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BRADYKININOGEN AND BRADYKININ IN THE CARDIOVASCULAR SHOCK PRODUCED BY PROTEOLYTIC ENZYMES*

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Abstract—As a model for the study of cardiovascular shock, where the participation of bradykinin and related peptides could be expected, trypsin, kallikrein, and a crystalline proteinase of bacterial origin (Nagarse) have been employed. Experimental conditions were found in which hydrolysis of bradykininogen *in vivo* with concomitant appearance of bradykinin-like peptides in the blood could be demonstrated. These results substantiate the idea that the release of peptides of the bradykinin type is an important factor in the production of the hypotension by proteolytic enzymes. The extent of the mobilization of kinin peptides during the experimental procedure was assayed by the determination of alterations in the amount of their precursor—bradykininogen—in plasma, since only a small percentage of the precursor destroyed could be recovered as the active peptide.

ACTIVATION of proteolytic enzymes from plasma is a constant feature observed in several varieties of cardiovascular shock.[1–3] Proteases, on the other hand, as has been demonstrated by Rocha e Silva since 1940,[4] are able to produce acute systemic arterial hypotension accompanied by characteristic signs of cardiovascular shock. Furthermore, polypeptides of the bradykinin family, which are released *in vitro* by the action of proteolytic enzymes upon plasma globuline, produce sustained hypotension when injected intravenously into animals.[5, 6] These facts might suggest that proteases owe their hypotensive effect to the release of bradykinin or related peptides.

In this paper an attempt has been made to correlate the cardiovascular changes produced by the intravenous injection of such proteases with alterations of the content of peptides of the bradykinin type and of their precursor, bradykininogen, in blood. Besides the esterolytic activity of plasma as measured by its capacity for splitting benzoyl-arginine-methyl ester (BAMe), other biochemical and hematological parameters of interest were measured.

For this study, three kinin-releasing enzymes have been employed: (1) trypsin, which Rocha e Silva[7] had demonstrated to produce shock in dogs, rabbits, and cats and was, along with the venom of *Bothrops jararaca*, the first enzyme used to show

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release of bradykinin from plasma globulines *in vitro*:[5] (2) kallikrein, a substance discovered by Frey and Kraut,[8] easily demonstrated in urine, pancreas, saliva, and salivary glands, which releases the polypeptide kallidin[9] (lysyl-bradykinin) and which is also a shock-producing substance;[10] (3) a crystalline bacterial proteinase prepared from the culture medium of *Bacillus subtilis*, Nagarse,[11] which Prado *et al.*[12] have shown to be a kinin-releasing enzyme and to cause a fall in blood pressure when injected intravenously into rats and dogs.

EXPERIMENTAL

Animal experiments. Dogs under pentobarbital anesthesia (30 mg/kg) were used. The carotid blood pressure was recorded with a mercury manometer and registered on a smoked drum. Polyethylene tubes were inserted into the femoral artery and vein. The enzymes were injected through the femoral vein and the blood samples taken, before and at various intervals after enzyme administration, from the femoral artery. As a routine the trachea was cannulated and artificial respiration applied whenever necessary.

The following tests and assays were carried out in blood samples before and after the enzyme administration: clotting time, esterolytic activity, plasma bradykininogen (BKG), plasma bradykinin, plasma histamine, hematocrit, and plasma protein.

Bradykininogen, hematocrit, and protein. Blood samples (4 ml), were collected with a siliconized syringe moistened with heparin (Liquémine, Roche); 1 ml was transferred to a Winthrobe tube for hematocrit values; the remaining volume was then centrifuged. Plasma BKG was estimated by the amount of bradykinin liberated from denatured plasma by an excess of trypsin, according to a previously described method.[13, 14] Bradykininogen concentration was expressed in units per milliliter plasma; one unit of BKG was defined as the amount of precursor able to liberate one unit of standard bradykinin as used in the Department of Pharmacology, Faculty of Medicine, Ribeirão Prêto, kindly supplied by Dr. Hanna Rothschild and prepared according to Prado *et al.*;[15] one unit of the standard corresponds to 0·44 μg of synthetic bradykinin BRS-640 (Sandoz Labs.).[16]

Proteins were determined by the Mokrasch and McGilvery[17] modification of the biuret method. Crystalline bovine albumin was used as a standard, and readings were done in a Klett-Summerson photocolorimeter with a green filter (540 mμ).

