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Liberation of a bradykinin-like substance in the circulating blood of dogs by trypsin, chymotrypsin and nagarse

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1. Using the blood-bathed organ technique with cat jejunum as the test organ, it was shown that kinin was regularly released by infusions of trypsin, chymotrypsin and nagarse into the blood superfusing the tissue or into the jugular vein of dogs.

2. Intravenous injections of the three enzymes, in doses which did not change plasma bradykininogen (BKG) level, induced generation of kinin in the circulating blood.

3. Nagarse in kinin-liberating concentrations did not liberate angiotensin in the circulating blood.

4. The responses of the cat jejunum strip, and the systemic hypotension induced by the three enzymes, were enhanced by treatment of the dog with a bradykinin potentiator.

5. Injection of the enzymes in the blood perfused hind leg of the dog caused a fall in the vascular resistance. In this preparation, bradykinin potentiating factor (BPF) potentiated trypsin, but not the effects of chymotrypsin and nagarse injections.

6. It is suggested that the blood pressure lowering effect of low doses of trypsin is due mainly to kinin release. Chymotrypsin and nagarse, in addition to having a direct action on the vascular smooth muscles, also have indirect hypotensive effects due to the liberation of kinin.

Rocha e Silva, Beraldo & Rosenfeld (1949) coined the name bradykinin for the hypotensive peptides obtained by incubation of plasma with either *Bothrops jararaca* venom or trypsin. Increasing intravenous doses of trypsin produce haemodynamic effects ranging from a transitory hypotension to an irreversible shock (Rocha e Silva, 1939). Histamine and bradykinin might participate in these trypsin effects (Corrado, Reis, Carvalho & Diniz, 1966), but to detect a definite change in plasma bradykininogen, doses of trypsin higher than 6 mg/kg had to be used (Diniz, Carvalho, Reis & Corrado, 1967). Rocha e Silva, Reis & Ferreira (1967) detected plasma kinin when chymotrypsin was incubated with guinea-pig plasma (in the presence of kininase inhibiting agents) but not with dog, rat, rabbit or human plasma. Nagarse,

a protease obtained from *Bacillus subtilis*, has been shown (Prado, Prado & Jurkiewicz, 1964) to be a kinin releasing enzyme ; when given intravenously to rats and dogs it causes hypotension. Nevertheless, only doses of nagarse higher than 6 mg/kg induced a consistent reduction in plasma bradykininogen and the appearance of bradykinin in the circulation of the dog (Corrado *et al.*, 1966).

Recently, Huggins & Thampi (1967), showed that nagarse liberated angiotensin-like material from heat-treated horse plasma and questioned the classification of this enzyme as a kininogenin.

In the present paper, the ability of small doses of trypsin, chymotrypsin and nagarse to liberate kinins is evaluated. The participation of kinin in the blood pressure lowering effects of these enzymes was inferred from their potentiation by dimercaptopropanol (BAL) (Ferreira & Rocha e Silva, 1962) and by the bradykinin potentiating factor (BPF) (Ferreira, 1965 ; Ferreira & Rocha e Silva, 1965). The actions of the enzymes were studied before and during an infusion of BPF using a blood-perfused hind leg. Whether kinin-releasing amounts of nagarse could liberate angiotensin in the circulating blood of the dog was also investigated.

Methods

Liberation of kinin in dog's blood by trypsin, chymotrypsin and nagarse

Blood-bathed organ experiments. Forty dogs, male and female, weighing 6–15 kg were used. They were anaesthetized with pentobarbitone (30 mg/kg, intravenously) and the trachea was cannulated to facilitate respiration. At the beginning of the superfusion of the assay tissues (see below), heparin (1,000 i.u./kg) was given intravenously.

Using the blood-bathed organ technique of Vane (1964) and perfusing the organs in cascade by the same stream of blood maintained at 37° C, the cat jejunum strip was used to detect the bradykinin-like material (see Ferreira & Vane, 1967) and rat colon to detect angiotensin (see Regoli & Vane, 1964). The enzymes, trypsin, chymotrypsin or nagarse were infused (i) directly into the externalized circulation or (ii) indirectly by intravenous infusion into the jugular vein. Blood was sampled continuously from a cannula in the carotid artery and pumped by a roller pump at 10 ml./min over the test organs. The blood then collected in a small reservoir and returned to the animal by gravity through a cannulated jugular vein. By altering the point at which the enzymes were infused into the external circuit, the duration of their contact with the blood before reaching the test organ could be varied from almost zero (infusing directly upon the assay tissue) up to a period of 20 sec. Infusions of bradykinin were made directly to the external circuit at a point to give 10 sec contact with the blood. The movements of the assay tissues were recorded on a smoked kymograph with auxotonic levers (Paton, 1957) giving a 16 : 1 magnification under an initial load of 1–4 g.

