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POSSIBLE PHYSIOLOGICAL ROLE OF NEW PEPTIDE FRAGMENTS RELEASED FROM BOVINE HIGH MOLECULAR WEIGHT KININOGEN BY PLASMA KALLIKREIN

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Abstract—The possibility of a physiological role for two new peptides, Fragment 1·2 and Fragment 2 (histidine-rich peptide), released from bovine plasma high molecular weight (HMW) kininogen by the action of plasma kallikrein was examined. The former peptide, which was liberated at the same rate as bradykinin, and the latter peptide, which was derived from Fragment 1·2 on subsequent incubation with plasma kallikrein, showed negligible kinin-like activities. However, the peptides strongly inhibited kinin formation in bovine plasma exposed to glass ballotini, and prolonged the calcium clotting-time of citrated rat plasma in a manner similar to hexadimethrine bromide. Also, they inhibited the activation of Hageman factor by kaolin. The above three types of experiments showed the potency of Fragment 1·2 to be approximately three times that of Fragment 2 on a molar basis. Neither fragment showed inhibition of already activated Hageman factor, nor of the esterase activities of plasma kallikrein, plasmin and thrombin. These results suggest that liberation of these polypeptides from HMW kininogen represents a negative feedback mechanism for the contact activation of Hageman factor.

A potent vasoactive peptide, bradykinin, is known to be released by plasma kallikrein from bovine high molecular weight (HMW) kininogen [1]. Plasma kallikrein is present in plasma as an inactive precursor and is activated directly by activated Hageman factor [2], which is also in an inactive form in plasma and thought to be activated by contact with negatively charged surfaces [3, 4]. Thus when Hageman factor is activated, bradykinin is released. Furthermore, active Hageman factor acts on Factor XI (PTA) [5] and plasminogen proactivator [6], thus initiating blood clotting and fibrinolysis as well.

Recent findings on genetic deficiency in plasma prekallikrein show another role of plasma kallikrein in activation of Hageman factor [7]. Thus prekallikrein is required for activation of Hageman factor, indicating a positive feedback mechanism. An accelerated enzyme system is known to be suppressed by several proteinase inhibitors in plasma [8]. Also, there are several recent reports on a deficiency of HMW kininogen which strongly indicate that it is also essential to the surface activation of Hageman factor [9–13].

As previously reported, on incubation of bovine HMW kininogen with plasma kallikrein, two new peptides besides bradykinin are liberated [14, 15]. One of the peptiides, Fragment 2, contains an unusually high level of histidine and was, therefore, previously called "histidine-rich peptide"; its complete amino acid sequence of 41 residues has been established [14], and a part of the work on its biological activity was reported [16]. The other fragment, named Fragment 1, is a glycopeptide also with a high content of histidine [17]; the entire sequence comprising 69 residues has recently been established [18]. During these studies, we found that these two peptides were not directly produced from HMW kininogen, but a larger fragment, consisting of 110 amino acid residues, named Fragment 1·2, is released concomitantly with bradykinin and subsequently cleaved into Fragments 1 and 2 [18, 19]. Moreover, recent studies have indicated that Fragment 1·2 is located adjacent to the C-terminus of the bradykinin moiety of HMW kininogen [20].

The present work was undertaken to determine whether these peptides, Fragment 1·2 and Fragment 2, played a physiological role in plasma kallikrein–kinin and blood clotting systems. We have found that Fragments 1·2 and 2 have the following effects *in vitro*: (1) inhibition of kinin formation in glass-exposed plasma, (2) prolongation of clotting time and (3) inhibition of formation of active Hageman factor. But these peptides had negligible kinin-like activities and did not interfere at high levels with kinin assays.

MATERIALS AND METHODS

Biological preparations

Isolated guinea pig ileum. Contraction of the ileum, suspended in a 5-ml organ bath at 37° in Tyrode solution aerated by 95% O_2 and 5% CO_2, was recorded by an isotonic transducer (Medical Electronics Commercial, ME-4012). Standard bradykinin and samples were added to the organ bath every 5 min, and contact time was 90 sec.

Isolated rat uterus. Virgin rats weighing 120–200 g were injected with 7.5 mg i.p. and s.c. of hexesterol (Hexron, Teikoku Zoki) 12–24 hr before assay. One horn of the separated uterus was suspended at 28° in a 5-ml organ bath filled with aerated Munsic solution [21]. Contraction of the uterus was measured as described above.

