Flaujeac Factor Deficiency

RECONSTITUTION WITH HIGHLY PURIFIED BOVINE HIGH MOLECULAR WEIGHT-KININOGEN AND DELINEATION OF A NEW PERMEABILITY-ENHANCING PEPTIDE RELEASED BY PLASMA KALLIKREIN FROM BOVINE HIGH MOLECULAR WEIGHT-KININOGEN

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ABSTRACT Flaujeac trait is the functional deficiency of a plasma protein of the intrinsic coagulation, kinin-forming, and plasma fibrinolytic pathways. The Flaujeac factor in man has been isolated and tentatively identified as a kininogen of high molecular weight (HMW).

Highly purified bovine HMW-kininogen, but not bovine low molecular weight kininogen, repaired Flaujeac factor deficiency. The two subspecies of this molecule, HMW-kininogen a and HMW-kininogen b, also corrected Flaujeac factor deficiency. When bovine HMW-kininogen was incubated with bovine plasma kallikrein, kinin-free HMW-kininogen, bradykinin, and a glycopeptide fragment (peptide 1–2; 12,584 daltons) were rapidly released. None of these fragmentation products corrected Flaujeac factor deficiency alone or in mixtures. The function of HMW-kininogen appeared to depend upon the structural integrity of the native molecule.

When injected in concentrations of 2 pmol–8 nmol/0.1 ml, peptide 1–2 caused increased vascular permeability in rabbits, rats, or guinea pigs. The enhanced permeability was maximal within 1–2 min and terminated in 5–10 min, differing from that of bradykinin or histamine. Injected together in equimolar amounts, peptide 1–2 and bradykinin produced a synergistic permeability response which was immediate in onset as well as prolonged in duration.

Peptide 1–2 is a rapidly acting, highly basic glycopeptide which mediates increased vascular permeability in a complementary and synergistic manner with bradykinin.

INTRODUCTION

The deficiency of a plasma protein which participates in the intrinsic coagulation, kinin-forming, and plasma fibrinolytic pathways was reported recently by three laboratories (3–5). They were recognized independently and identified by eponyms as Fitzgerald (3, 6), Flaujeac (4, 7–9), and Williams (5, 10) traits. We have exchanged reagents with Dr. Waldmann (3, 6) and Dr. Colman (5, 10) and have reported that mutual correction of the coagulation assays did not take place when mixtures were made of all possible combinations of the Fitzgerald, Flaujeac, or Williams plasma (8,9) suggesting the deficiency of a common plasma protein. Normal plasma corrected the activated partial thromboplastin time (APTT)[1] in each instance, excluding the possibility of a circulating anticoagulant which might be responsible for the functional abnormalities.

This work was presented in part at the Western Section meeting of the American Federation for Clinical Research, 6 February 1976, Carmel, Calif. Preliminary reports have been published (1, 2).

Dr. Matheson was supported by a National Research Service Individual Award of the U. S. Public Health Service (F32-HL-05170-01). Dr. Wuepper was recipient of a U. S. Public Health Service Research Career Development Award AM-40086-06.

Received for publication 29 March 1976 and in revised form 16 August 1976.

[1] *Abbreviations used in this paper*: APTT, activated partial thromboplastin time; HMW, high molecular weight; LMW, low molecular weight; PF/dil, permeability factor of dilution.

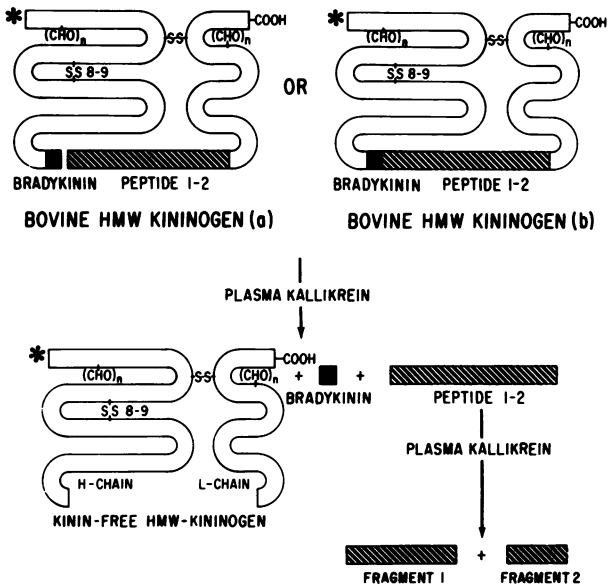

FIGURE 1 Fragmentation of bovine HMW-kininogen with bovine plasma kallikrein. (Redrawn from Kato, H., et al.) (22). See text for details.

Donaldson and her co-workers recognized a fourth example of this deficiency (11, 12) by employing Fitzgerald plasma as the reference source. A fifth case has been identified, with Flaujeac plasma as the source of deficient plasma (13).

We have previously isolated and characterized the Flaujeac factor, shown a deficiency of kininogen antigen in the proposita's plasma, and inhibited the function of the factor with antiserum to human kininogen (4,9). Of the two general forms of human kininogen in human plasma, we recognized that partially purified high molecular weight (HMW) kininogen, but not low molecular weight (LMW) kininogen, reconstituted the Flaujeac plasma (4,9). This observation has already been confirmed with plasma from another patient with the same functional deficiency (11). This activity has been retained by human HMW-kininogen purified more than 5,000-fold.[2] In this collaborative study, we have tested very highly purified bovine kininogens and their fragments, substantial segments of which have been subjected to amino acid sequence analysis, for their ability to reconstitute Flaujeac factor deficient plasma.

The existence of kininogens of high molecular weight was first described by Jacobsen (14) and Jacobsen and Kriz (15). In contrast to LMW-kininogen, the HMW-kininogen showed a preferential susceptibility to plasma kallikrein. Attempts to purify HMW-kininogen revealed it to be quite labile and recoverable only in low yield. A HMW-kininogen of 76,000 mol wt and LMW-kininogen of 49,000 mol wt have been successfully purified from bovine plasma by Yano et al. (16, 17), and Komiya et al. (18–20).

As depicted in Fig. 1, bovine HMW-kininogen is a 76,000 mol wt glycoprotein with a masked N-terminal residue and C-terminal leucine. Two HMW-kininogen subspecies, a and b, which have the same number and composition of tryptic peptides have been recovered. They differ only by a single proteolytic cleavage at the C-terminal end of the bradykinin moiety without loss of bradykinin or any other fragments (19).