The systemic blood pressure was measured in the femoral artery by means of a mercury manometer and recorded on a kymograph. The hypotension was measured by calculating the area of the blood pressure fall in the 5 min following administration of the enzymes (Ferreira & Rocha e Silva, 1965).

Bradykininogen. The bradykininogen (BKG) concentrations of arterial blood samples were determined both before and during the enzyme-induced hypotensions,

by adding trypsin to them and measuring the kinin released according to the method of Diniz, Carvalho, Ryan & Rocha e Silva, 1961 (see also Diniz & Carvalho, 1963).

Potentiation of the enzyme effects

The action of BPF (0·5–1 mg/kg) or dimercaptopropanol (5–10 mg/kg), previously shown to potentiate the hypotension induced by bradykinin and to inhibit blood kininases (Ferreira, Corrado & Rocha e Silva, 1962 ; Erdös & Wohler, 1963 ; Ferreira, 1966), was studied on the effects of the enzymes. All agents were injected in the jugular vein.

Measurement of changes in resistance to blood flow in the intact hind limb following close arterial injection of the enzymes (trypsin, chymotrypsin and nagarse)

Ten dogs were anaesthetized with pentobarbitone (30 mg/kg), the femoral artery was sectioned and a cannula inserted in the central cut end directed the blood through a roller pump before returning it to the animal via a cannula in the distal cut end. The pump maintained a constant flow rate which was adjusted at the beginning of the experiment to give an initial perfusion pressure equal to the systemic arterial blood pressure. Enzymes were injected into the distal cannula

TABLE 1. *Liberation of kinin by trypsin, chymotrypsin and nagarse infused* (a) *into the external circulation, and* (b) *intravenously*

(*a*) External circuit

Trypsin (μg/ml.)	Chymotrypsin (μg/ml.)	Nagarse (μg/ml.)	Bradykinin equivalent (ng/ml.)
5 (I)	20 (V)		1
10 (II)	10 (VI)	10 (XVI)	2
	10 (VII)		2
	20 (XI)		2
	20 (XII)		2
5 (III)	5 (III)	10 (XVII)	4
10 (IV)	10 (IV)	10 (XVIII)	4
10 (V)	10 (VI)	10 (XIX)	4
	10 (VIII)		4
	10 (IX)		4
	20 (XIII)		4
	20 (XIV)		4
	40 (XV)	10 (XX)	8

(*b*) Intravenous infusion

Trypsin (mg/min)	Chymotrypsin (mg/min)	Nagarse (mg/min)	Bradykinin equivalent (ng/ml.)
2 (XXIII)	4 (II)	4 (XXIII)	1
1 (XXIV)		2 (XXIV)	1
1·5 (XXI)	6 (XXI)		2
2 (II)	6 (XIII)		2
2 (XXII)	2 (VII)		2
4 (XIII)	1 (XXV)		4
	2 (XXVI)		4
	2 (XXVII)		4
	2 (XXVIII)		8

The enzymes were infused (*a*) into the external circulation of the blood cascade system or into the jugular vein (*b*), as described in the text. In (*a*) the final concentration of the enzymes (μg/ml.) is given and in (*b*) the rate of their intravenous infusion (mg/min). The bradykinin equivalent is the concentration of the peptide in the blood bathing the assay tissues which produced a response matching that elicited by the infusion of enzyme. The roman numerals indicate the experiment number.

and changes in the perfusion pressure were measured by means of a mercury manometer connected to a side arm of the same cannula and recorded on a kymograph. In some experiments, the proximal cannula was lengthened by a piece of siliconed tubing, kept warm at 37° C in a water bath. In this way, the enzymes could be maintained in contact with the blood for up to 1 min before reaching the vessels of the hind limb.

Drugs

Angiotensin (Ciba); bradykinin (BRS, Sandoz and Parke Davis); alpha-chymotrypsin 3 × crystallized (Mann Research Lab.); dimercaptopropanol-BAL (Mann Research Lab.); nagarse (Teikoku Chemical Ind. Co. Ltd.); trypsin 2 × crystallized (Mann Research Lab.).

Results

Liberation of kinin

Enzyme infusions into the external circulation

Cat jejunum superfused with Krebs solution. This preparation was sensitive to bradykinin (1–2 ng) causing it to contract. Trypsin, chymotrypsin and nagarse in concentrations up to 80 μg/ml., however, did not elicit a response. The enzymes could not reach such high concentrations in the blood bathing the tissues during the intravenous infusions.