Esterolytic activity and coagulation time. Blood samples of 3·5 ml were drawn; for determination of plasma esterolytic activity, 2·5 ml was immediately transferred to 12-ml centrifuge tubes with 0·1 ml of 2·5% sodium oxalate solution, and 1 ml was used for determination of the coagulation time by the method of White and Lee;[18] time was recorded from the moment at which blood was aspirated into the syringe.

Esterolytic activity was estimated by the method of Brown,[19] with BAMe as substrate.

Kinin and histamine. For the detection of plasma kinin released in these experiments, 5 ml of blood was collected into a siliconized syringe and transferred to siliconized centrifuge tubes containing 20 ml chilled alcohol acidified with 0·5 ml of 10% acetic acid. In these conditions the proteins were immediately precipitated and the enzymatic reactions blocked. The supernatant was separated by centrifugation at 1500 rev/min during 15 min in an International centrifuge at 5°, and then transferred to 50-ml round-bottom flasks. The precipitate was washed twice with 10 ml

alcohol. The combined supernatants were evaporated to dryness under reduced pressure at 40° and the residue dissolved in 4 ml saline. The smooth-muscle-stimulating activity was determined on the guinea pig ileum either by the 4-point assay[10] or by the more common procedure of twice bracketing one dose of the standard between two doses of the unknown. The perfusing fluid contained atropine ($3{\cdot}5 \times 10^{-4}$ m-moles/liter) and diphenhydramine ($1{\cdot}7 \times 10^{-3}$ m-moles/liter). The recovery of bradykinin added to the precipitated blood, by this method, is approximately 70%.[21] After each determination, the material was subjected to digestion by chymotrypsin (100 μg/ml buffered at pH 7·8 with Tris), and assayed for the eventual presence of other smooth-muscle active substances. Occasionally the extract was also tested by the relaxing effect on rat duodenum preparations.

Blood samples were also drawn for histamine determination by Code's[22] method. The results were given respectively in units of bradykinin or micrograms histamine per milliter plasma, calculated from the hematocrit values.

Enzymatic activities of Nagarse, trypsin, and kallikrein preparation. Enzymes used were trypsin (Mann Research Laboratories, twice crystalized); kallikrein (Padutin, Bayer, 10 units/mg, an impure preparation of kallikrein obtained from hog pancreas), Nagarse (*B. subtilis* crystalline proteinase from Nagarse Co. Ltd., Amagasaki, Japan), kindly supplied by Professors Leal Prado (São Paulo) and M. Bacilla (Paraná); chymotrypsin (crystalline, Armour).

Substrates and assay methods

Casein (Pfanstiehl), enzyme assayed according to Northrop *et al.*[23] BAMe (Mann), esterase activity measured by the method of Brown,[19] with small adaptations. Bradykininogen, dog plasma globulin fraction, precipitated in 35–45% saturated ammonium sulfate solution, dialyzed, and diluted to 35–40 units of BKG/ml. The substrates were prepared either according to Horton[24] (partially denaturated globulins) or Van Arman[25] (completely denaturated globulins). Kinin-releasing activity was measured by incubation of 0·025–1·0 ml of enzyme solutions in conditions of excess of substrate (2 ml) buffered at pH 7·8 with 0·1 M Tris buffer in a total volume of 3 ml during 5 min, at 37°. The reaction was stopped by addition of 10 ml hot alcohol, the suspension boiled 5 min, and the excess liquid removed under reduced pressure at 37°. The dry residue was suspended in 5 ml saline solution, centrifuged, and the kinin dissolved and assayed in the guinea pig ileum preparation.

