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Inhibitory effects of aprotinin on kallikrein and kininases in dog's blood

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Summary

1. The blood-bathed organ technique was used in dogs to estimate kinin generation in the blood. Strips of cat jejunum were used as assay tissues.

2. Infusions of kallikrein at 0·5–8 mU/ml into the blood in the extracorporeal circuit led to a generation of kinin at 0·6–10 ng/ml. The kinin generated was at the same concentration after incubation of kallikrein with blood for 15 s or 120 seconds. Intravenous infusions of kallikrein at (8–125 mU/kg)/min led to similar blood concentrations of kinin. These infusions induced substantial falls in blood pressure.

3. Aprotinin inhibited the generation of kinin by kallikrein, but the concentration needed *in vivo* was 20,000 times higher than would be expected from the definition of the units.

4. After intravenous injection of large doses of aprotinin, the kallikrein-inhibiting activity passed off within 40–60 minutes. At the same time, there was a gradual reduction in kininase activity, so that the half life of bradykinin in blood increased from a mean of 13 s to 40 seconds. This effect reached a maximum 1–3 h after injection of aprotinin.

5. It is suggested that a metabolite of aprotinin is responsible for the kininase inhibition and that this effect may limit the usefulness of aprotinin in man.

Introduction

Aprotinin (Trasylol), a kallikrein inhibitor isolated from bovine lungs, is a basic peptide containing fifty-eight amino-acids. It inhibits enzymes of different substrate specificity, such as trypsin, chymotrypsin, various kallikreins, plasmin and some undefined cell proteinases (Trautschold, Werle & Zickgraf-Rüdel, 1967). Its activity is expressed in terms of kallikrein inhibitor units (KIU) and 1 KIU (0·14–0·15 μg) is the amount of inhibitor which produces 50% inhibition of 2 kallikrein units (KU) under standard conditions *in vitro* (incubation for 30 min at pH 8 and 37° C; Werle & Kaufmann-Boetsch, 1960).

In dogs intravenous aprotinin is tolerated in doses of up to 10^6 KIU/kg. Aprotinin is less well tolerated in rats, especially when they have undergone surgery. High doses in rats may lead to fatal 'shock-like reactions' (see Trautschold *et al.*, 1967). Occasionally, repeated high doses in patients with a history of allergy have led to

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'signs of intolerance' (see Trautschold *et al.*, 1967), probably due to histamine release (Haberland, 1967).

We have studied the interactions between kallikrein and aprotinin in the circulation of the dog. Because aprotinin is a peptidase inhibitor we have also investigated its activity on the enzymes which inactivate bradykinin in blood and lungs.

Methods

The blood-bathed organ technique (Vane, 1964, 1969) was used to assay bradykinin in the blood stream. Cat jejunum was opened longitudinally and stored in Krebs solution at 4° C for 2–7 days. On the day of the experiment, longitudinal strips (about 2 mm wide and 6–8 cm long) were cut as previously described (Ferreira & Vane, 1967a). Two strips of cat jejunum were superfused at 10 ml/min in cascade with Krebs solution at 37° C whilst the dog was being prepared. In some experiments, two banks of assay tissues were used, with two strips of cat jejunum in each. Longitudinal movements of the strips were detected by auxotonic levers (Paton, 1957) of 16:1 magnification writing on smoked paper on a kymograph. The initial load on the tissues was 1–2 g.

Seventeen mongrel dogs of either sex weighing 8–22 kg were anaesthetized with halothane. Anaesthesia was then maintained with chloralose (100 mg/kg i.v.) and supplemented when necessary with pentobarbitone (5–10 mg/kg, subcutaneously or intravenously). The trachea was cannulated and the lungs were ventilated mechanically. Polyethylene cannulae were tied into a femoral or carotid artery and a catheter was passed down the jugular vein into the right atrium for removal of blood. A femoral vein was cannulated for replacement of the blood.