The vasoactive peptide bradykinin and a recently described glycopeptide, peptide 1–2, are located within an intrachain disulfide loop (19, 21–23). The N-terminal portion of the peptide 1–2 is connected with the C-terminus of bradykinin within the loop in the HMW-kininogen molecule (22).

Upon incubation of bovine plasma kallikrein with bovine HMW-kininogen a or HMW-kininogen b, bradykinin, kinin-free HMW-kininogen, and the glycopeptide fragment (peptide 1–2, 12,584 daltons) are rapidly and simultaneously released in equimolar amounts as shown schematically in Fig. 1 (21, 22). Upon prolonged incubation with bovine plasma kallikrein, peptide 1–2 is subsequently cleaved into a glycopeptide (fragment 1; 8,000 daltons) (21,23) and a "histidine-rich peptide" (fragment 2; 4,584 daltons) (24). Bovine HMW-kininogen from which both the bradykinin and the peptide 1–2 moieties have been enzymatically removed by plasma kallikrein has been designated "kinin-free HMW-kininogen." Further study of this residual of the parent molecule has demonstrated it to be a 66,000 mol wt glycoprotein consisting of two polypeptide chains (heavy (H) and light (L) chains) linked by a single disulfide bridge (Fig. 1) (22).

Amino acid composition and carbohydrate content of all the fragments have been determined (21), and the sum of the total amino acid residues of kinin-free HMW-kininogen, peptide 1–2, and bradykinin coincides with that of the whole bovine HMW-kininogen molecule. By contrast, bovine LMW-kininogen is not susceptible to plasma kallikrein, and incubation of LMW-kininogen with a glandular kallikrein releases only bradykinin with no counterpart for peptide 1–2 (22).

We have now tested bovine kininogens and their native cleavage products to learn whether they are corrective in human HMW-kininogen deficient plasma and what molecular role these proteins play in coagulation, fibrinolysis, and induction of increased vascular permeability. Moreover, it has been possible to study the two molecular forms of bovine HMW-kininogen, a and b. In addition, we now report that the bovine peptide 1–2 fragment is a mediator of increased

[2] Matheson, R. T., D. R. Miller, and K. D. Wuepper. Unpublished observations.

vascular permeability in its own right. Permeability was studied in several animal species which were all responsive. The duration of effectiveness of peptide 1–2 differed from that of bradykinin or histamine, and an equimolar mixture of pepetide 1–2 and bradykinin resulted in a prolonged, complementary, and synergistic reaction of increased vascular permeability. These studies establish a third permeability-enhancing activity of the kinin-forming system in mammalian species in addition to bradykinin and permeability factor of dilution (PF/dil).

MATERIALS

Reagents and chemicals. Synthetic bradykinin (BRS 640, Sandoz Ltd., Basel, Switzerland), histamine dihydrochloride (Fisher Scientific Co., Pittsburgh, Pa.), chlorpheniramine maleate, (Schering Corp., Bloomfield, N. J.) and ristocetin (Abbott Laboratories, Chicago, Ill.) were purchased from their suppliers. Rabbit brain "cephalin", a platelet substitute in coagulation assays, was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Human plasmas. Flaujeac factor deficient plasma was obtained from the proposita, Mme. Flaujeac. Nine parts blood was mixed with one part 3.8% citrate or acid-citrate dextrose, and stored at −70°C until used. Normal pooled plasma from 28 donors was treated identically.

Biological reagents. Human fibrinogen, 90% coagulable (Grade L, AB Kabi, Stockholm, Sweden), soluble collagen, and adenosine diphosphate (Sigma Chemical Company) were purchased from their suppliers. Bovine thrombin, topical, and epinephrine hydrochloride were obtained from Parke, Davis and Co., Detroit, Mich.

Purified bovine proteins. HMW-kininogen, LMW-kininogen, kinin-free HMW-kininogen, and peptide 1–2. Highly purified HMW-kininogen was prepared from fresh bovine plasma by the previous method (18), and LMW-kininogen was purified according to the revised method by precipitation with zinc acetate (25). Bovine plasma kallikrein was prepared as previously described (26, 27). The specific activity of the kallikrein was 23.5 TAME U/μg protein.

Preparation and isolation of peptide fragments produced from bovine HMW-kininogen by highly purified bovine plasma kallikrein was accomplished as described (21, 22). Briefly, bovine HMW-kininogen was incubated with bovine plasma kallikrein (0.1%, wt:wt) in 0.2 M ammonium bicarbonate buffer pH 8.0, at 37°C (21), and the digest applied to a column of Sephadex G-75 (Superfine, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The kinin-free HMW-kininogen, which did not release kinin upon incubation with plasma kallikrein, was eluted in the void volume fraction, and one major peak with the absorbance at 280 nm (peptide 1–2) and bradykinin were separated. The isolated peptide 1–2 and kinin-free kininogen showed single bands, respectively, on sodium dodecyl sulfate-gel electrophoresis (21).

The highly purified HMW-kininogen, LMW-kininogen, kinin-free HMW-kininogen, and peptide 1–2 were dialyzed against distilled water and lyophilized. The proteins were solubilized 1 mg/ml in sterile normal saline and stored in aliquots at −70°C until used.

Ultracentrifugation. Preparative ultracentrifugation was carried out in linear gradients of 10–37% sucrose dissolved in 0.15 M NaCl buffered with Tris-HCl 0.05 M, containing 0.001 M EDTA. 200-μg samples in 0.2 ml were layered onto the gradients and spun in a Beckman ultracentrifuge model L5-65 with an SW-50 rotor (Beckman Instruments Inc., Palo Alto, Calif.) at 45,000 rpm for 16 h at 5°C. Fractions of 15 drops were collected and assayed for protein content and permeability activity.

Assay methods. The kaolin APTT has been described (9). The ability of highly purified bovine HMW-kininogen, LMW-kininogen, and cleavage products of HMW-kininogen to correct the prolonged APTT of Flaujeac plasma was assessed by the addition of 50-μl samples of serial dilutions of the purified protein solutions to 50 μl of the kaolin/cephalin mixture and 50 μl Flaujeac plasma. After incubation for 30 min at 37°C, the mixture was recalcified with 50 μl of 0.05 M $CaCl_2$ and the clotting time was recorded.

The ability of the same highly purified proteins to correct the fibrinolytic defect of Flaujeac plasma was measured with a kaolin-activated euglobulin lysis assay as described (9).