Administration of proteases. In a first series of experiments the endovenous injection of the enzyme solutions was done in small doses to produce blood pressure alterations reversible in 15 to 20 min. Adequate doses were found to be approximately 3 mg/kg for trypsin and kallikrein and 1·5 mg/kg for Nagarse. After the return of blood pressure to the initial level, a second dose, double the amount of the first, was injected. A partial recovery of blood pressure was observed in most of the experiments within 1 to 2 hr. Nagarse, however, at this dosage level (3 mg/kg) caused a transient hypotension lasting for 30–45 min in most of the dogs; kallikrein (6 mg/kg) proved to be lethal to most of the dogs, death occurring after approximately 5 hr. Trypsin (6 mg/kg), on the contrary, produced a fall in blood pressure, reversible in about 1 hr, no deaths being observed. To produce an irreversible hypotension, 12 mg trypsin/kg was usually needed. From 1 to 1·5 hr after the injection of 12 mg trypsin/kg, 6 mg kallikrein/kg, or 3 mg Nagarse/kg, when blood pressure was partially recovered,

double doses of the enzyme solution were again administered intravenously (24 mg trypsin, 12 mg kallikrein, or 6 mg Nagarse, per kg). These doses always produced a rapid and irreversible fall of blood pressure which killed the animals in 15–20 min in spite of the use of artificial respiration.

A second and third series of experiments were performed, in which the injected single dose of enzymes killed the animals in 5 to 7 hr and 15 to 20 min respectively.

Samples of blood for determination of bradykininogen, bradykinin, BAMesterase activity, hematocrit, and plasma proteins were collected in all cases. Control blood samples were withdrawn before the protease injection or after 10, 30, 60, 120, and 180 sec for kinin assay. Samples for other biochemical or hematological determinations were removed after 2, 15, and 30 min or more, if necessary, after the enzyme administration. In some experiments the histamine content of plasma was also determined.

The total amount of blood removed in an experiment never exceeded 2·0 per cent of the body weight of the animal.

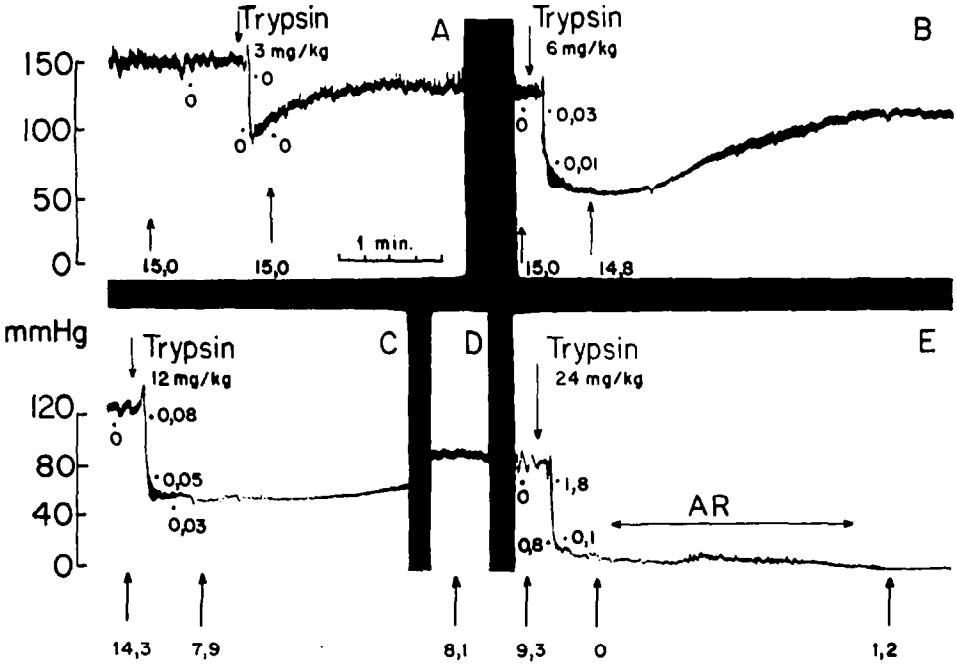

FIG. 1. The effect of increasing doses of trypsin on arterial blood pressure of the dog. Animal under pentobarbital sodium anesthesia. Numbers indicated by points refer to the values of kinin expressed in units of bradykinin per milliliter plasma, and numbers indicated by arrows (pointing up) to the levels of BKG (U/ml plasma). Breaks in the records indicate respectively lapses of 20, 45, 50, and 60 min between A and B, B and C, C and D, and D and E. At AR, artificial respiration. Observe that the death of the animal occurred after the BKG reached a value near zero.