Rat systemic blood pressure. The systemic arterial pressure of rats weighing 375–400 g was measured by a high pressure transducer (Nihon Kohden MPU-0.5) connected to a thin cannula (PE-60) inserted into the femoral artery under pentobarbital sodium anesthesia (50 mg/kg, i.p.), and recorded on an ink-writing oscillograph. Another PE-60 cannula was put into the carotid artery for intra-arterial injection. Bradykinin and sample solutions (0.1–0.5 ml) were injected through the cannula.

Vascular permeability increase in rabbit skin. Five min after intravenous injection of pontamine sky blue (50 mg/kg) in 0.43% saline solution (1.2 ml/kg), 0.1 ml of the peptide fragments or bradykinin solution was injected into the shaved abdominal skin of albino rabbits under pentobarbital sodium anesthesia (30 mg/kg, i.p.). The diameter of the blueing spot was measured 30 min after the intradermal injection.

Kinin formation by glass activation

Bovine blood, collected in sodium citrate (0.38% final concentration) at the slaughterhouse, was centrifuged at 1000 *g* at room temperature. The bovine plasma thus obtained was kept at −20° until use. Glass contact was strictly avoided during preparation of the plasma. The bovine plasma (0.5 ml) was incubated with glass ballotini (Jencons Ltd., No. 14 H102/1, approximately 0.1 mm in diameter) (0.5 g/0.5 ml of plasma) in a plastic tube containing 1,10 phenathroline (10^{-4} g/ml) and 0.1 M phosphate buffer (pH 7.4) to 2.0 ml final volume. Incubation was started when glass ballotini were added, and 0.05-ml aliquots of the incubation mixture were taken at 10-min intervals and added to the organ bath. The kinin formed was assayed on the rat uterus. Synthetic bradykinin was used as a standard.

Given doses of hexadimethrine bromide, Fragment 1·2 and Fragment 2 were preincubated for 16–50 min with glass ballotini before the addition of plasma. Incubation was started upon the addition of plasma. Incubation with the inhibitors was carried out in parallel with control incubation. The per cent of inhibition was calculated from the maximum kinin levels formed within 60 min in the presence or absence (control) of test agents by the following equation:

$$\text{Inhibition per cent} = \frac{\text{kinin of control plasma} - \text{kinin with test agents}}{\text{kinin of control plasma}} \times 100$$

Calcium clotting time

A slight modification in the method described in Ref. 22 was made. Blood was collected from Sprague–Dawley rats weighing 400–500 g into a plastic tube (Falcon No. 2006) containing 0.1 vol. of 3.8% sodium citrate solution. Plasma was obtained by centrifugation at 1000 *g* for 10 min. Fifty μl of 0.15 M NaCl or test agents in 0.15 M NaCl, and 100 μl of rat plasma were mixed in a glass tube (10 × 105 mm) and kept at 37° for exactly 2 min. The time interval from the addition of 50 μl of 0.05 M $CaCl_2$ until clot formation was regarded as the clotting time. The end point of clot formation was inspected by gently tilting the tubes every 15 sec at 37°.

Radiochemical assays for Hageman factor activation and for esterolytic activities of other plasma enzymes

Formation of active Hageman factor or prekallikrein activator. Citrated human plasma was diluted (1:200) with 0.15 M NaCl solution in a plastic tube (Falcon No. 2006) containing 1 mg/ml of kaolin and incubated for 1 hr at 37° [23]. The test agents were mixed with kaolin before addition of diluted plasma.

Measurement of the activity of active Hageman factor. Twenty μl of the above solution and 10 μl of 0.5 M Tris, pH 8.0, were incubated for 10 min at room temperature with or without partially purified human plasma prekallikrein fraction (20 μl, 0.2 TAME units/ml) [23], and the [^{3}H]TAME (100,000 dis./min) was added as a substrate for plasma kallikrein activated by active Hageman factor. After a 30-min incubation at room temperature, the split [^{3}H]MeOH was counted in a scintillation counter (Mark II, Nuclear-Chicago). Each sample was run in duplicate and the difference of the mean counts between mixtures with and without prekallikrein was calculated as the active Hageman factor formed (HFa). Inhibition per cent was calculated as:

$$\text{Inhibition per cent} = \frac{\text{HFa of control plasma} - \text{HFa with test agent}}{\text{HFa of control plasma}} \times 100$$