Vascular permeability and PF/dil formation. Bioassay of vascular permeability followed a modification of the method of Miles and Wilhelm (28). Albino guinea pigs (400–600 g), rabbits (2,000–2,500 g), and rats (300–500 g) were injected intravenously with 50–70 mg/kg body weight of Evans blue dye in a 5.0% solution in saline. 0.05- or 0.10-ml test solutions were then injected intracutaneously. The animal was sacrificed 30 min after the injections were begun and the diameter of blueing on the reflected skin was measured by transillumination. Three to eight animals were used for each experiment, and duplicate injections were performed from rump to neck or neck to rump to offset regional variability that occurs in some animals. Arithmetic means were calculated by measuring the largest diameter of blueing and its perpendicular, and the results expressed as mean permeability diameter. Measurements were performed in a single blind manner, and unless otherwise stated, the results were those found in rabbits.

Tests for PF/dil formation were performed as described elsewhere (29). 20-μl samples of plasma combined with varying amounts of the purified bovine peptides were diluted to 2.0 ml with sterile saline in a new glass tube, covered with parafilm, (American Can Co., Greenwich, Conn.) and inverted 40 times each min on a Lab Tek sample mixer (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) for 15 min. 0.1-ml sample volumes were then assayed for permeability in the rabbit as above.

The time course of permeability was determined by injecting a series of single doses at time intervals before dye injection. 1 min after blueing, the animals were sacrificed and mean permeability diameters were measured. Equimolar amounts of histamine, bradykinin, and a mixture of peptide 1–2 and bradykinin were injected simultaneously in each animal for comparative studies.

The effect of chlorpheniramine maleate on permeability was evaluated by injecting peptide 1–2, 0.1–1.0 μg (8 pmol–0.8 nmol), or histamine dihydrochloride, 0.01–1.0 μg (60 pM-3 nM histamine base), in blued rabbits. After 30 min, 10 mg chlorpheniramine maleate was administered intravenously, and the intracutaneous injections were repeated at different sites. 30 min after the second series of injections the animals were sacrificed and permeability diameters were measured as above.

Disc-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed in 7% acrylamide (Eastman Kodak Co., Rochester, N. Y.) according to the method described by Davis (30). Gels were stained for protein with Coomassie brilliant Blue in 10% trichloroacetic acid. Unstained gels were cut into 1.3-mm segments and the proteins eluted into 0.2 ml of 0.05 M Tris buffer in 0.15 M NaCl for 48 h at 4°C.

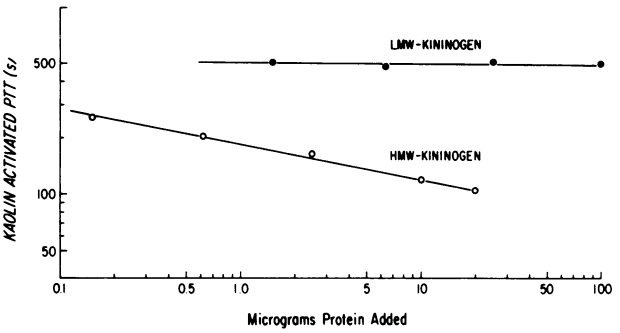

FIGURE 2 Correction of Flaujeac plasma by purified bovine HMW-kininogen. Normal human APTT = 65 s. A dose-related response was given by 0.1–20 μg of HMW-kininogen. LMW-kininogen gave no correction.

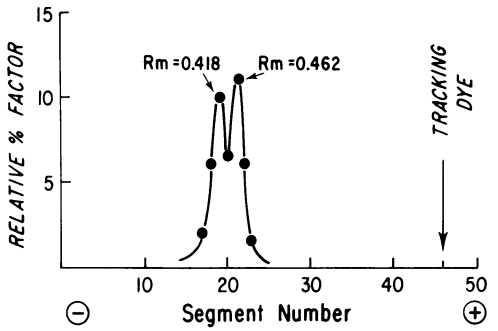

FIGURE 4 Disc gel electrophoresis of purified bovine HMW-kininogen. Two zones of correction migrated with the two subspecies of HMW-kininogen identified in a parallel gel. Rm, relative mobility

Smooth muscle contraction. The ability of peptide 1–2 to contract smooth muscle was measured by bioassay on the rat uterus or guinea pig ileum. Peptide 1–2, 1–100 μg, was added to a 10-ml muscle bath containing a rat uterine horn or guinea pig ileum suspended in oxygenated Tyrode's solution at 37°C. Bradykinin or histamine served as positive controls for each preparation respectively.

Granulocyte chemotaxis. The ability to attract human granulocytes was measured in a radioassay by employing ^{51}Cr-labeled granulocytes in a double micropore filter system (31). Gey's balanced salt solution and endotoxin-stimulated serum served as negative and positive controls, respectively. Peptide 1–2 was assayed for chemotactic activity in concentrations of 0.05–50 μg/ml. Each concentration of peptide 1–2 was tested in triplicate on two separate occasions.

Platelet aggregometry. Peptide 1–2 was tested for its ability to cause aggregation of human blood platelets in concentrations of 0.1–10 μg/0.45 ml platelet-rich-normal plasma (platelet Aggregometer, Chrono-log Corp., Broomall, Pa.). The ability of peptide 1–2 to alter the platelet aggregation response patterns of known aggregating agents was assessed by incubating 1.0-μg amounts of peptide 1–2 with adenosine diphosphate 2.0 μM/50 μl, soluble collagen 16 μg/50 μl, epinephrine hydrochloride 2.5 μmol/50 μl, or ristocetin 0.6 mg/50 μl for 10 min at 27°C, after which 50 μl of each mixture was added to 0.45-ml volumes of platelet-rich-plasma and aggregometry performed. In separate experiments, 1 μg of peptide 1–2 was incubated with 0.45 ml platelet-rich-plasma for 10 min at 27°C before the addition of the same aggregating agents.

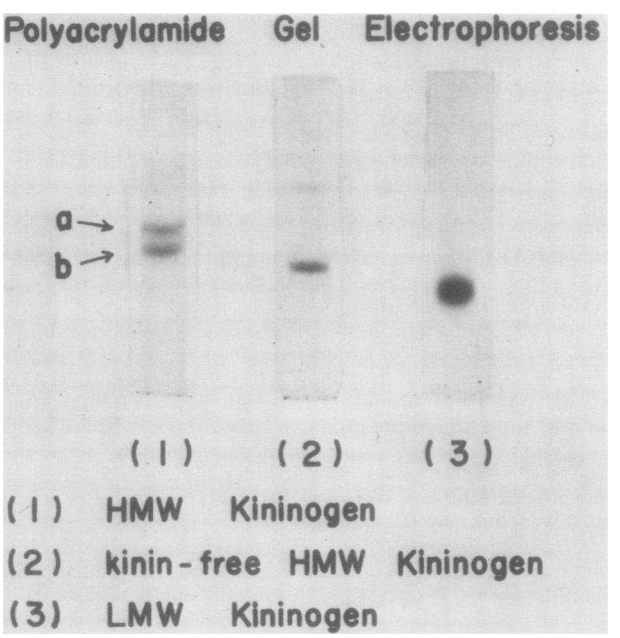

FIGURE 3 Acrylamide gel electrophoresis of bovine HMW-kininogen (showing subspecies a and b), kinin-free HMW-kininogen, and LMW-kininogen.