When the operative procedure was over, heparin (1,000 IU/kg) was injected intravenously into the dog and the strips of cat jejunum were superfused with arterial blood instead of Krebs solution. In experiments with two banks of assay tissues, blood from the femoral artery was used to superfuse one and venous blood, taken from a catheter introduced into the right atrium through a jugular vein, was used for the other. The rate of flow was kept constant at 10 ml/min by a two-channel roller pump. After superfusion of the tissues, the blood was collected in a reservoir and returned intravenously to the dog by gravity.

Infusions were made either directly into the blood bathing the assay tissues (IBB) or intravenously into the dog for sufficient time (4 min) to give a plateau response of the isolated organs. In some experiments the half life of bradykinin in the circulating blood was determined. An incubation circuit (Ferreira & Vane, 1967b) was used to prolong the contact of the infused substances with the blood in the extracorporeal circulation. This consisted of a coil of silicone tubing interposed between the animal and the blood-bathed organs, kept at 37° C by a water bath. The coil contained 20 ml blood so that at a flow rate of 10 ml/min infusions of drugs made into the beginning of the coil were in contact with the blood for 2 min before reaching the assay tissues. Shorter contact times were obtained by infusing the drug at marked points in the coil.

Guinea-pig isolated lungs were perfused through the pulmonary artery with Krebs solution, as described by Piper & Vane (1969a, b). Eight guinea-pigs of either sex weighing 350–450 g were used. In some experiments aprotinin was infused into

the Krebs solution perfusing the isolated lungs ; in others the guinea-pig was anaesthetized with pentobarbitone sodium (30 mg/kg i.p.) and aprotinin was injected intravenously 30, 60 or 90 min before removing the lungs. Lobes of lungs isolated from dogs were perfused with Krebs solution containing 3% dextran.

In all lung perfusion experiments the perfusate from the lungs dripped into a funnel from which it immediately superfused the strips of cat isolated jejunum. By infusing bradykinin either into the pulmonary artery or into the perfusate from the lungs, bradykinin inactivation in the lungs could be estimated.

The substances used were synthetic bradykinin (Parke-Davis), pig pancreatic kallikrein (Glumorin, Bayer) and aprotinin (Trasylol A, Bayer). The composition of the Krebs solution (mM) was: NaCl, 118 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·17 ; glucose, 5·6 ; $NaHCO_3$, 2·1. It was gassed with 95% oxygen and 5% CO_2.

Results

When superfused with Krebs solution the cat jejunum strips were sensitive to bradykinin in concentrations of 1–5 ng/ml. Neither kallikrein (1–10 mU/ml) nor aprotinin (5–20 KIU/ml) contracted the cat jejunum or changed its response to

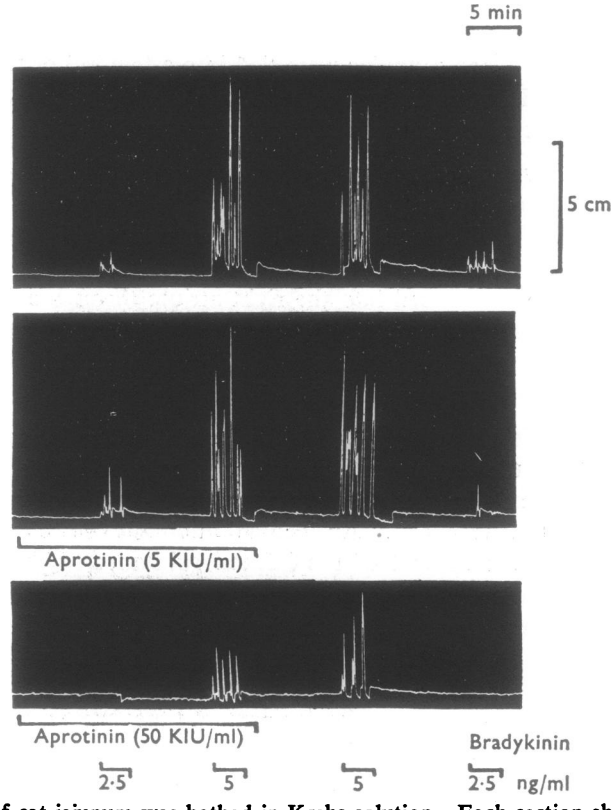

FIG. 1. A strip of cat jejunum was bathed in Krebs solution. Each section shows contractions induced by bradykinin (2·5, 5, 5 and 2·5 ng/ml). Aprotinin (5 KIU/ml) had no effect on the contractions induced by bradykinin, but aprotinin (50 KIU/ml) decreased them. Time 5 min; vertical scale 5 cm.