RESULTS

(I) *Effects of the administration of increasing doses of trypsin.* The results of a typical experiment are shown in Fig. 1 where plasma BKG, bradykinin, and blood pressure changes are presented. Other biochemical and hematological findings are shown in Fig. 2 which shows the changes in hematocrit, blood clotting time, esterolytic activity,

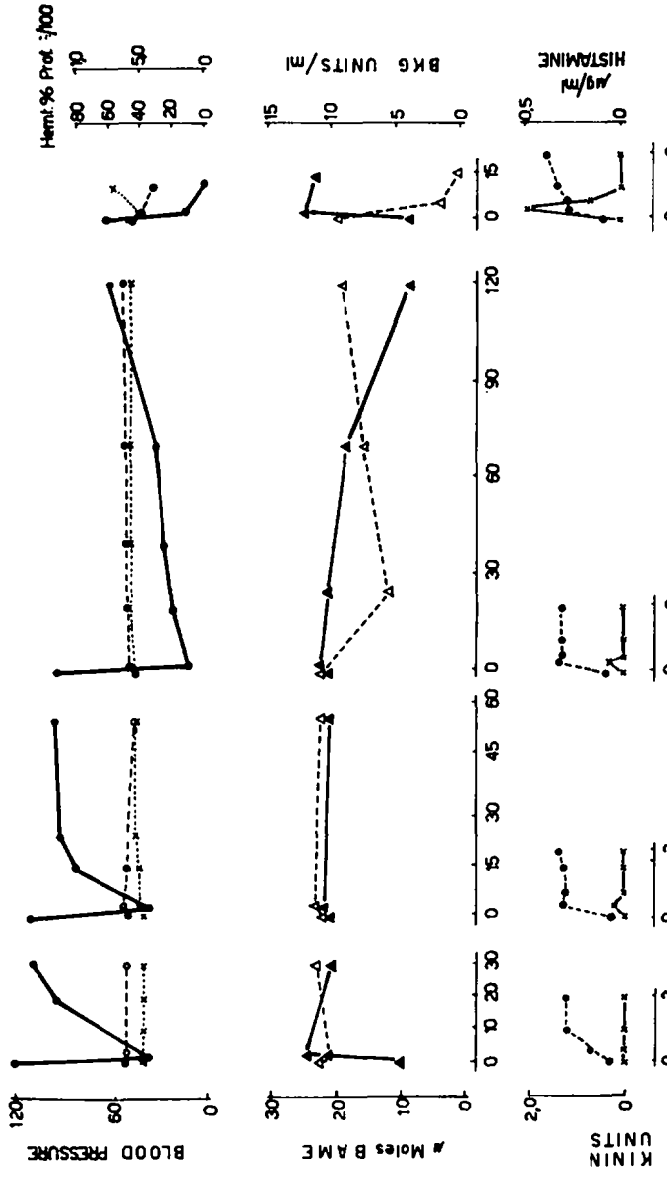

FIG. 2. Effects of intravenous injection of trypsin in the dog. Trypsin in doses of 3, 6, 12 and 24 mg/kg weight of animal was injected into a dog. Blood pressure (●——●) falls immediately after the injection; hematocrit (Hemt., ×·····×) and plasma proteins (Prot., ○- - -○) expressed in g/100 ml of plasma changed only after an injection of 24 mg trypsin/kg weight. Middle part of the figure shows the modifications of BKG (△- - -△) expressed in U/ml plasma and hydrolysis of BAMe (▲——▲) (μmoles hydrolyzed/ml plasma/hr). Kinin (×——×) and histamine (●- - -●) contents of plasma are shown in the lower section of the figure. Kinin was detected in plasma only during the first minutes after the injection.

protein content, BKG values, bradykinin and histamine levels. A significant alteration of the BKG content was observed only after injection of 12 and 24 mg trypsin/kg; after the administration of this dose, BKG practically disappeared from the circulation. Kinin activity was detected in the blood only after the injection of doses of trypsin higher than 12 mg/kg; a peak of this substance in plasma was reached 10–30 sec after the enzyme injection. The concentration of the active substance decreased rapidly and could not be detected 3 min later. Clotting time increased immediately after the administration of low doses of trypsin (3 and 6 mg/kg).

Histamine appeared in the circulation immediately after the administration of 3 mg trypsin/kg and, in contrast to bradykinin, remained in the circulation for a longer time. Esterolytic activity increased immediately after the injection of the lowest dose of trypsin. At peak activity, 30–35 μmoles BAMe was hydrolyzed per ml plasma per hr.