RESULTS

Correction of abnormal kaolin-activated partial thromboplastin time of Flaujeac plasma with highly purified bovine HMW-kininogen. Freshly prepared bovine HMW-kininogen significantly corrected the prolonged kaolin APTT of Flaujeac plasma in a dose-dependent manner in the range of 0.1–10.0 μg (Fig. 2). By contrast, LMW-kininogen, in added amounts of 1.0–100 μg failed to affect the prolonged APTT. Upon completion of this study polyacrylamide gel electrophoresis of the purified bovine HMW-kininogen confirmed the earlier observation (19) that two proteins were present (Fig. 3). These two proteins differ by a single proteolytic cleavage contained by HMW-kinonogen a and not by HMW-kininogen b. These results suggested that the proteins were in their native state and had not undergone degradation. Upon incubation with plasma kallikrein and electrophoresis, both subspecies converted to the more acidic fragment, kinin-free HMW-kininogen (Fig. 3) (21, 22).

The two species of HMW-kininogen were resolved by polyacrylamide gel electrophoresis. The gel was cut into 1.3-mm segments and eluted into a buffer solution. The amount of Flaujeac factor present in each sample relative to that in pooled normal human plasma as determined by coagulation assay was ex-

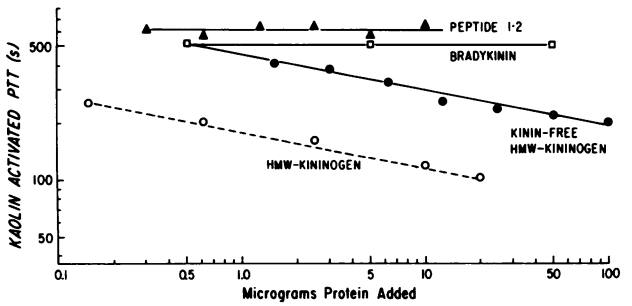

FIGURE 5 Failure of bovine HMW-kininogen cleavage products to correct Flaujeac plasma. Kinin-free HMW-kininogen gave correction 0.6 of 1% as effective as HMW-kininogen. Peptide 1–2 and bradykinin gave no correction.

pressed as "Relative % Factor." Two zones of correction of the APTT of Flaujeac factor deficient plasma were recognized at relative mobilities of 0.42 and 0.46 (Fig. 4). These mobilities corresponded exactly with the two subspecies of HMW-kininogen identified in a parallel gel which was stained for protein. Since the stained peaks and recovered activity were of equal magnitude, HMW-kininogen a and HMW-kininogen b appeared equipotent.

Failure of bovine HMW-kininogen cleavage products to correct the prolonged APTT of Flaujeac plasma. The results of attempted reconstitution of the prolonged APTT of Flaujeac plasma by the three cleavage products of bovine HMW-kininogen are shown in Fig. 5. Compared to native bovine HMW-kininogen, kinin-free HMW-kininogen gave partial correction but peptide 1–2 and bradykinin were completely ineffectual. In a separate experiment, an equimolar mixture of the three fragments did not contribute more than the bovine kinin-free HMW-kininogen alone. It may be seen that 0.6 μg of native HMW-kininogen was as effective as 100 μg of kinin-free HMW-kininogen (Fig. 5). Attempts were then made to determine whether the kinin-free HMW-kininogen retained partial corrective ability or whether residual native HMW-kininogen in the preparation was responsible for the correction.

Samples of bovine HMW-kininogen or bovine kinin-free HMW-kininogen were applied to gradients of sucrose and sedimented overnight. Fractions were collected and tested for correction of the APTT with Flaujeac plasma. Corrective ability was determined relative to that of pooled normal human plasma and expressed as "Relative Activity." The fractions which corrected Flaujeac plasma sedimented identically (Fig. 6). However, the bulk of the protein in the native HMW-kininogen differed from the lighter sedimenting kinin-free HMW-kininogen fragment, indicating that a small amount of undegraded native HMW-kininogen was responsible for the activity of this solution, and that bovine kinin-free HMW-kininogen did not correct the abnormal APTT of Flaujeac plasma. The second smaller protein peak in Fig. 6 (above) has not yet been identified. It was not associated with any corrective activity and may have represented an aggregate of HMW-kininogen.

Reconstitution of fibrinolytic activity of Flaujeac plasma with bovine HMW-kininogen. In the fibrinolytic assay, Flaujeac plasma differed from normal in that lysis times were as long as the reagent control. This was corrected by mixing with normal human plasma (1:1) (Table I). The addition of HMW-kininogen to Flaujeac plasma reconstituted its ability to give kaolin-activated lysis in a dose-dependent manner. This was not due to plasmin in the HMW-kininogen preparation as shown by a control in which plasma was excluded. LMW-kininogen did not correct Flaujeac deficient plasma (Table I).

Failure of bovine HMW-kininogen cleavage prod-

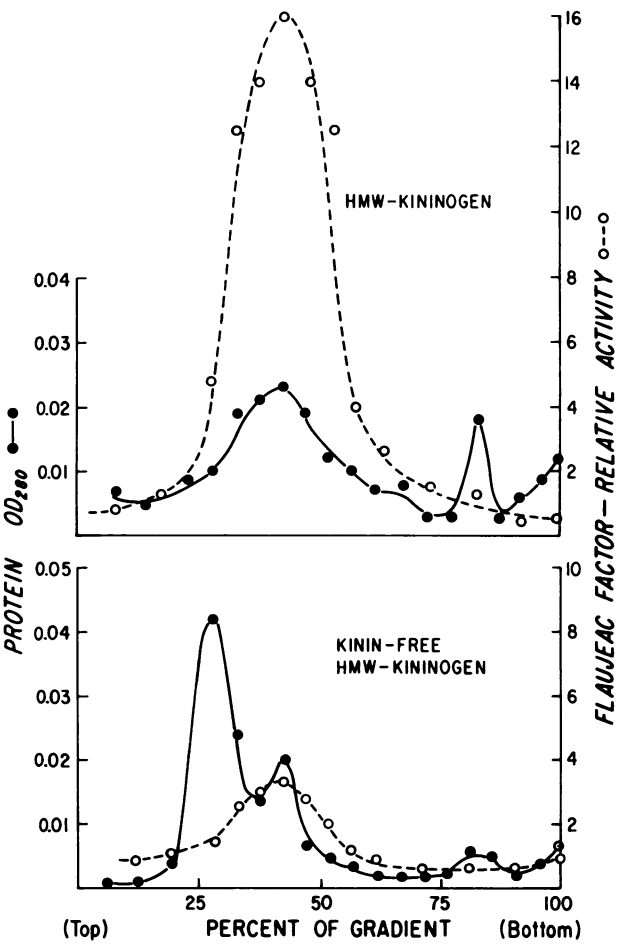

FIGURE 6 Sucrose gradient ultracentrifugation of bovine HMW-kininogen and kinin-free HMW-kininogen. Flaujeac factor activity (O – – – O) sediemented identically with native HMW-kininogen.