bradykinin. In higher doses, aprotinin (25–50 KIU/ml) reduced the contractions of the cat jejunum induced by bradykinin (Fig. 1).

When bathed in blood, cat jejunum strips were sensitive to bradykinin (2–10 ng/ml) and infusions of kallikrein into the extracorporeal circuit now caused contractions of the tissue through generation of a kinin in the blood. Kallikrein (2–8 mU/ml) was infused into the incubating circuit so that it was in contact with the blood for 15, 30, 60 and 120 s before reaching the assay organs (two experiments). The contractions of the strips of cat jejunum showed that the same concentration of kinin was reaching the assay tissues whether the kallikrein was incubated with the blood for 15 or 120 s (Fig. 2). Thus an equilibrium between kinin generation and inactivation must be established in the blood in less than 15 seconds.

Table 1 compares the rates of infusion of kallikrein into the external circuit with the concentrations of kinin (estimated as bradykinin) detected by the cat jejunum. The assay was made by infusing bradykinin at rates chosen to bracket the contractions induced by the kallikrein infusions. The kallikrein and the bradykinin infusions were made at the nearest convenient point to the assay tissues, which gave a contact time with blood of 15 seconds. After this 15 s contact the concentration of kinin formed by kallikrein would be constant (see earlier) but the bradykinin concentrations would be approximately halved, since the half life of bradykinin in dog's