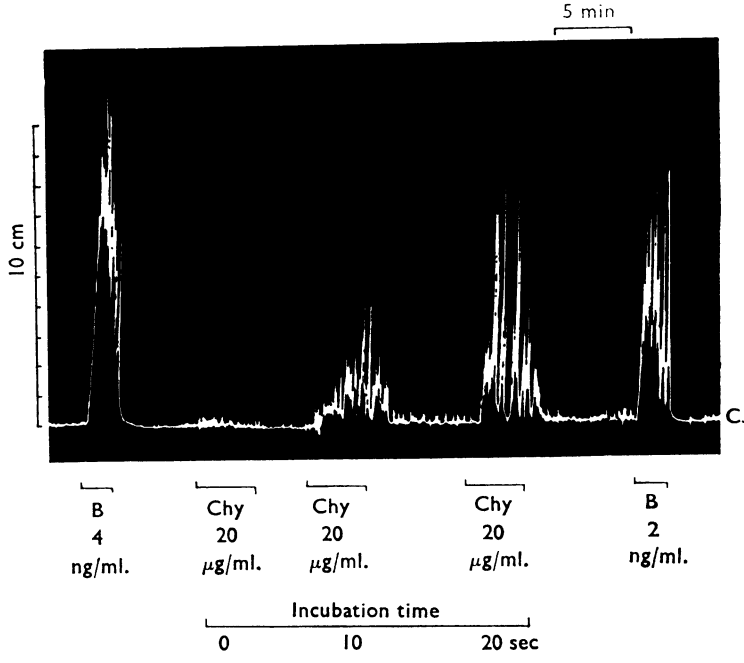


FIG. 1. Influence of the time of contact (incubation) of chymotrypsin with blood on the liberation of kinin. The infusion of chymotrypsin (Chy) at three points in the external circulation induced bigger contractions in the cat jejunum strip (CJ) commensurate with the increase in incubation time. B, Bradykinin.

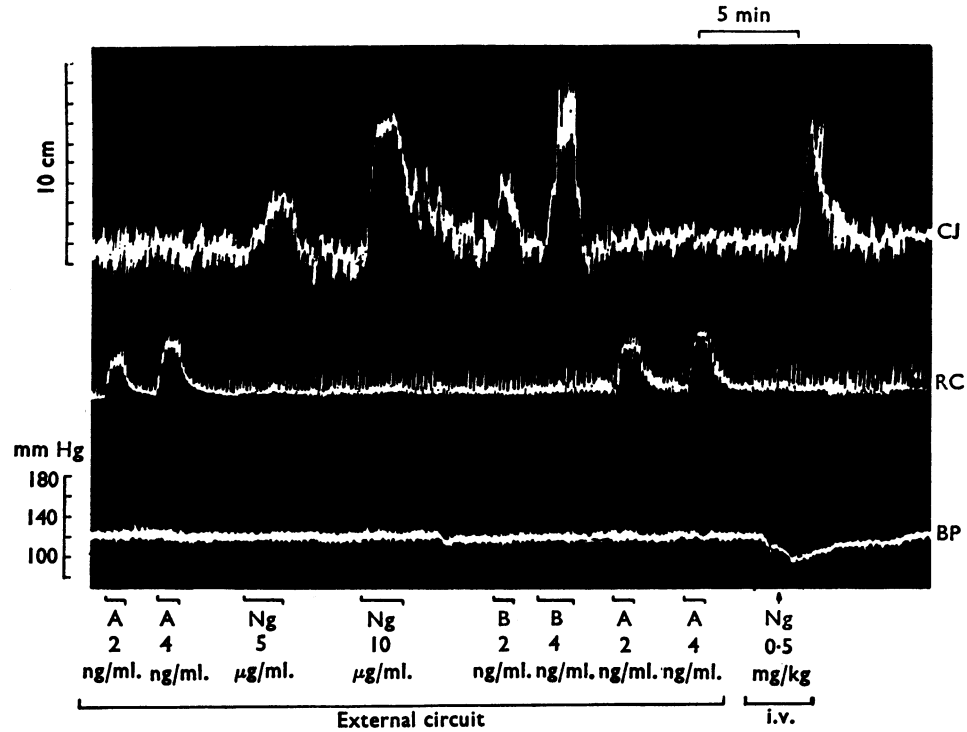


FIG. 2. Liberation of kinin by nagarse. The infusion of nagarse (Ng) into the external circulation produced contractions of the cat jejunum strip (CJ) without affecting the rat colon (RC). Intravenous injection (i.v.) (arrow) gave a similar result. Dog (7 kg). BP, Blood pressure ; A, angiotensin ; B, bradykinin. Experiment XIX, Table 1.