TABLE I
Reconstitution of Kaolin-Activated Euglobulin Lysis with Bovine HMW-Kininogen

Plasma source	Additions	Euglobulin lysis Time (min) Exp. 1	Exp. 2
Saline	—	>120.	80.0
Normal	—	6.2	6.1
Flaujeac	—	110.	70.2
Flaujeac + normal (1:1)	—	7.7	9.2
Flaujeac	HMW-Kininogen 25 μg	13.9	13.3
Flaujeac	HMW-Kininogen 2.5 μg	34.1	21.8
Flaujeac	HMW-Kininogen 0.25 μg	47.9	23.6
Flaujeac	HMW-Kininogen 0.025 μg	63.9	47.2
—	HMW-Kininogen 25 μg	NT*	81.2
Flaujeac	LMW-Kininogen 25 μg	NT	78.1
—	LMW-Kininogen 25 μg	NT	45.6

* NT, not tested.

ucts to correct fibrinolysis. Bovine kinin-free HMW-kininogen, peptide 1–2, or synthetic bradykinin were tested singly and in a mixture containing equimolar amounts of these fragments and found to be ineffective in reconstituting the fibrinolytic defect in Flaujeac plasma (Table II). In addition they did not enhance lysis by normal plasma (Table II).

Correction of absent PF/dil formation in Flaujeac plasma with bovine HMW-kininogen. Flaujeac plasma, which failed to form the PF/dil at a 1:100 dilution in rabbits was reconstituted in a dose-dependent manner by bovine HMW-kininogen (Table III). LMW-kininogen demonstrated no correction.

Although peptide 1–2 and bradykinin did not reconstitute the absent PF/dil formation of Flaujeac plasma, these fragments combined in equimolar amounts with kinin-free HMW-kininogen caused substantial correction, a reaction to be explained below.

Biologic activity of a fragment of bovine HMW-kininogen, peptide 1–2. Further investigation of the cleavage products released from bovine HMW-kininogen by plasma kallikrein revealed the peptide 1–2 to be an effective mediator of increased vascular permeability in the rabbit (Fig. 7). Serial dilutions of peptide 1–2 in sterile normal saline were injected intracutaneously in rabbits previously blued with intravenous Evans blue due. Dilutions of bradykinin and histamine dihydrochloride were used for comparative studies and served as positive controls. Sterile normal saline, a negative control, produced no more than 1–1.5 mm diameter blueing due to the trauma of injection. In the range of 2 pmol–0.8 nmol (0.025–10 μg/0.1 ml injection) peptide 1–2 produced permeability in a dose-dependent manner which was intermediate in effect between that produced by bradykinin or histamine base in rabbit skin (Fig. 7).

After the demonstration of its permeability activity, a sample of peptide 1–2 was layered onto a gradient of sucrose and sedimented for 16 h. Fractions collected from the gradient were diluted 1:20 in sterile normal saline for determination of protein content and permeability activity. A single protein peak sedimented in a range consistent with the known molecular weight of peptide 1–2, *viz.* 12,584 daltons (Fig. 8, top). As shown in Fig. 8 (bottom) the vascular permeability

TABLE II
Failure of Cleavage Products of Bovine HMW-Kininogen to Correct the Kaolin-Activated Euglobulin Lysis of Flaujeac Plasma

Plasma source	Additions	Euglobulin lysis Time (min)
Normal	KF-HMW-Kininogen* 25 μg	7.5
Normal	Fragment 1–2 10 μg	8.2
Normal	Bradykinin 5 μg	10.0
Normal	KF-HMW-Kininogen 10 μg + Fragment 1–2 2 μg + Bradykinin 0.2 μg	10.0
Flaujeac	KF-HMW-Kininogen 25 μg	58.9
Flaujeac	Fragment 1–2 10 μg	56.8
Flaujeac	Bradykinin 5 μg	61.0
Flaujeac	KF-HMW-Kininogen 10 μg + Fragment 1–2 2 μg + Bradykinin 0.2 μg	54.7

* KF-HMW-Kininogen, kinin-free high molecular weight kininogen.

TABLE III
Reconstitution of the PF/dil Phenomenon with Bovine HMW-Kininogen

Plasma source	Additions	Mean diameter Blueing
		mm
—	Saline	2 f*
Normal	—	8
Flaujeac	—	1 f
Flaujeac	HMW-Kininogen 10 μg	8
Flaujeac	HMW-Kininogen 1 μg	6
Flaujeac	HMW-Kininogen 0.1 μg	4
Flaujeac	HMW-Kininogen 0.01 μg	3 f
—	HMW-Kininogen 10 μg	4
—	HMW-Kininogen 1 μg	2 f
Flaujeac	LMW-Kininogen 10 μg	2 f

* f, Faint blueing.

activity sedimented identically with the peptide 1–2 and was clearly distinct from that induced by increasing concentrations of sucrose alone.

When assayed in the skin of the rat or guinea pig, peptide 1–2 produced increased vascular permeability in a dose-dependent manner similar to that found in the rabbit (Fig. 9). Peptide 1–2 appeared slightly more potent in the rat skin.

Synergism of peptide 1–2 and bradykinin. Since bradykinin and peptide 1–2 are released simultaneously in equimolar quantities from bovine HMW-kininogen by plasma kallikrein, the permeability activities of equimolar combinations of bradykinin and peptide 1–2 were determined and compared to those of bradykinin or peptide 1–2 alone in the same animal. The combination of bradykinin and peptide 1–2 produced a synergistic increase in vascular permeability activity (Fig. 10). The addition of an equimolar amount of peptide 1–2 to bradykinin produced permeability equal to that expected from a 10–20-fold increase in bradykinin alone. The synergistic increase in permeability persisted even with the addition of amounts of peptide 1–2 which were less than one-tenth of the threshold permeability dose of the peptide alone (2 pmol).