TABLE 1. ARTERIAL BLOOD PRESSURE AND HEMATOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY TRYPSIN* IN THE DOG

	Minutes after trypsin injection						
	0	2	60	120	180	240	300
Arterial blood pressure (mm Hg)	160	30	80	110	80	40	20
Clotting time (sec)	150	1200	IN†			IN	IN
Blood protein (g/100 ml plasma)	7·0	7·0	7·0			7·8	7·8
Hematocrit (%)	56	63	72			70	72
Esterolytic activity, BAMe (μmoles hyd./hr/ml plasma	5	28	19	14			8
Bradykininogen (U/ml plasma)	13	8	6	6	5	5	5

	Seconds after trypsin injection					
	0	10	30	60	120	180
Bradykinin (U/ml plasma)	0·0	0·30	0·09	0·03	0·01	0·01
Histamine (μg/ml plasma)	0·0	0·10	0·17	0·20	0·20	0·20

* 12 mg/kg.
† IN: The blood became incoagulable.

This pattern of response to the injection of increasing doses of trypsin is remarkably reproducible when the same sequence of enzyme injections is followed. The same type of response and approximately the same percentual alterations in hemodynamic, hematological, and biochemical data were found in all five experiments done.

(II) *Effects of a single intravenous dose of trypsin* (12 *mg/kg*). Approximately half the BKG content of the plasma was consumed immediately after the injection of

this dose of trypsin; simultaneously, an increase in BAMe esterolytic activity was observed, and small amounts of bradykinin and histamine were detected in the plasma. The death of the animals occurred 4–5 hr after the trypsin injection (Table 1).

(III) *Effects of the injection of trypsin* (24 *mg/kg*). After this dose of trypsin an abrupt fall of blood pressure was observed, and the animal died within 10–15 min. It can be seen that BKG disappeared almost completely from the blood, while esterolytic and kinin and histamine levels were markedly increased in plasma.

(IV) *Effects of the injection of increasing doses of kallikrein.* Kallikrein is also able to release kinin and to induce concomitant disappearance of bradykininogen from plasma. However, no activation of BAMe esterase or release of histamine in the plasma of kallikrein-treated animals could be observed. Blood-clotting times were often reduced after kallikrein administration. The main vascular, hematological, and biochemical changes observed in a typical experiment are shown in Figs. 3 and 4.

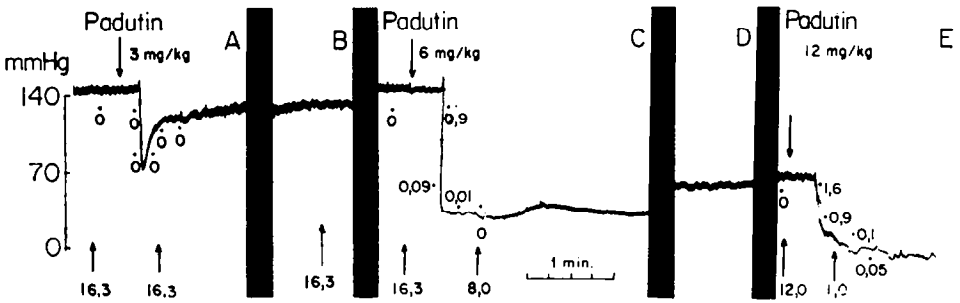

FIG. 3. Arterial blood pressure changes induced by kallikrein (Padutin) in the dog. Dog under pentobarbital sodium anesthesia; 3·0, 6·0, and 12·0 mg kallikrein/kg were successively injected into the vein. BKG levels and kinin values of plasma as indicated in Fig. 1. Breaks in the records indicate respectively lapses of 10, 30, 50, and 20 min between A and B, B and C, C and D, and D and E.

(V) *Effects of the injection of the crystalline proteinase (Nagarse) from* B. subtilis. In the dog, Nagarse evoked reactions similar to those elicited by trypsin. The bacterial enzyme was able to produce irreversible shock associated with the disappearance of BKG from plasma and release of kinin into the circulation. Nagarse also activated plasma BAMe esterase and increased the clotting time. The bacterial proteinase preparations used were, however, considerably more active than the twice-crystallyzed sample of trypsin employed in our experiments. As little as 6 mg Nagarse/kg was sufficient to kill a dog in 15 min; this can be compared with the dose of 24 mg trypsin/kg required to accomplish the same effect. Nagarse, in contrast to trypsin, released only small amounts of histamine into the plasma. Figures 5 and 6 show results of experiments done with stepwise increased doses of Nagarse.