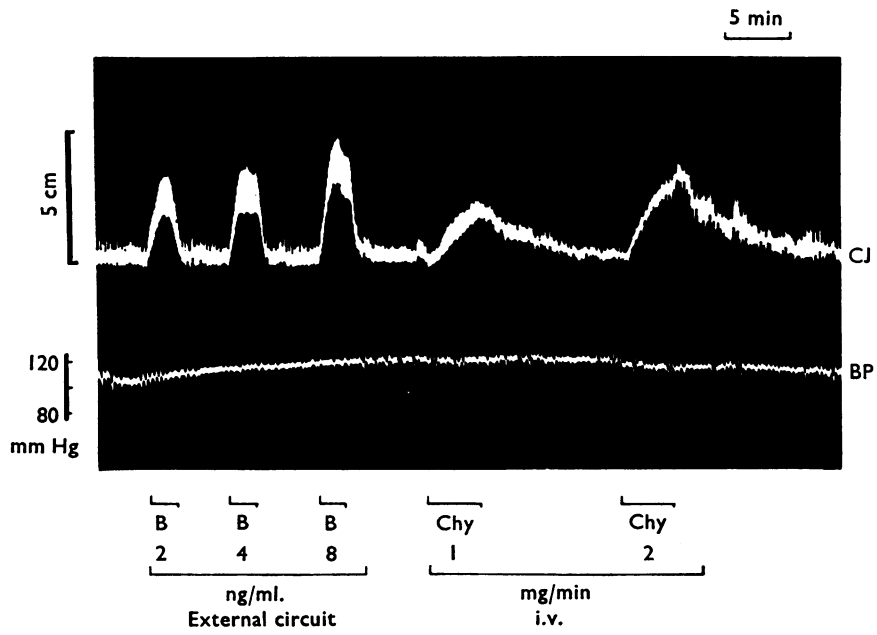


FIG. 3. Liberation of kinin by intravenous infusion of chymotrypsin in the dog (10 kg). Note that intravenous (i.v.) infusion of chymotrypsin (Chy) liberated active material on the cat jejunum strip (CJ) without affecting the blood pressure (BP). B, Bradykinin. Experiment XXVII, Table 1.

Cat jejunum superfused with the dog's blood. When the enzymes were infused into the external circulation, concentrations of 5–20 μg/ml. regularly induced contractions (Table 1). Moreover, increasing the duration of contact (incubation time) of the enzyme with blood increased the response of the assay organ. For example, when chymotrypsin was infused close to the tissues or at points in the external circulation so that it mixed with blood for periods of 0, 10 and 20 sec respectively, the responses were greater as the contact time increased (Fig. 1). Increasing the concentration of the enzymes in the blood bathing the tissues also increased the responses, but the amount of kinin released by the same concentration varied from animal to animal (Fig. 2 and Table 1). No sensitization of the assay tissues after infusions of enzymes was observed.

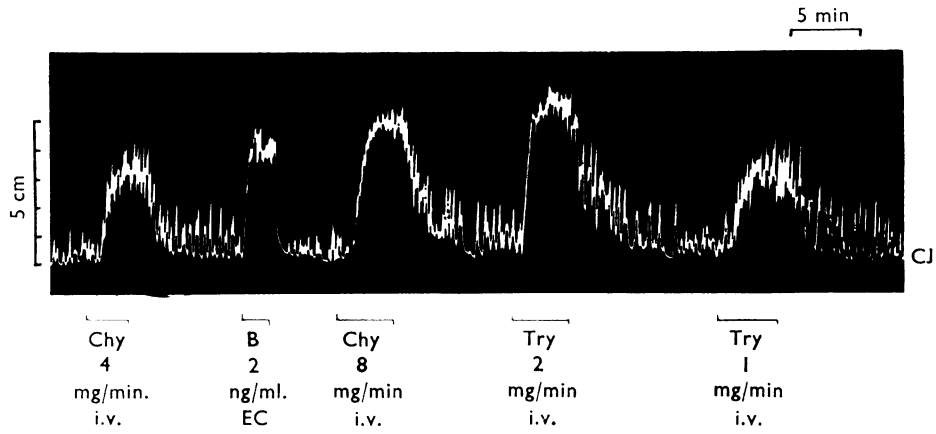


FIG. 4. Liberation of kinin by intravenous infusion of trypsin (Try) and chymotrypsin (Chy) in the dog (8 kg). Note that the response to bradykinin (2 ng/ml.) was intermediate between the responses elicited by 4 and 8 mg/min of chymotrypsin and 1 and 2 mg/min of trypsin. CJ, Cat jejunum strip. Experiment XXI. Table 1. EC, External circuit i.v., intravenously.