Time course of permeability reactions. The duration of increased vascular permeability was determined by injecting the Evans blue dye after the completion of a series of timed sequential intracutaneous injections. The animal was sacrificed immediately after blueing and the diameters of dye leakage were measured. A representative time-course study is shown in Fig. 11. The onset and duration of permeability due to peptide 1–2 was distinct from that of either histamine or bradykinin. The enhanced permeability of peptide 1–2 was maximal within 1–2 min and terminated in 5–10 min. Histamine induced permeability peaked within 5–10 min and was of 18–20 min

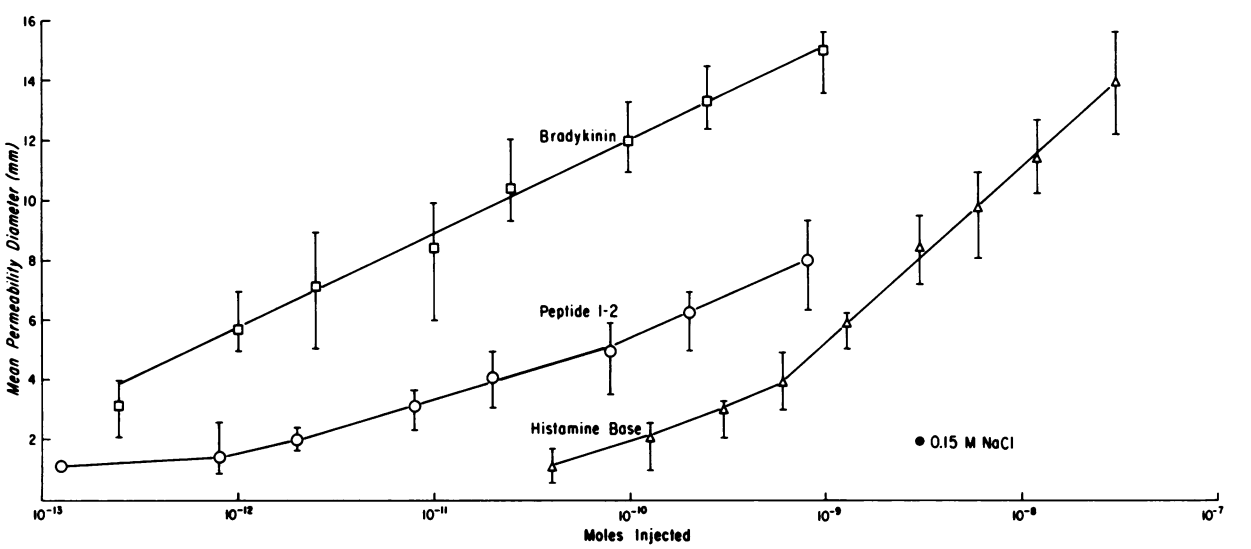

FIGURE 7 Comparison of bovine peptide 1–2 (○ —— ○), bradykinin (□ —— □), or histamine (△ —— △) permeability responses in the rabbit. Brackets denote the range of response; symbols represent the mean of 3 to 8 determinations at the stated dosage levels.

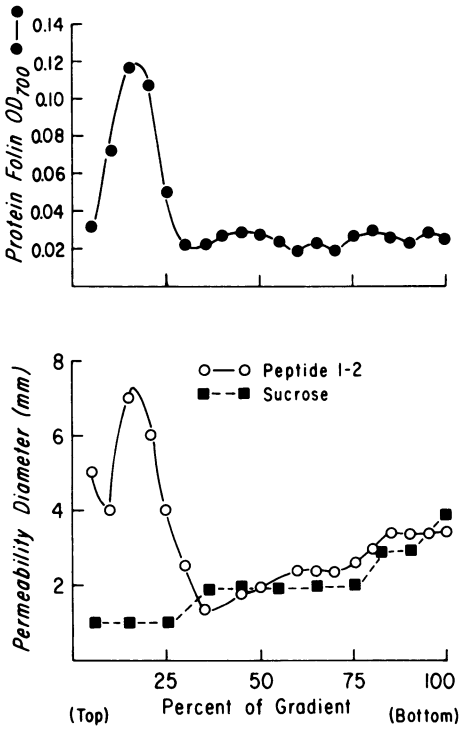

FIGURE 8 Sucrose gradient ultracentrifugation of bovine peptide 1–2. Protein (● —— ●) and permeability activity (○ —— ○) from the same gradient. The permeability activity of a gradient of sucrose (■ - - - ■) is shown.

duration. Injected together in equimolar amounts, peptide 1–2 and bradykinin produced permeability which was maximal within 5 min, was enhanced over that of bradykinin alone, and was significantly prolonged, lasting 30–40 min.

Chlorpheniramine effect on peptide 1–2 induced permeability. To further compare the permeability induced by peptide 1–2 with that of histamine, the susceptibility of permeability by peptide 1–2 to inhibition by a histamine antagonist was evaluated. Peptide 1–2 induced permeability was partially inhibited by 10 mg of intravenous chlorpheniramine maleate

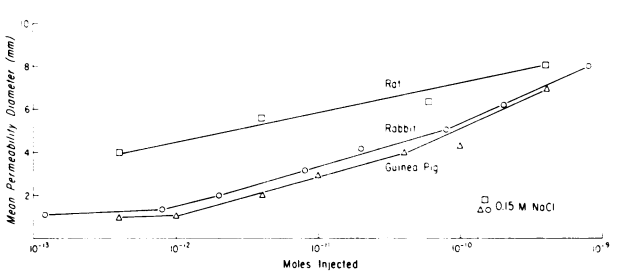

FIGURE 9 Permeability responses to bovine peptide 1–2 in the rat (□ —— □), rabbit (○ —— ○), or guinea pig (△ —— △).

(Fig. 12), showing a decrease in permeability equivalent to that of a 10-fold decrease in the dose of fragment injected. By contrast, histamine-induced permeability was completely inhibited in the same animal by chlorpheniramine maleate (Fig. 12).

Further investigation of peptide 1–2. Attempts were made to delineate further biologic activities of peptide 1–2. In concentrations up to 10 μg/ml, peptide 1–2 did not cause smooth muscle contraction in a rat uterine horn responsive to 5 ng/ml of bradykinin. Nor did equal concentrations of the fragment contract a guinea pig ileum responsive to histamine.