Assay of the effect of test agents on the activated Hageman factor. Twenty μl of activated Hageman factor solution (as described above in the section on the formation of active Hageman factor) from control plasma was preincubated with the test agents for 10 min at room temperature. Then partially purified prekallikrein was added and assayed in the same manner described in the preceding section. [^{3}H]-TAME assay used here is more precisely described in Ref. 23, which is a modification of an original radiochemical assay developed by Beaven *et al.* [24], and reviewed in Ref. 25.

TAME-esterase activities of plasma kallikrein, thrombin and plasmin. The esterase activities of these plasma enzymes were determined by [^{3}H]TAME assay. Human plasma kallikrein, human plasmin or bovine thrombin was incubated in 0.1 M Tris buffer, pH 8.0, with [^{3}H]TAME for 30 min at room temperature. The released [^{3}H]MeOH was counted as described above. The test agents were preincubated with esterases for 10 min at room temperature before the addition of [^{3}H]TAME. Each sample was run in duplicate and the mean counts in dis./min were calculated.

Peptide fragments and other agents used

Fragment 1·2 and Fragment 2 were prepared from highly purified bovine plasma HMW kininogen [26] incubated with highly purified bovine plasma kallikrein by the method previously described [18, 19]. Hexadimethrine bromide (Aldrich, Wisc.), kaolin

Table 1. Biological activities of Fragment 1·2 and Fragment 2 in comparison with bradykinin*

	Fragment 1·2	Fragment 2
Isolated rat uterus (contraction)	0.0001	0.00025–0.001
Isolated guinea pig ileum (contraction)	<0.0001	<0.0004
Systemic blood pressure in rat (decrease)	0.001 ~ 0.01	0.002 ~ 0.003
Vascular permeability increase in rabbit skin (increase)	0.001	0.001

* Values indicate the potencies in comparison with bradykinin (= 1) on a weight basis.

(Fisher, N.J.), bovine thrombin (Parke–Davis, Mich.), bradykinin (Protein Research Foundation, Minoh, Osaka, Japan) and [^{3}H]Tosyl-L-arginine methyl ester ([^{3}H]TAME. 125 mCi/m-mole, Calatomic, Calif.) were purchased. Fragment 1·2, Fragment 2, HMW kininogen and hexadimethrine bromide were dissolved in 0.15 M NaCl solution as 1 mg/ml, and bradykinin as 100 μg/ml. [^{3}H]TAME solution was prepared as 100,000 dis./min/10 μl in distilled water. All solutions were stored at $-20°$ until used. Plasmin was prepared from human plasminogen (KABI), activated by streptokinase (Varidase, Lederle-Japan, Tokyo) as follows: plasminogen [3 casein units (cu)] and streptokinase (10 units) in 500 μl of 0.02 M phosphate buffer, pH 7.4, and 500 μl glycerol were mixed in a plastic tube and stored at room temperature for 48 hr.

RESULTS

Biological activities of Fragment 1·2 and Fragment 2 on pharmacological preparations

As shown in Table 1, the peptide fragments showed nearly negligible biological effects on several preparations which are very sensitive to ng amounts of bradykinin.

Whereas bradykinin contracts the rat uterus at a threshold dose of 3×10^{-10} g/ml, Fragment 1·2 and Fragment 2 showed only slight contractions in 100,000- and 400,000-fold larger doses, respectively, on a weight basis (two preparations). On the guinea pig ileum, bradykinin showed contraction at the minimal dose level of 10^{-9} g/ml, whereas these peptides caused only slight contraction at a dose level 20,000-fold higher (two preparations). The fragments neither potentiated nor inhibited the responses of the rat uterus or the guinea pig ileum to bradykinin.

The peptide fragments decreased the systemic blood pressure of anesthetized rats as bradykinin did, when injected intra-arterially, but the potencies were very low again, compared with the threshold doses of bradykinin (10–20 ng/kg). Three to 30 μg/kg of Fragment 1·2 was required to produce the same effect and the larger dose (30 μg/kg) lowered the pressure by only 8 mm Hg. Thus, the potency of Fragment 1.2 was only 0.01, when compared with bradykinin. Fragment 2 also showed low potency. Three to 11 μg/kg of the peptide decreased the pressure by only 8–28 mm Hg. Therefore, the activity of Fragment 2 was 0.003 of bradykinin.