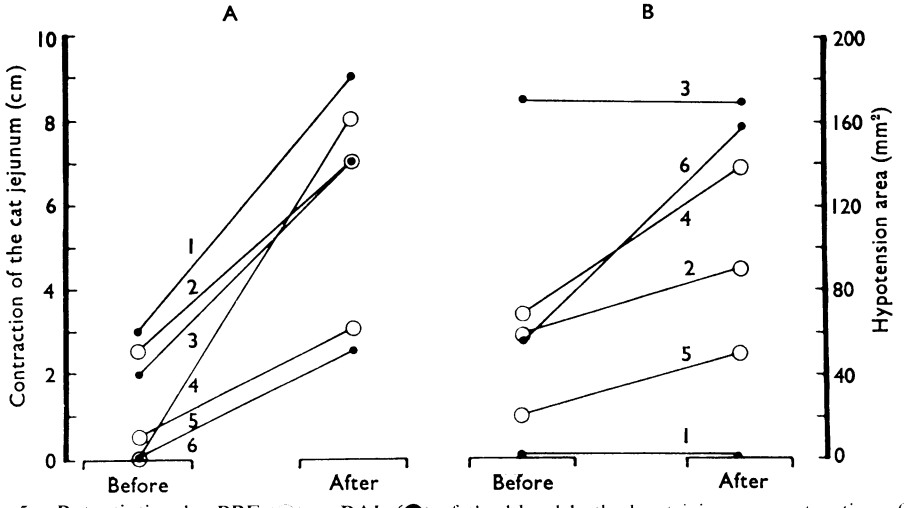


FIG. 5. Potentiation by BPF (○) or BAL (●) of the blood bathed cat jejunum contractions (A) and hypotension in the dog (B) induced by chymotrypsin injections. The doses of the agents were: chymotrypsin 2 mg/kg; BPF, 1 mg/kg; BAL, 10 mg/kg. The figures indicate the results in the same experimental animals.

In three experiments, the kinin-releasing activity of trypsin and chymotrypsin was compared by infusions in the external circuit. Similar potencies were obtained in experiments III and IV in which either enzyme (5 and 10 μg/ml.) liberated material equivalent to a bradykinin concentration of 4 ng/ml. Trypsin was more effective than chymotrypsin in dog V ; trypsin (10 μg/ml.) and chymotrypsin (20 μg/ml.) liberated the equivalent of 4 and 1 ng/ml. of bradykinin respectively (Table 1a).

In five experiments, nagarse (10 μg/ml.) liberated a bradykinin-like substance in concentrations ranging from 2 to 8 ng/ml. (bradykinin equivalent) without evidence of release of angiotensin. A record of one of these experiments is shown in Fig. 2. An infusion of nagarse into the external circuit to give a concentration of 10 μg/ml. induced a response of the cat jejunum equivalent to 4 ng/ml. of bradykinin. Although the rat colon was also sensitive to angiotensin, 4 ng/ml. no response was observed. The same result was obtained when nagarse was injected intravenously.

Intravenous infusions

Table 1b shows the results of intravenous infusions of the enzymes in eleven dogs. Trypsin, chymotrypsin and nagarse, in doses varying from 1·5 to 6 mg/min, released kinin into the circulating blood. In three experiments (not listed) intravenous infusions of the enzymes did not liberate detectable amounts of kinin, though the cat jejunum was sensitive to 2–4 ng/ml. of the peptide. In these experiments, however, injections of the enzymes into the external circulation contracted the assay tissues.

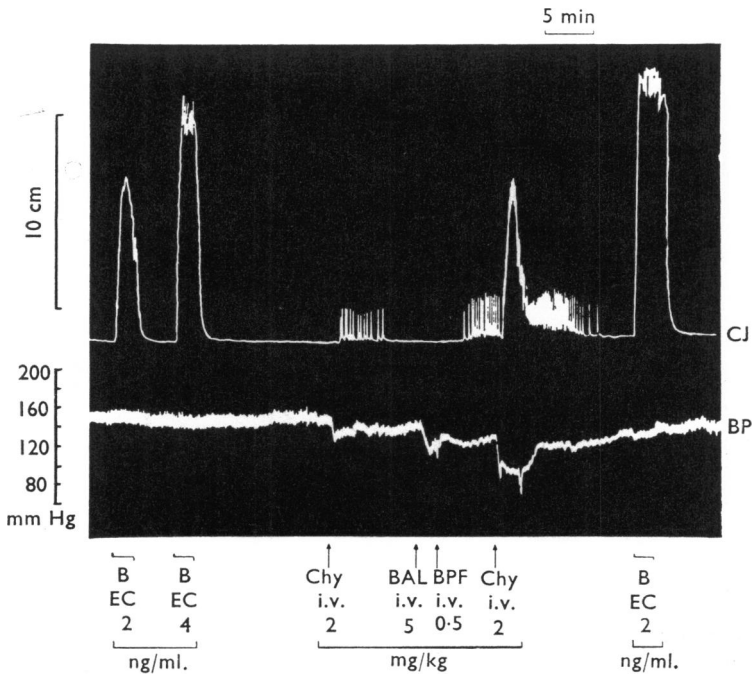


FIG. 6. Potentiation by BAL and BPF of the liberation of kinin induced by chymotrypsin. CJ, Cat jejunum strip ; BP, blood pressure. Bradykinin (B) was infused in the external circuit (EC). The injections (arrows) of chymotrypsin (Chy) and of bradykinin potentiators were given intravenously (i.v.). Dog, 12 kg.