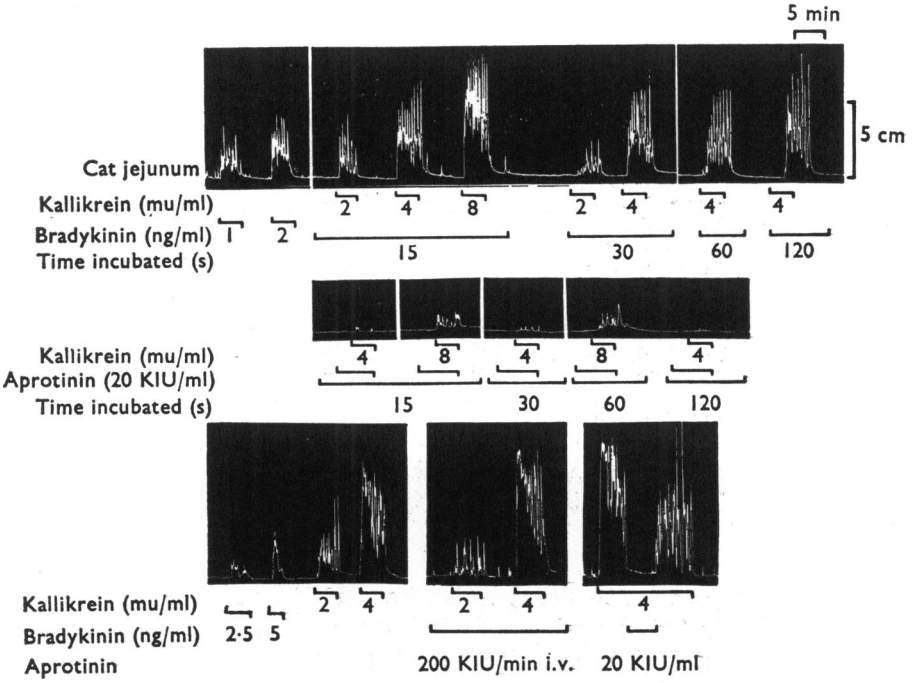

FIG. 2. A cat jejunum was bathed in arterial blood from an 18·0 kg dog. The top tracings show contractions induced by bradykinin (1 and 2 ng/ml; first section) and by kallikrein (2, 4 and 8 mU/ml) incubated for 15, 30, 60 or 120 seconds. The middle tracings show that aprotinin (20 KIU/ml) inhibits the generation of kinins by kallikrein (4 or 8 mU/ml) incubated for 15, 30, 60 or 120 seconds. The bottom tracings show that intravenous aprotinin (200 KIU/min) hardly affects the generation of kinins by kallikrein IBB (2 and 4 mU/ml) but aprotinin (20 KIU/ml) given directly into the blood bathing the cat jejunum inhibits kinin generation for the duration of the infusion only. Time 5 min; vertical scale 5 cm.

blood is 11–16 s (McCarthy, Potter & Nicolaides, 1965; Ferreira & Vane, 1967b; see also Table 2). Thus, the estimates in Table 1 of kinin concentrations induced by kallikrein infusions are likely to be double the actual concentration reaching the assay tissues.

Effects of aprotinin on kallikrein activity

Kallikrein (1–10 mU/ml) was infused to give a 15 s contact with the blood in the external circuit. Aprotinin was also infused in the incubating circuit so that it was in contact with the blood for 15–120 seconds. These infusions of aprotinin by themselves had no effect on the cat jejunum preparations in concentrations up to 20 KIU/ml, but, as in Krebs solution, higher concentrations reduced the contractions induced by bradykinin.

TABLE 1. *Concentrations of kinin generated by infusions of kallikrein into blood or intravenously*

		Infusion into bathing blood		Intravenous infusion		
Dog	Weight (kg) and sex	Kallikrein concentration (mU/ml)	Estimated kinin generated by kallikrein at 1mU/ml (ng/ml)	Kallikrein intravenous infusion rate (mU/kg)/min	Estimated kinin generation by this dose (ng/ml)	Estimated kinin generation by kallikrein at (25 mU/kg)/min (ng/ml)
1	14·5 ♀	4	2·5	27	2	1·8
2	18·5 ♂	4	1·8	–	–	–
3	13·0 ♂	8	1·0	125	5	1·0
		0·5				
4	22·0 ♂	1	3·8	–	–	–
		2				
6	15·0 ♂	2	3·5	53	5	2·4
7	12·5 ♀	0·5	0·6	8	0·3	1·0
8	8·5 ♂	2	2·0	24	2·5	2·6
9	8·0 ♂	2	1·0	–	–	–
10	11·0 ♀	0·25	3·2	7·3	1·0	3·4
13	15·0 ♂	8	1·0	–	–	–
14	20·0 ♀	4	1·0	40	2	1·2
		8				
		0·5				
15	11·5 ♂	1	1·0	8·7	2	5·7
		2				
16	13·0 ♂	8	2·0	61	8	3·2
17	16·0 ♀	4	2·5	25	4	4·0
Mean	–	–	1·9	–	–	2·6

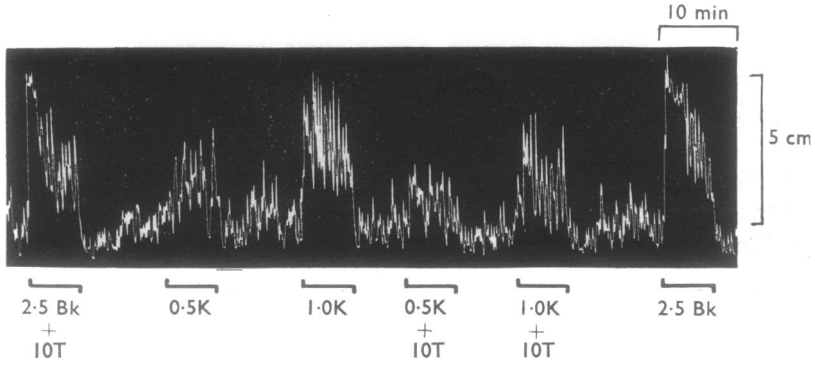

FIG. 3. A strip of cat jejunum was bathed in arterial blood from a 22 kg dog. Contractions were obtained either with bradykinin (2·5 ng/ml; 2·5 BK) or with kallikrein (0·5 and 1·0 mU/ml; 0·5 K and 1·0 K). The kinin generation by kallikrein was inhibited by aprotinin (10 KIU/ml; +10T). Time 10 min; vertical scale 5 cm.