Proteolytic activity in vitro. It was of interest to examine whether the effects *in vivo* observed after injection of trypsin, kallikrein, and Nagarse bore relation to the proteolytic activity of these enzymes *in vitro* on Horton's and Van Arman's substrates. The results of these experiments are summarized in Table 2. Tryspin is the most active kinin-releasing enzyme *in vitro*. The kallikrein preparations used were not pure. They show a probable contamination with chymotrypsin and kininases which could explain the weak kinin-releasing activity on Horton's substrate.

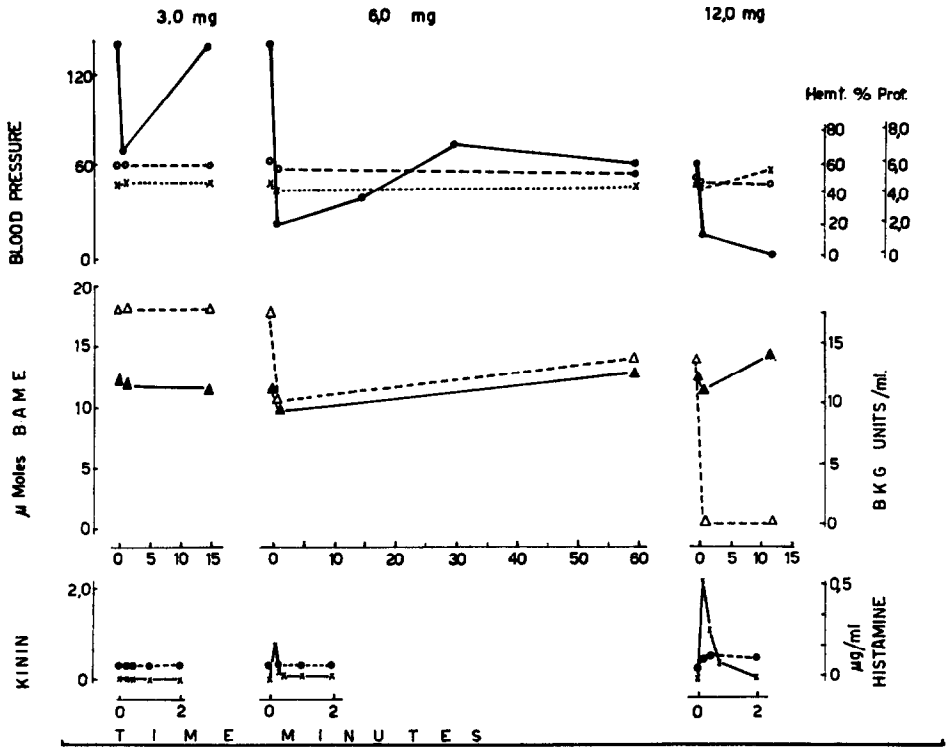

FIG. 4. Effects of intravenous injection of kallikrein (Padutin). A dog was injected successively with doses of 3·0, 6·0, and 12·0 mg weight/kg of a kallikrein preparation. Blood pressure (●——●), hematocrit (×····×), plasma protein (○····○), BKG (△---△), and BAMe esterase values were observed during the experiment. Kinin (×——×) and histamine (●---●) contents of plasma were also followed. Note the simultaneous fall of BKG and rise of kinin in plasma after injection of the enzyme preparation. In contrast to trypsin, histamine is not released by kallikrein.