The kinin releasing activity of trypsin infused intravenously was compared with that of chymotrypsin and nagarse. Trypsin was more potent than chymotrypsin (dog II, XIII, XXI) or nagarse (dog XXIII and XXIV, Table Ib).

Figure 3 shows that chymotrypsin infused into the circulating blood of the dog at the rate of 1 to 2 mg/min may also release kinin, though in quantities insufficient to lower the systemic blood pressure. A similar result was obtained in seven other experiments. A comparison between trypsin and chymotrypsin infusions is shown in Fig. 4; chymotrypsin (4 mg/min) gave a response equivalent to 1 mg/min of trypsin.

The intensity of the hypotension induced by infusions of trypsin and nagarse (1 to 4 mg/min) varied from dog to dog and was not related to the amount of kinin detected in the circulating blood.

Potentiation of the enzyme effects

Figure 5 shows that the contractions of the assay tissues induced by chymotrypsin injections (1 mg/kg) were enhanced after the treatment of the animal with a bradykinin potentiator (BAL or BPF). These substances also increased the hypotension induced by chymotrypsin in four out of six experiments. In the two experiments

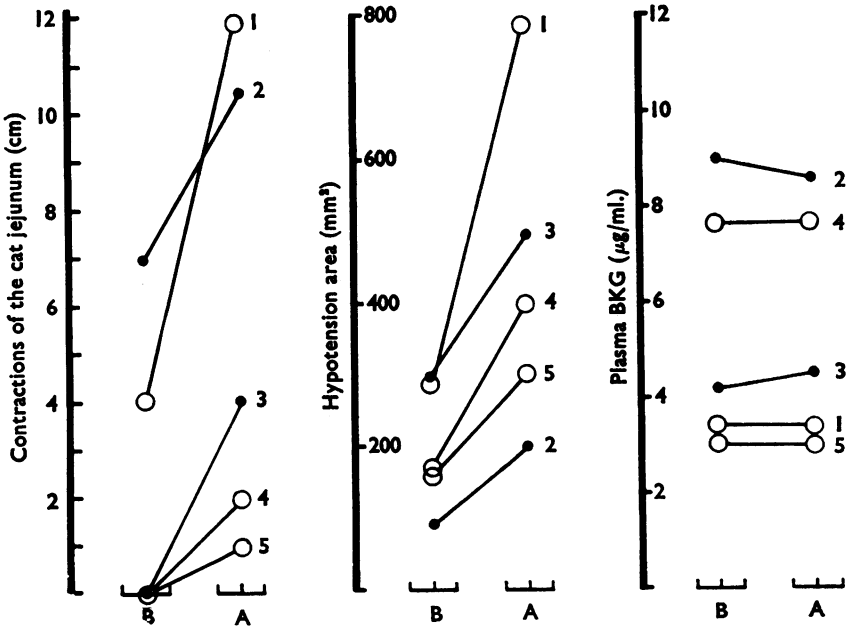
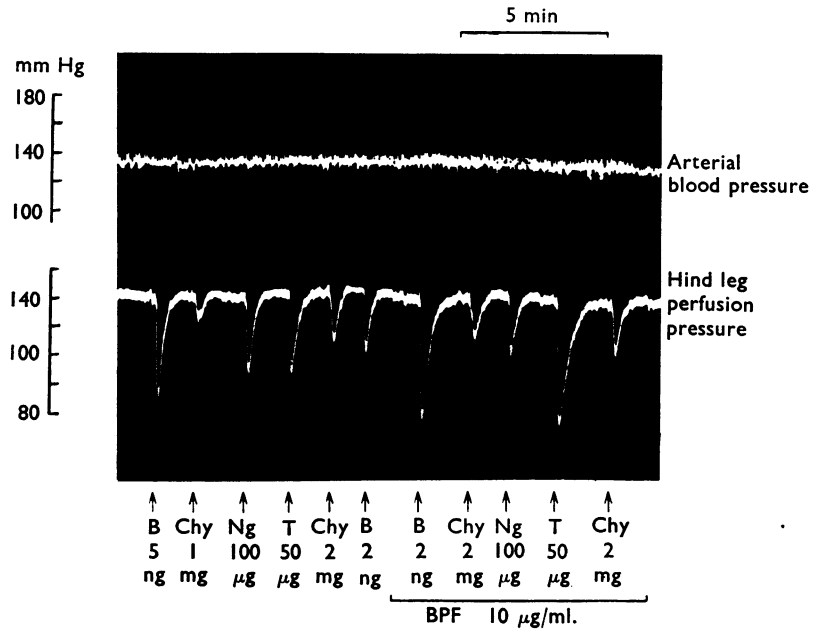


FIG. 7. Variation of bradykininogen (BKG) concentration in plasma and potentiation of the blood-bathed cat jejunum responses and hypotension induced by intravenous injections of trypsin and nagarse by BPF. The points show the results of the enzyme injections in five experimental animals before (B) and after (A) the treatment with BPF (1 mg/kg). The doses of trypsin (○) and nagarse (●) were 0·4 mg/kg and 1 mg/kg respectively. Left panel presents the responses of the blood-bathed cat jejunum measured in cm. The middle panel shows the variation of systemic blood pressure expressed in mm². The right panel indicates the measurements of plasma bradykininogen (BKG) in µg of bradykinin/ml. of blood sampled before (B) and after (A) the enzyme injection, when the maximum effect was observed.

in which there was no potentiation, either the enzyme did not promote hypotension (experiment 1), or the fall of blood pressure was very marked before the administration of the potentiator (experiment 3).