With ^{51}Cr-labeled human granulocytes in chemotactic assays, peptide 1–2 (0.05–50 μg/ml) did not cause increased directional movement of cells when tested in three separate experiments. In standard aggregometry determinations peptide 1–2 (0.1–10 μg/0.45 ml platelet-rich-plasma) did not cause aggregation of platelets, nor did it alter response patterns to adenosine diphosphate, ristocetin, epinephrine, or collagen when preincubated with the platelet-rich-plasma or with the aggregating agents themselves.

DISCUSSION

In the data reported here, bovine HMW-kininogen, a monomeric plasma glycoprotein of mol wt 76,000 significantly corrected the activated partial thromboplastin time of Flaujeac plasma in a dose-dependent manner, while LMW-kininogen did not. The presence of two bovine HMW-kininogen subspecies, a and b, was confirmed, and they were found to be equipotent in their ability to reconstitute this plasma. To date, it has not yet been determined whether the two subspecies represent a postsynthetic modification of the HMW-kininogen molecule or an artifact which results during the purification process. These subspecies have been shown to have the same number and composition of tryptic peptides (19) and appear to differ only in that a single proteolytic cleavage has taken place at the C-terminal end of the bradykinin moiety. This limited proteolytic cleavage within an intrachain disulfide loop in HMW-kininogen leads to an alteration in charge characteristics (Figs. 1, 3, 4). The data reported here also showed bovine HMW-kininogen significantly repaired the fibrinolytic and PF-dil forming defects of Flaujeac plasma in a dose-dependent fashion while LMW-kininogen did not.

In addition to analysis of native bovine HMW-kininogen, we studied the fragmentation products of HMW-kininogen released by plasma kallikrein. When highly purified bovine HMW-kininogen was incubated with bovine plasma kallikrein (0.1% wt:wt) equimolar amounts of bradykinin, kinin-free HMW-kininogen (21), and peptide 1–2 were rapidly released (22);

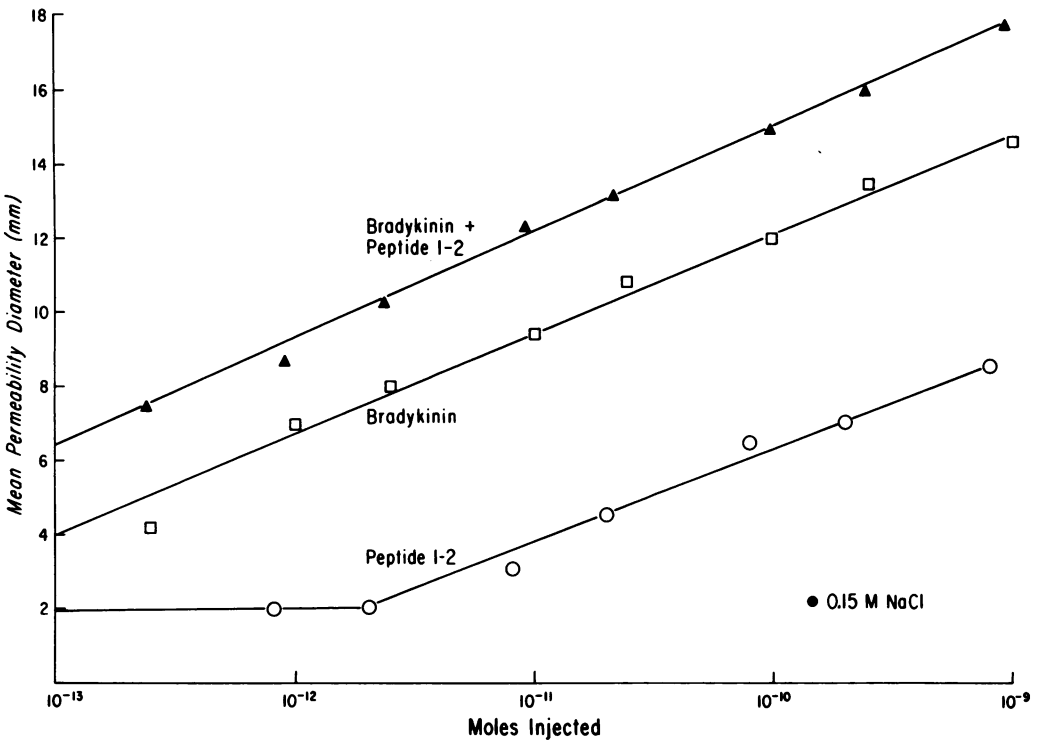

FIGURE 10 Synergistic permeability activity of bradykinin and bovine peptide 1–2 (▲——▲) in the rabbit. The addition of an equimolar amount of peptide 1–2 to bradykinin produced permeability equal to that of a 10-fold increase in bradykinin alone.

the reaction went to completion in 30 min. Peptide 1–2 was so designated since upon prolonged incubation with plasma kallikrein, it was subsequently cleaved into a glycopeptide (fragment 1) (28), and a "histidine-rich peptide" (Fragment 2) (24) in a reaction which required 18 h to complete (22). Bradykinin is positioned in an intrachain disulfide loop (19) within the kininogen. Within the same disulfide loop the N-terminus of peptide 1–2 is linked to the C-terminal residue of bradykinin (21). Thus, plasma kallikrein produces three peptide bond cleavages within bovine HMW-kininogen to generate kinin-free HMW-kininogen, peptide 1–2, and bradykinin. Amino acid composition studies support the contention that the kinin-free HMW-kininogen, bradykinin, and peptide 1–2 comprise the entire bovine HMW-kininogen molecule (21).

Bovine kinin-free HMW-kininogen appeared to give partial correction of Flaujeac plasma but peptide 1–2 and bradykinin were ineffectual. The apparent activity of the kinin-free HMW-kininogen was shown (Fig. 6) to be due to a small amount of undegraded native HMW-kininogen (less than 1%) in the kinin-free HMW-kininogen solution. Equimolar combinations of the three fragments gave no more correction than bovine kinin-free HMW-kininogen alone.