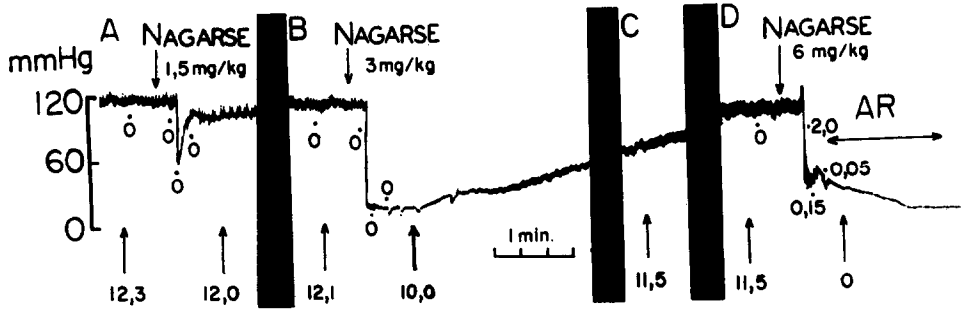

FIG. 5. Arterial blood pressure after nagarse injection; 1·5, 3·0 and, 6·0 mg enzyme/kg were injected successively into the vein of a dog under pentobarbital sodium anesthesia. BKG levels and kinin values of plasma as in Fig. 1. Breaks in the records indicate respectively lapses of 12, 20, and 15 min between A and B, B and C, and C and D. At AR artificial respiration.

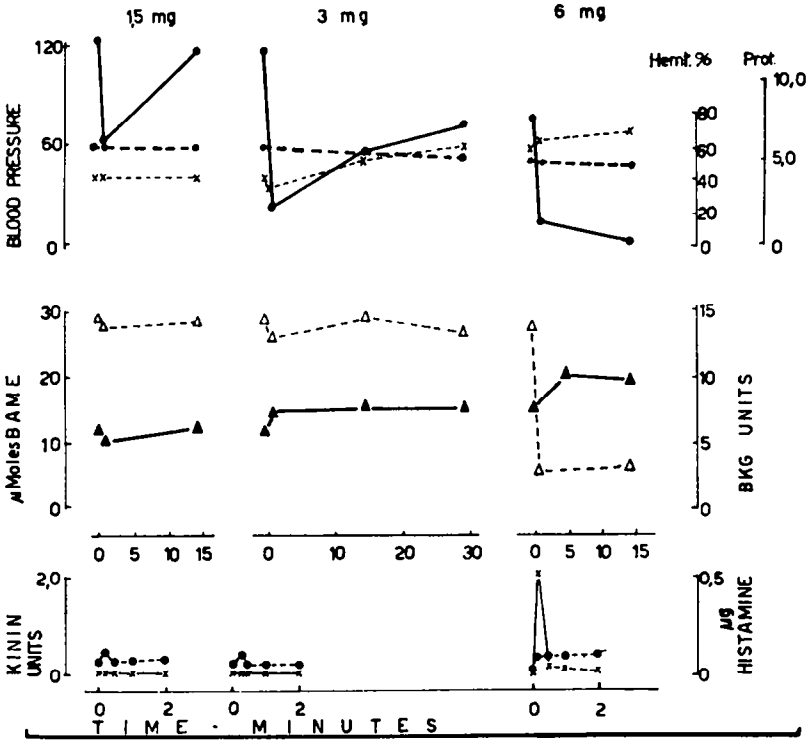

FIG. 6. Effects of injection of nagarse in the dog. Increasing doses of Nagarse, 1·5, 3·0, and 6·0 mg/kg weight, were injected into a dog. Blood pressure (●——●), hematocrit (× - - - ×), plasma proteins (●- - - ●), BKG (△- - - △), BAMe esterase activity (▲——▲), kinin (×——×), and histamine content (●. . . . ●) of plasma are expressed as shown in Fig. 1. Observe the abrupt fall of BKG and the sudden increase of plasma kinin in plasma.

TABLE 2. HYDROLYSIS OF SEVERAL SUBSTRATES BY TRYPSIN, NAGARSE, AND KALLIKREIN PREPARATIONS

Enzyme	Substrate			
	Casein*	BAMe†	Horton‡	Van Arman‡
Trypsin	0·26	16·0	5·8	11·0
Nagarse	0·76	1·2	4·6	0·6
Kallikrein	0·11	0·93	0·67	trace

* Milligrams tyrosine released/mg enzyme/min.

† Micromoles hydrolyzed/mg enzyme/min.

‡ Units of bradykinin released/mg enzyme/min. Conditions of assay as described in Methods.