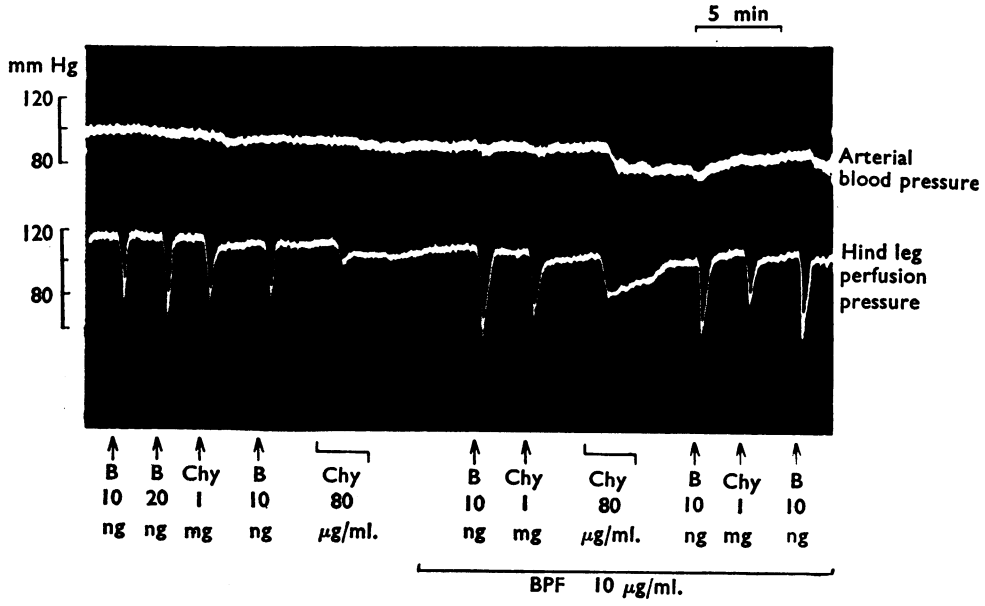


FIG. 8. Action of BPF on the effects of trypsin, chymotrypsin and nagarse on the perfusion pressure of the hind leg of the dog. The tracings were obtained in two different experimental animals. The injections of bradykinin (B), chymotrypsin (chy) nagarse (Ng) and trypsin (T) indicated by the arrows were made into the blood near the distal portion of the femoral artery. The infusions (——) were made in a lengthened external circuit which increased the contact with the blood for 40 sec. The perfusion flow was 50 ml./min in both experiments.

The potentiation of the effects of chymotrypsin is illustrated in a dog treated with BAL and BPF (Fig. 6). The effectiveness of the potentiators is shown by the increase in the response of the cat jejunum to the control infusion of bradykinin. The hypotensive action of chymotrypsin was also potentiated.

The hypotension and the cat jejunum contraction induced by intravenous injections of trypsin (0·4 mg/kg) and nagarse (1 mg/kg) were also potentiated by BPF (Fig. 7). This figure also shows that before and during the hypotension produced by the enzymes there was negligible variation in the plasma BKG. Similar results were obtained in two experiments with chymotrypsin.

Measurement of changes in resistance to blood flow in the intact hind limb following close arterial injection of the enzymes

Trypsin (25–100 μg), chymotrypsin (0·4–1 mg) and nagarse (120–500 μg) lowered the resistance of the vessels of the hind leg of the dog perfused at constant flow. An infusion of BPF, which was effective in enhancing vasodilation induced by bradykinin, also potentiated that due to trypsin but not that due to chymotrypsin or nagarse (Fig. 8, upper panel). When chymotrypsin was infused (not injected) into the external circulation, allowing contact of the enzyme with blood for an extra period of 40 sec, BPF potentiated the fall in vascular resistance (Fig. 8, lower panel). Note that the injections of chymotrypsin were not potentiated.

Discussion

Shock produced by proteolytic enzymes, such as trypsin, chymotrypsin or nagarse might constitute models for other kinds of shock such as that produced by anaphylaxis and by endotoxins. The demonstration that the shock is due, at least in part, to the release of kinins depends on detection of free kinins in the circulating blood. The methods currently used to extract the active materials from samples of blood before and during shock are time consuming and may be subject to error due to the release of kinin during the processing of the samples.