The generation of kinin by kallikrein was inhibited by aprotinin. The inhibition was rapid (Fig. 2) and was not increased by preincubating the aprotinin with the blood for 2 min rather than 15 seconds. In most experiments, an aprotinin concentration (20 KIU/ml) was used which was high enough to ensure almost complete inhibition of kallikrein activity, but lower concentrations were used in two experiments. In another part of the experiment of Fig. 2 (not illustrated), an IBB infusion of aprotinin at 15 KIU/ml gave an inhibition of kinin generation by kallikrein (8 mU/ml) to that previously given by 2 mU/ml, that is, a 75% inhibition. In the other experiment, with a kallikrein concentration of 1 mU/ml, the concentration of aprotinin needed to produce a 50% inhibition of kinin generation was 10 KIU/ml (Fig. 3).

Several types of experiment suggested that aprotinin had a relatively short life as a kallikrein inhibitor in the circulation. First, when given into the extracorporeal circulation, the inhibitory effects of aprotinin passed off as soon as the infusion was stopped (see last panel of Fig. 2). Second, infusion rates of 20–40 KIU/min into the dog had little or no inhibitory effect on intravenous infusions of kallikrein. Infusion rates of aprotinin as high as 150 KIU/min had to be given into the dog to reduce or abolish the activity of kallikrein (200–800 mU/min i.v.; Fig. 4). An interesting feature of this experiment was that although the hypotensive action of kallikrein was inhibited the generation of kinins by kallikrein infusions into the extracorporeal circuit was not (Fig. 4). Furthermore, during the aprotinin infusion, bradykinin injections (10–20 μg i.v.) had a greater effect both on the blood pressure and on the blood-bathed cat jejunum.

Single intravenous injections of aprotinin (2,000 KIU/kg) decreased the sensitivity of the blood-bathed cat jejunum to bradykinin for several minutes. The contractions of the cat jejunum induced by bradykinin infusions were larger than the controls, suggesting either a potentiation of bradykinin activity or an inhibition of its destruction during its contact with blood. Later, from 30 to 180 min after the aprotinin injections there was also a much larger contraction of the strips of cat jejunum when kallikrein was infused into the external circuit.

To find out whether the increase in sensitivity to kinin was caused by preservation of kinin in the blood by reduction of kininase activity, the half life of bradykinin in the blood and the influence of aprotinin on it were measured, using the incubating circuit.

Influence of aprotinin on the half life of bradykinin in blood

The half life of bradykinin in the circulating blood, as determined in four dogs, varied from 11 to 16 seconds. When aprotinin was infused intravenously (150 KIU/min) or into the external circuit (20 KIU/ml) or even after a single injection of aprotinin intravenously (2,000 KIU/kg) the half life of bradykinin in the blood stream was prolonged. Table 2 shows the prolongation in four dogs which had received aprotinin (2,000 KIU/kg). The increase in the half life of bradykinin lasted for several hours after the aprotinin injection (Fig. 5). This was in contrast to the inhibitory effect of aprotinin on kallikrein activity, which lasted for only 20–60 minutes. As found by Ferreira & Vane (1967b) the half life of bradykinin was the same in venous and arterial blood. The prolongation of the half life by aprotinin was also similar in venous and arterial blood.