In the rabbit skin vascular permeability test, the activity of both peptides was so low that the potency was only 0.001, when compared with bradykinin on the weight basis.

Kinin formation by glass activation

After the addition of glass ballotini to the incubation mixture, kinin formation occurred slowly in bovine plasma and reached a plateau after approximately 30 min. The maximum levels of kinin formed were 512–1200 ng/ml of plasma. In the presence of hexadimethrine bromide, a known inhibitor of Hageman factor activation [27], the time to reach the maximum in kinin formation was delayed and the levels were suppressed. The inhibition with 15 μg/ml of the agent was 73.2 $\pm$ 5.9 per cent of the control kinin formation (Fig. 1).

When Fragment 1·2 or Fragment 2 was preincubated with glass ballotini, the rate of kinin formation slowed and its maximum level was lowered, depending on the concentration of the fragments. Fifty μg/ml of Fragment 1·2 and Fragment 2 inhibited kinin formation 86.2 $\pm$ 2.3 and 99.2 $\pm$ 0.8 per cent respectively (see Fig. 1). Preincubation time did not significantly affect the inhibition rate. There was no marked difference in the potencies of the two peptides. Thus, Fragment 1·2 is considered to be three times as potent as Fragment 2, since the ratio of the molecular weights is three (12,600 and 4,600 respectively). On a weight basis, hexadimethrine bromide was three times as effective as the peptide fragments.

Calcium clotting time of citrated rat plasma

The calcium clotting time of citrated rat plasma without the addition of the test agents was deter-

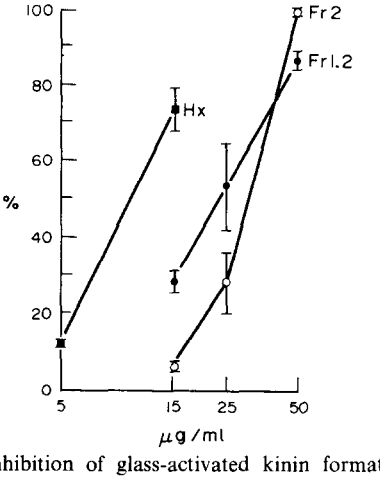

Fig. 1. Inhibition of glass-activated kinin formation by Fragment 1·2, Fragment 2 and hexadimethrine bromide. Ordinate: per cent inhibition of the formed kinin level by test agents. Abscissa: final concentration of the test agents in the incubation mixture. Fr 1·2: Fragment 1.2; Fr 2: Fragment 2; Hx: hexadimethrine bromide.

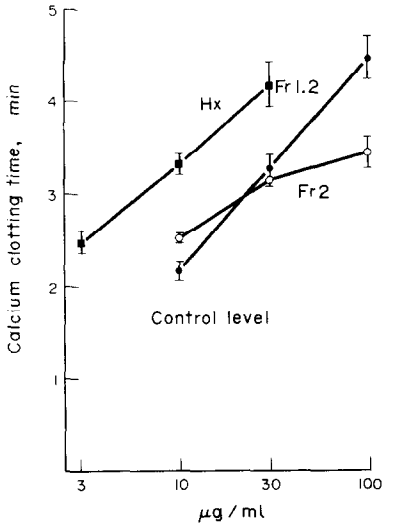

Fig. 2. Prolongation of calcium clotting time of citrated rat plasma by Fragment 1·2, Fragment 2 and hexadimethrine bromide. Each point indicates the mean of six experiments with vertical bars showing S.E.M. The concentration is expressed as the final concentration in the incubation mixture. The calcium clotting time in the absence of the test agent is shown as control level. Abbreviations are the same as in Fig. 1.

