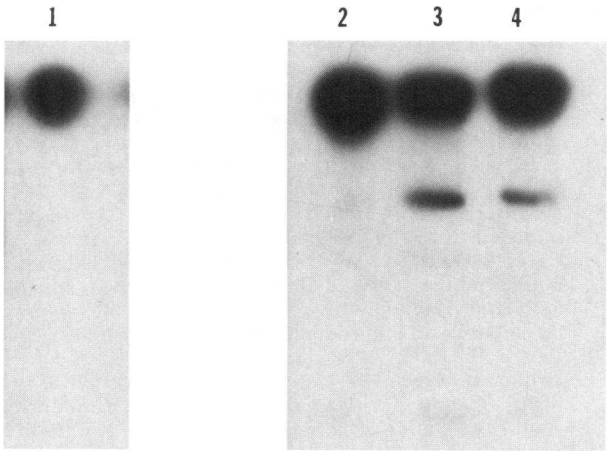

FIGURE 6 Autoradiograph of an SDS slab gel electrophoresis demonstrating prekallikrein cleavage by LPKA and HF_f. ^{125}I-PK (0.5 μCi/μg) was incubated for 30 min with buffer, LPKA, or HF_f. Track 1 had PK plus buffer, track 2 had PK plus buffer (reduced), track 3 had PK plus LPKA (reduced), track 4 had PK plus HF_f (reduced).

with sodium dodecyl sulfate (SDS) or SDS and 2-mercaptoethanol and electrophoresed on a slab gel electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). After electrophoresis, the gel was stained, destained, and dried on a Bio-Rad gel dryer. Kodak X-Omat R film was exposed to the dried gel. The resulting autoradiograph (Fig. 6) shows that the cleavage fragments of PK resulting from its proteolytic cleavage by either LPKA or HF_f are of similar molecular weights. The kallikrein form by both enzymes without reduction (not shown) had approximately the same mobility as that obtained with the PK that was incubated with buffer. However, upon reduction with 2-mercaptoethanol, the PK activated by both HF_f and LPKA dissociated into a heavy chain and a faint light chain (Fig. 6). Thus, the mechanism for the cleavage of prekallikrein appears to be the same for both enzymes.

LPKA cleavage of PK results in a proteolytic active enzyme. The proteolytic activity of the kallikrein formed during the incubation of prekallikrein with LPKA was ascertained by its ability to generate bradykinin from plasma kininogen. The generation of kinin was determined by incubating 50 μl of an LPKA preparation with 5 μl of prekallikrein (70 μg/ml) for 30 min at 37°C, followed by the addition of either 200 μl of heat-inactivated plasma or 200 μl of HMW kininogen (100 μg/ml). The incubation was continued for 5 min at 37°C after which the entire mixture was added to a 5-ml organ bath containing a guinea pig ileum. The resulting contraction of the ileum was compared with the contraction induced by known concentrations of bradykinin. Thus, the kallikrein formed by LPKA was not only capable of cleaving Bz-Pro-Phe-Arg-*p*NA (see Table I), but also capable of generating biologically active kinin (Table II).

DISCUSSION

We have previously shown that supernatants from passively sensitized antigen-challenged human lungs are capable of directly cleaving three proteins of the HF-dependent systems (PK, HF, kininogen) (12–14, 29). Thus, there appears to be a family of proteases in the human lung that interact with the HF-dependent systems. In this manuscript we describe one of these proteases, the LPKA, which was partially purified, and totally separated from the other two proteases by sequential chromatography on SP-Sephadex, DEAE-Sephacel, and Sepharose 6B (Figs. 1–3). LPKA was shown to differ from the known activators of PK (HF_a, HF_f) in several respects: molecular weight, synthetic substrate specificity, antigenic specificity, and inhibitor specificity. LPKA has a mol wt by gel filtration of ~175,000 (Fig. 3), while HF_a and HF_f have molecular weights of ~100,000 and 40,000 respectively (24). Unfortunately the molecular weight could not be determined on molecular sieve gel with a Sephadex matrix, which would normally be used with this size protein, because the LPKA molecule appeared to adhere to the Sephadex matrix even in the presence of 1 M NaCl. It is unlikely that the molecular weight of 175,000 represents one of the forms of HF bound to a carrier protein, since that complex would possess the property of maintaining its proteolytic activity, while losing its antigenicity (see below). D-Pro-Phe-Arg-*p*NA is a substrate for HF_a and HF_f (25), but not for LPKA (Table I). Corn trypsin inhibitor does not affect the activity of LPKA (Fig. 5) but completely inhibits the ability of both HF_a and HF_f to activate PK (27). Finally, LPKA is immunologically distinct from HF_a and HF_f (Figs. 4 and 5). Taken together, the data indicate that LPKA is unrelated to HF.

TABLE II
Activation of Prekallikrein by LPKA

	^{125}I-Prekallikrein cleaved	Bradykinin equivalent generated from HMWK
		ng/ml
Buffer	−	0
Buffer + prekallikrein	−	*2.5
LPKA + prekallikrein	+	‡500

* The result of a small amount of kallikrein contaminating the prekallikrein.
‡ No bradykinin equivalence was observed when HMW kininogen (*HMWK*) was omitted.

The data lead us to suggest that the cleavage fragments of prekallikrein resulting from its cleavage by LPKA and HF_f are of similar molecular weights (Fig. 6). The data further indicate that this cleavage by LPKA converts prekallikrein to its active form, kallikrein. The generated kallikrein is not only capable of cleaving the synthetic substrate Bz-Pro-Phe-Arg-*p*NA, but also capable of cleaving its natural substrate HMW kininogen, to generate biologically active kinin (Table II).

Activation of the HF-dependent systems appears to occur during IgE-mediated reactions in man. We have reported the consumption of coagulation factors during systematic human anaphylaxis (30). The coagulation system is also activated in the late-phase skin reaction (31). Furthermore, coagulation Factors XII, XI, and IX are consumed during systematic anaphylaxis in rabbits (32). Thus, it seems clear that, in man and animals, activation of the HF-dependent systems occurs during IgE-mediated events. What has not been clear, however, is the mechanism whereby these systems become activated in vivo. Our demonstration of the IgE-mediated release of a PK activator, an HF activator, and a kininogenase, may well provide a mechanism by which this could occur.

We earlier suggested that certain chemical mediators, derived from basophils and mast cells, may participate not only in immediate-type inflammatory events, but also in important aspects of the entire inflammatory process (33–36). Other reports suggest the participation of basophils and/or mast cells in subacute or chronic phases of inflammation. These reports show deposition of fibrin in and around inflammatory lesions, such as those of cutaneous basophil hypersensitivity (37–40). It is possible that basophils and mast cells, through the release of LPKA, the HF activator (13, 35), or the kininogenase (13, 33, 34) may activate the HF-dependent systems, and thus participate in inflammatory processes.

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