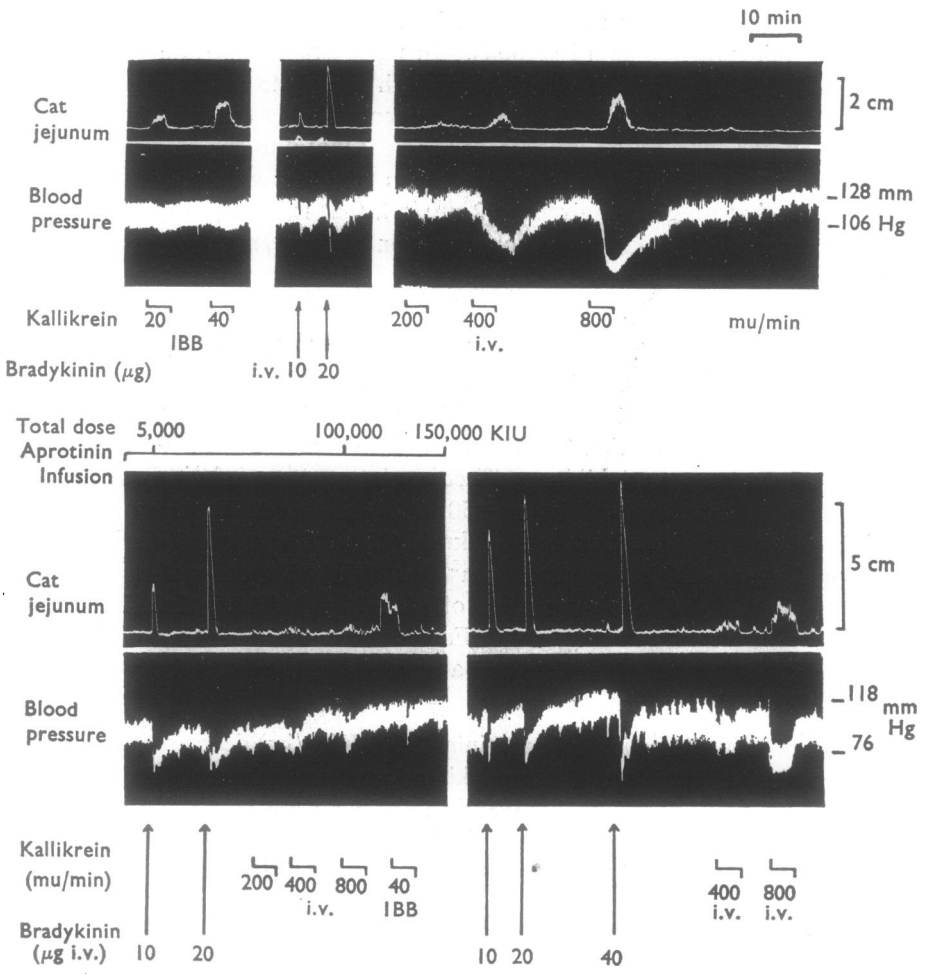

FIG. 4. A strip of cat jejunum (top record) was bathed in arterial blood from a 15 kg dog: the lower record is of mean blood pressure. Kallikrein infusions into the bathing blood (20 and 40 mU/min IBB) caused contraction of the cat jejunum, due to generation of kinins. There was also a transient contraction when bradykinin (10 and 20 μg) was injected intravenously. Infusions of kallikrein intravenously (200, 400 and 800 mU/min i.v.) caused contractions of the cat jejunum and increasing falls in blood pressure. Aprotinin was then infused intravenously to a total dose of 150,000 KIU. The effects of bradykinin (10 and 20 μg i.v.) were potentiated and those of kallikrein (200, 400 and 800 mU/min i.v.) were diminished. Thirty minutes after the aprotinin infusion was stopped (last panel), the effects of bradykinin were still potentiated, but the inhibition of the kallikrein activity was less. Time 10 min; vertical scales 2 and 5 cm and mmHg. (1 mmHg≡1.333 mbar.)