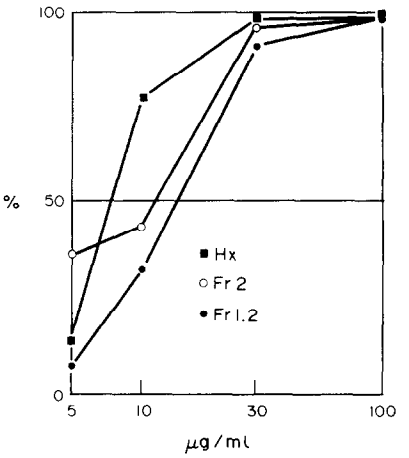

Fig. 3. Inhibition of the formation of active Hageman factor by Fragment 1·2, Fragment 2 and hexadimethrine bromide. Each point shows the mean from four human plasma samples. Ordinate: per cent inhibition of the formation of active Hageman factor determined as the activity of prekallikrein activation. Abscissa: concentration of the test agents in the incubation mixture for activation of Hageman factor. Abbreviations are the same as in Fig. 1.

mined through eight experiments to be 101 ± 12 sec. This is the control level depicted in Fig. 2. The clotting times were markedly prolonged by Fragments 1·2 and 2, as well as hexadimethrine bromide. There was not much difference between the two peptides, although the Fragment 2 curve had a lower slope. However, by rough estimate, Fragment 1·2 was three times as potent as Fragment 2 on a molar basis, as seen also in kinin formation. The potencies of both fragments were about one-third that of hexadimethrine bromide on a weight basis (Fig. 2).

Inhibition of activation of Hageman factor

Figure 3 depicts the inhibition of the formation of Hageman factor. The formation of active Hageman factor or prekallikrein activator (PKA) of human plasma by kaolin was inhibited significantly by Fragments 1·2 and 2 as well as by hexadimethrine bromide. The inhibition was dose dependent in the concentration range of 5–30 μg/ml. Almost complete inhibition was found at 100 μg/ml of all three agents. On a weight basis, the two peptides had about the same effect as already seen for kinin formation and blood clotting. The average count of the formed active Hageman factor of control plasma was determined from four individuals to be 13,932 ± 2,990 dis./min.

In the same series of experiments, as shown in Table 2, no inhibition of the formation of active Hageman factor was observed after addition of HMW kininogen (50 μg/ml), the mother protein of these fragments. Nor did bradykinin, concomitantly released with Fragment 1·2, inhibit the activation of Hageman factor up to a concentration of 50 μg/ml.

Effect on already activated Hageman factor, plasma kallikrein, plasmin and thrombin. There was no significant difference between the counts with or without Fragment 1·2, Fragment 2 or HMW kininogen (as shown in Table 3) if they were added to a mixture of already activated Hageman factor and prekallikrein up to a concentration of 100 μg/ml. Moreover, these peptide fragments did not show any effect on the esterolytic activities of human plasma kallikrein, human plasmin and bovine thrombin, as shown in the same table.

Table 2. Effect of HMW kininogen and bradykinin on the formation of active Hageman factor*

Control	HMW kininogen (50 μg/ml)	Bradykinin (10 μg/ml)	Bradykinin (50 μg/ml)
18,776		20,214	
11,150			11,600
24,476	25,334		
27,420	30,772		
99†	280† (485)‡		

* Numbers represent amounts of active Hageman factor formed expressed as dis./min of [^{3}H]MeOH.

† Dis./min formed without kaolin.

‡ Value in parentheses was found in the absence of plasma but in the presence of HMW kininogen.

Table 3. Effect of Fragment 1·2, Fragment 2 and HMW kininogen on already activated Hageman factor and on plasma esterases*

	Control	Fragment 1·2 (100 μg/ml)	Fragment 2 (20 μg/ml)	Fragment 2 (100 μg/ml)	HMW kininogen (100 μg/ml)
Already activated Hageman factor (+ preKK)					
PreKK†					
0.001 units	8,226	7,652		9,688	10,126
0.004 units	22,222		23,474		
Plasma esterases					
Human plasma kallikrein†					
0.001 units	11,286	15,910		13,230	17,488
0.0012 units	11,104		12,236		
Plasmin					
0.005 cu‡	6,442	7,080		7,344	7,556
0.01 cu	14,784	15,624		12,900	16,178
Thrombin					
0.001 NIH units	8,458	6,844		6,980	7,712
0.01 NIH units	46,932	51,210		47,224	48,892

* Numbers indicate means of duplicates of counts of released [^{3}H]MeOH in dis./min.
† Partially purified prekallikrein and plasma kallikrein [23] expressed as a TAME unit.
‡ Casein unit.