The dynamic bioassay method of Vane (1964) was used to detect bradykinin. This method uses tissues which are highly sensitive to the agents which are expected to be released.

A direct action of the enzymes on the assay tissues could be ruled out because trypsin, chymotrypsin and nagarse in concentrations at least four or eight times larger than those which induced responses after contact with blood did not affect them when bathed with Krebs solution. Furthermore, the responses of the cat jejunum strip to the same concentration of enzymes increased with increase in the time of contact of the enzymes with blood. In addition treatment of the animal with a bradykinin potentiator (BPF or BAL) enhanced the responses of the cat jejunum strip to the same intravenous injection of the enzymes.

The sensitization to bradykinin of some isolated smooth muscle preparations by trypsin or chymotrypsin, as first described by Edery (1964), was not observed in the blood-bathed cat jejunum preparation in the conditions of our experiments.

Trypsin, chymotrypsin and nagarse infused either into the external circulation or intravenously into the intact animal liberated kinin. The results of the " external circulation " experiments showed that a concentration of trypsin, chymotrypsin and nagarse of the order of 10 μg/ml. in the blood bathing the tissues was able to release

the equivalent of 1–4 ng/ml. of bradykinin. When the enzymes were infused intravenously in doses ranging from 1 to 6 mg/min, a concentration of kinin of the order of 1 to 4 ng/ml. was observed in the circulating blood. This indicates that the concentration of enzymes in the arterial blood during the intravenous infusions reached the values attained when they were infused into the external circulation. As mentioned above, concentrations of enzymes four to eight times larger did not induce responses in the Krebs-bathed cat jejunum. In three experiments, the enzymes infused intravenously did not produce detectable amounts of kinin. Since injections in the external circulation did induce responses, the ineffectiveness of the intravenous infusions were probably due to a dilution effect which prevented the enzymes reaching a kinin liberating concentration in the arterial blood of the animal.

The ability of trypsin to generate bradykinin when incubated with dog's plasma *in vitro* is well established (Rocha e Silva *et al.*, 1949 ; Rocha e Silva *et al.*, 1967). *In vivo*, it is possible to detect circulating bradykinin only with doses higher than 12 mg/kg (Corrado *et al.*, 1966). Our results confirm the *in vitro* experiments and also show that doses of trypsin (0·4 mg/kg) which did not modify the plasma BKG concentration caused a detectable release of kinin into the circulating blood. The potentiation by BPF of the effects of injections of trypsin on the systemic arterial blood pressure, and on the peripheral resistance in the perfused hind leg, suggest that they were due mainly to kinin liberation. The results did not, however, exclude the possibility of a direct action of trypsin on the vascular smooth muscle or the concomitant liberation of other mediators, such as histamine (Dragstedt & Rocha e Silva, 1941 ; Corrado *et al.*, 1966).

Recently we have shown that chymotrypsin liberates kinin when incubated with guinea-pig plasma *in vitro*, but not with the dog, rat, rabbit, and human plasma (Rocha e Silva *et al.*, 1967). In the present paper, it was shown that chymotrypsin liberates kinin into the circulating blood and that its haemodynamic effects might be partially due to bradykinin release. The failure to detect the liberation of kinin from dog's plasma by chymotrypsin *in vitro* might be due to a slow release rate counteracted by a quick destruction in this system, coupled with a less sensitive assay procedure. That chymotrypsin has a slower kinin releasing activity compared with trypsin can be inferred from the hind leg perfusion experiments. In the few seconds which were necessary for the enzymes to go from the injection point to the vascular region, trypsin but not chymotrypsin liberated kinin, but if the latter enzyme was in contact with the blood for an extra 40 sec before it reached the vascular bed kinin was liberated.

Prado *et al.* (1964) classified nagarse as a kininogenin on the grounds that it liberated a bradykinin-like substance when it was incubated with heated horse plasma. Huggins & Thampi (1967), utilizing the same substrate, questioned the classification of nagarse as a kininogenin because they were also able to show a liberation of angiotensin-like material. Our results indicate that kinin generating doses of nagarse do not liberate detectable amounts of angiotensin in the circulating blood of dogs.

Corrado *et al.* (1966) detected circulating kinin only after administration of nagarse to dogs in doses of 6 mg/kg which definitely promoted a fall of plasma BKG. Our results show kinin liberation with a dose of 1 mg/kg which induced no BKG variation.

BPF did not enhance the effects of chymotrypsin and nagarse given directly into the hind leg circulation, but potentiated the responses of the cat jejunum strip when the enzymes were given intravenously. BPF also potentiated the fall of systemic blood pressure induced by injections of chymotrypsin and nagarse. Consequently the hypotensive effects of nagarse and chymotrypsin can be interpreted as being at least in part the result of a kinin release.

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