The inability of the three fragmentation products of bovine HMW-kininogen, alone or in equimolar combination to correct Flaujeac factor deficient plasma suggested that the function of this molecule may be

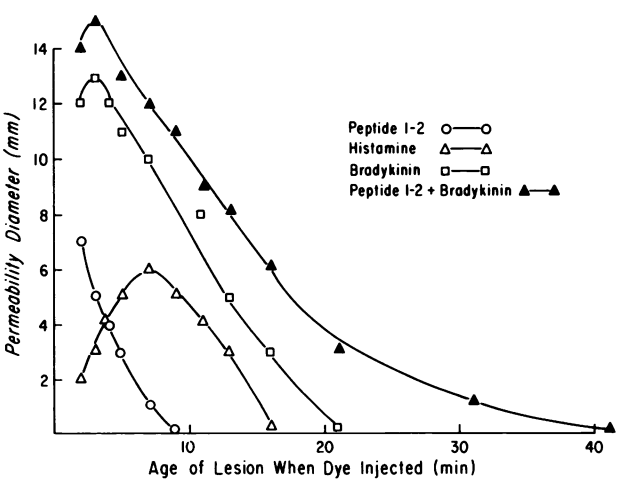

FIGURE 11 Time course of permeability produced by equimolar doses of bovine peptide 1–2 (○——○), histamine base (△——△), bradykinin (□——□), and bradykinin + peptide 1–2 (▲——▲) in the rabbit.

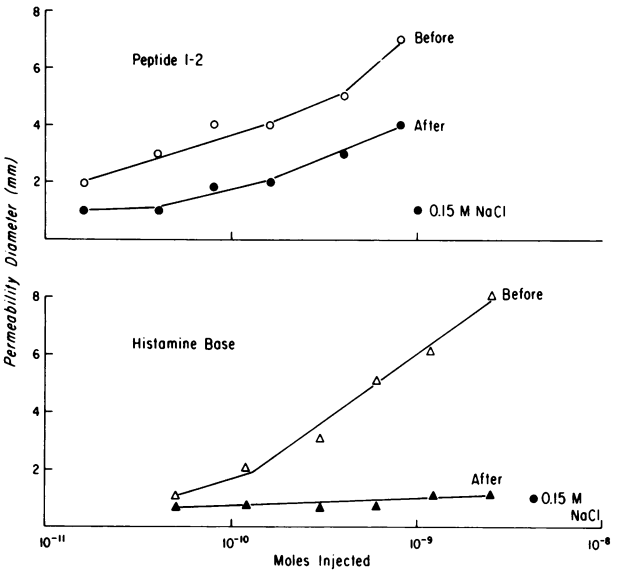

FIGURE 12 Chlorpheniramine effect on permeability caused by bovine peptide 1–2.

dependent upon their combined presence in the native molecule. Since peptide 1–2 is a highly basic fragment, it might subserve a strong binding function, possibly in association with negatively charged surfaces, and exert conformational effects upon one or both of the other two components of the contact activation system; namely, Hageman factor and Fletcher factor (prekallikrein). Additional experiments are planned to test this hypothesis.

Coincident with the presentation of this work in abstract form (1), McGregor et al. presented data on the reconstitution of a similar functional deficiency by using bovine proteins (32). They studied a fixed quantity of bovine HMW-kininogen, 100 μg, in the activated partial thromboplastin time test and also learned that it was corrective for this plasma. Similarly 100 μg bovine kinin-free HMW-kininogen gave a correction which was not statistically different from that of HMW-kininogen. These findings, which differ from those reported here by more than two orders of magnitude, are explained by examination of the dose-response relationship (Figs. 2 and 5). We have found that addition of 20–100 μg of bovine HMW-kininogen did not give shorter coagulation times than 20 μg, and that 100 μg was even slightly inhibitory. A thorough analysis of the dose response is thus necessary to compare the two molecular forms of kininogen. Our data support the contention that the partially corrective activity of the bovine kinin-free HMW-kininogen was due to the presence of trace amounts of intact HMW-kininogen in the preparation.

Particular attention was given to the newly-described bovine glycopeptide fragment (peptide 1–2) (21, 22) to learn whether this fragment had biological activity. There is no counterpart for peptide 1–2 in bovine LMW-kininogen (22). The complete amino acid sequence of bovine peptide 1–2 has been reported (23, 24). It is a highly basic 12,584 dalton glycopeptide rich in histidine and glycine. In this investigation, the isolated bovine peptide 1–2 was found to produce increased vascular permeability when injected intracutaneously in the rabbit, rat, or guinea pig. It produced increased permeability in the range of approximately 10 pmol–10 nmol which was dose dependent and intermediate in potency between bradykinin or histamine in rabbit skin.

Permeability induced by bovine peptide 1–2 was further distinguished from that of bradykinin or histamine in the rabbit by a more rapid onset of maximal activity and shorter duration of effect. The permeability caused by peptide 1–2 was only partially reduced by pretreatment with an antihistamine, whereas that of histamine was completely inhibited. More significantly, and simulating the simultaneous release of peptide 1–2 and bradykinin from bovine HMW-kininogen, intracutaneous injection of equimolar combinations of bovine peptide 1–2 and bradykinin produced a synergistic permeability response which was rapid in onset and of longer duration than that of bradykinin alone. Preliminary experiments in this laboratory have suggested that the 4,584 dalton fragment 2 moiety (released from peptide 1–2 upon prolonged incubation with plasma bovine kallikrein) has permeability properties similar to those of peptide 1–2. Fragment 1 did not have significant permeability increasing properties.

This is the first demonstration that a polypeptide fragment released by plasma kallikrein from HMW-kininogen increases vascular permeability and potentiates the action of bradykinin. Prostaglandins of the E series have been reported to potentiate bradykinin's effects (33, 34), and bradykinin potentiating factors have been isolated from the venom of reptiles. The mechanism of action of these venom potentiating factors is by interference with a dipeptide hydrolase (kininase II, angiotensin converting enzyme, carboxydipeptidase) (35) which is present in serum and is abundant on the membranes of endothelial cells throughout the body (36) and has also been recognized in the proximal tubule of the kidney (37). Work is now underway in our laboratories to explain the mechanism by which synergy takes place between peptide 1–2 and bradykinin.

Except for bradykinin, the fragmentation products produced from human HMW-kininogen by plasma kallikrein have not been characterized. Such investigation will require highly purified human HMW-kininogen. The ability of bovine HMW-kininogen to repair the defects of Flaujeac plasma suggests a degree

of functional and structural similarity with human HMW-kininogen. However, until human HMW-kininogen is better characterized and its fragmentation products identified, the data presented here must remain suggestive for the human kinin-forming system.

These observations do serve to substantiate further a functional role for a kininogen, HMW-kininogen, in the plasma pathways of coagulation and fibrinolysis and in the permeability reactions observed with diluted plasma. The new basic permeability agent, peptide 1–2, will require careful consideration in those settings in which plasma kallikrein may be activated in vivo and simultaneously release bradykinin and peptide 1–2 from HMW-kininogen. More work is required to explain the synergy between bradykinin and peptide 1–2.

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service grants HL-18618 and AM-05300, the Medical Research Foundation of Oregon, and the Harlow Foundation.

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