DISCUSSION

The proteases used in the experiments presented in this paper are different, regarding their origin and properties. They share the common ability to release kinin *in vitro* and cause a fall in blood pressure. When injected into dogs, these enzymes produce a

remarkably similar pattern of reaction. This suggests a common mechanism underlying the production of this type of reaction. It was demonstrated that the enzymes deplete bradykininogen stores of the plasma *in vivo* and release bradykinin or bradykinin-like substances into the circulation. The amount of plasma kinin recovered from these experiments probably does not reflect the amount of active polypeptide released. The half-life of these polypeptides in plasma is probably short. The half-life of bradykinin injected into dogs, according to Saameli and Eskes[27] is approximately half a minute, and a considerable destruction or leakage to the extravascular compartments can be expected in a short time. The information obtained by determination of the BKG values, before and after the enzyme injections, is more likely to give the exact measurement of the amount of bradykinin released during the fall of blood pressure. Therefore, in the event of pathological processes such as anaphylactic shock, acute pancreatitis, snake venom poisoning, and other conditions in which there is liberation or activation of proteolytic enzymes with trypsin-like action, the determination of the plasma levels of the precursor, instead of the active peptide, seems to be a more convenient procedure. We have observed in our experiments that a fall of BKG to approximately half the initial value was invariably followed by an irreversible hypotension, independent of the enzyme used. Whether the hypotension is due to some unknown toxic property of these enzymes is not easy to decide. However, the enormous amount of such potent vasoactive substances as the kinin peptides, released in these cases ($\approx$2·0 mg pure material in a 15-kg dog) in a short interval of time and probably at strategic areas of the cardiovascular system, might be the mechanism that triggers the irreversible fall of blood pressure.

The disappearance of the kinin precursor from blood has already been demonstrated in different modalities of shock in our laboratories[14, 27] and in others,[28, 29] and bradykinin has also been recovered from blood of animals submitted to peptone and anaphylactic shock.[3, 30] Kinins also have been estimated in blood of dogs at 3 or 4 min after kallikrein injection, by Binia and co-workers.[31] Despite the difference in doses and in methods employed, the amount of kinin recovered by these authors, 3–4 min after enzyme injection, is of the order of magnitude to be expected by our experiments.

It is interesting to compare some of the effects of the enzymes used in this study on the hydrolysis of BKG *in vivo* and *in vitro*. Thus, *in vitro*, Nagarse is more active upon casein than trypsin or the kallikrein preparation, and it is intermediate in its ability to release bradykinin from Horton's and Van Arman's substrates. On the other hand, Nagarse, when injected into the circulation, is more active than trypsin in depleting BKG. The data on BKG levels, given in units per milliliter plasma, were not corrected for the hematocrit values. The BKG fall, which occurred after the injection of higher doses of enzymes, is proably still more pronounced than the results presented. Nagarse and trypsin activate BAMe esterases of plasma and although no correlation between esterolytic activity and BKG levels of plasma could be found, the possibility that these enzymes act indirectly by activation of plasma proteases *in vivo* cannot be excluded. Kallikrein does not activate BAMe esterase *in vivo* and, although the preparation used in our experiments was not pure, kallikrein was almost as potent as Nagarse in destroying BKG *in vivo*.

As for the alterations of clotting time, with the exception of kallikrein-injected animals, where this parameter remains unaltered or slightly diminished, all animals

showed an increase in clotting time, which suggests that kallikrein acts more specifically upon BKG and has little effect on other substrates, such as clotting factors and BAMe esterase precursors. Trypsin and Nagarse probably exhibit a less especific and more extensive proteolytic action. Prothrombinolytic, fibrinogenolytic, and heparin-releasing activity of trypsin[32] are sufficiently known, and it is probable that analogous phenomena occur after Nagarse injection.

Histamine possibly plays an important role in the genesis of the hypotension caused by trypsin. Even after injection of a small dose of this enzyme (3 mg/kg), the amine was detected in the blood. The irreversible shock, however, is initiated only after considerable destruction of BKG has occurred. Nagarse and kallikrein apparently do not affect the histamine stores in the dog. On the other hand, it is possible that, besides histamine, kinin peptides might play some role in the hypotension after the administration of low doses of trypsin (3 and 6 mg/kg), an event which may also occur after the treatment with Nagarse or kallikrein. Thus, a small percentual reduction in BKG levels of the order of 5% to 10%, difficult to be detected by our method of determination, is more than sufficient to account for the liberation of enough polypeptide to elicit considerable effects on the arterial blood pressure. Finally, an additional possibility is that the enzymes themselves may have a direct action upon the smooth-muscle fibres.[6, 12, 33, 34]

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