DISCUSSION

Fragment 1·2 is released from HMW kininogen by plasma kallikrein along with bradykinin [15]. Fragment 2 is split later from Fragment 1·2, by plasma kallikrein. The fact that the peptide fragments are released simultaneously with bradykinin led us to assume that the three might share similar biological activities, such as smooth muscle stimulation, vasodilatation, vascular permeability increase and so forth.

However, bradykinin-like activities found in Fragment 1·2 and Fragment 2 were so low as to be negligible, as shown in Table 1. Furthermore, the biological activities of bradykinin were neither potentiated nor inhibited by the peptide fragments.

The kinin formation of mammalian plasma by contact activation with glass ballotini is known to be mediated by activation of Hageman factor [28, 29]. Fragments 1·2 and 2, like hexadimethrine bromide, markedly depressed kinin formation in bovine plasma if the glass ballotini were preincubated with the peptide fragments. This inhibition was not due to antagonism to bradykinin on the rat uterus preparation, since the fragments did not affect the response of bradykinin.

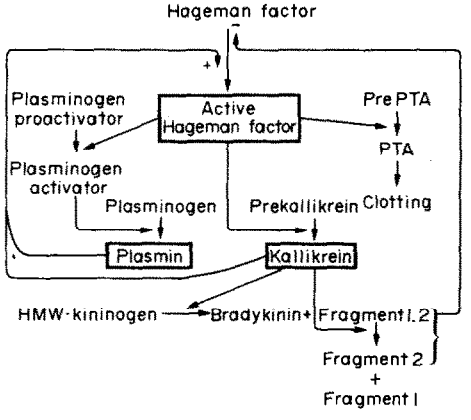

Fig. 4. Proposed scheme for a negative feedback mechanism on contact activation of Hageman factor.

Intrinsic blood coagulation is also known to be initiated by the activation of Hageman factor [29, 30], and calcium clotting time could be prolonged by any defect in coagulation factors. The addition of Fragments 1·2 and 2 was found to prolong the calcium clotting time of citrated rat plasma in a glass tube, as shown in Fig. 2.

When Fragment 1·2 or Fragment 2 was added before the formation of active Hageman factor, the release of [^{3}H]MeOH was suppressed. This suppression, however, did not exclude the possibility that the fragments would inhibit plasma kallikrein itself or already activated Hageman factor, since the Hageman factor activation was measured by the esterolytic activity of the plasma kallikrein formed. However, this possibility was unlikely, since these fragments inhibited neither active Hageman factor, itself, nor plasma kallikrein (Table 3). This inhibition, therefore, was due to inhibition of the formation of active Hageman factor in a manner similar to that shown by hexadimethrine bromide [23, 27].

These facts suggest the following hypothesis regarding activation of the kinin system as a scheme shown in Fig. 4. When Hageman factor is activated, it converts prekallikrein to kallikrein, which in turn acts on HMW kininogen to release bradykinin, which exerts potent biological activities on surrounding tissues [31, 32]. Plasma kallikrein further activates Hageman factor in a positive feedback mechanism as reported by several authors [4, 7, 29, 31]. Meanwhile, Fragment 1·2 and later Fragment 2 are released from HMW kininogen along with bradykinin, by the action of plasma kallikrein. These peptides may inhibit the further activation of Hageman factor. Thus, a negative feedback mechanism may occur during activation, besides the inhibition of active enzymes by plasma proteinase inhibitors [8]. Consequently, the kallikrein–kinin system may have a self-control system in its own cascade.

These observations, taken with generally accepted views of the mechanisms involving contact activation

of Hageman factor [28, 30], suggest that these peptides exert the observed effects by interfering with the surface activation of Hageman factor, presumably by blocking negative charges on the surface, in analogy to hexadimethrine bromide and other polyvalent, positively charged molecules [24, 27, 33].

On the other hand, recent reports on genetic deficiency in HMW kininogen suggested that HMW kininogen could be a cofactor in contact activation of the Hageman factor in addition to plasma kallikrein [9–13]. A functional role for the histidin-rich moiety (Fragment 1·2) of the HMW kininogen molecule remains to be studied.

Subsequent to submission of this manuscript, Fragment 1 was determined to have almost negligible activity in rat uterus contraction (<0.00025 of bradykinin on a molar basis), in inhibition of formation of prekallikrein activator and in prolongation of calcium clotting time.

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