TABLE 2. *Change in half life of bradykinin after aprotinin* (2,000 *KIU/kg*)

Dog	Weight (kg) and sex	Before aprotinin $t_{\frac{1}{2}}$(s)	Time after aprotinin injection 5–60 min $t_{\frac{1}{2}}$(s)	60–140 min $t_{\frac{1}{2}}$(s)	140–220 min $t_{\frac{1}{2}}$(s)	265–320 min $t_{\frac{1}{2}}$(s)
5	10·0 ♀	14	20	–	29	–
11	8·5 ♂	12	–	25	53	–
12	10·0 ♀	14	–	24	–	–
13	15·0 ♂	11	20	73	37	22

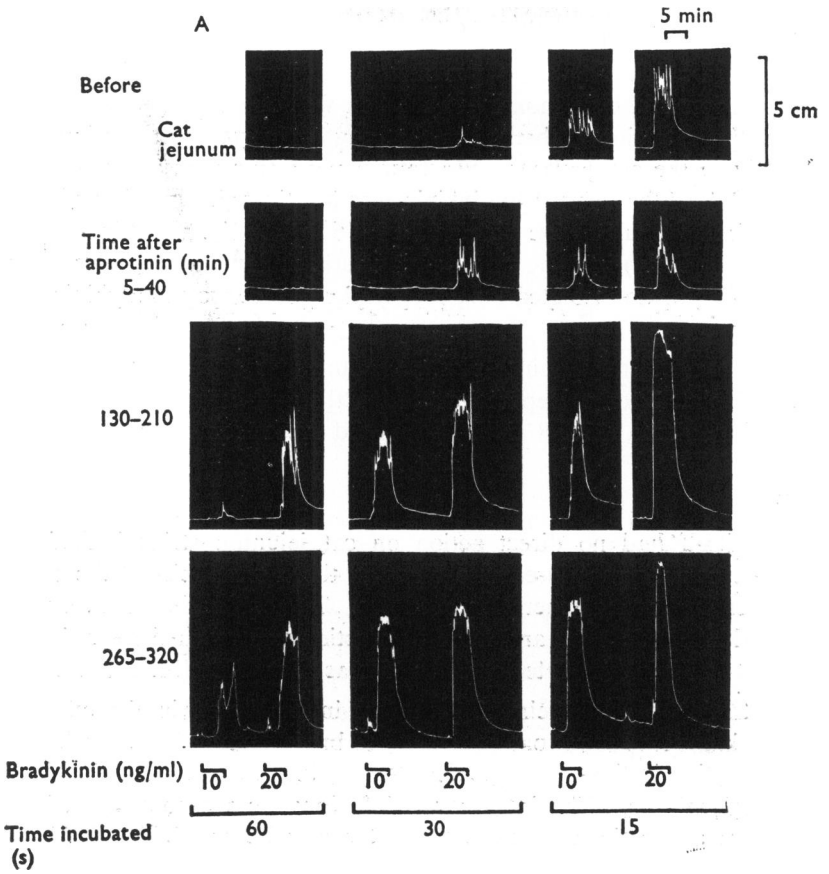

FIG. 5. The tracings (A) show contractions of a strip of cat jejunum bathed in arterial blood from a 15 kg dog. Each vertical section shows the effects of two concentrations of bradykinin (10 and 20 ng/ml) incubated for 60 s (left hand) 30 s (middle) and 15 s (right hand). The top horizontal section is before infusion of aprotinin (2,000 KIU/kg i.v.). Each assay of the half life of bradykinin takes 30–80 min, so each horizontal section thereafter is taken 5–40 min, 130–210 min and 265–320 min after the aprotinin infusion. Note the increasing preservation of bradykinin in the blood. Time 5 min; vertical scale, 5 cm. The graph (B) is plotted from this experiment and shows the variation in the half life of bradykinin with time after the aprotinin infusion.

Effects of aprotinin on lung bradykininase activity

Infusions of bradykinin were given into isolated perfused lungs. An estimate of the destruction in the pulmonary circulation was obtained by matching the contractions of the cat jejunum bathed in the lung perfusate with infusions of bradykinin directly to the assay tissues. In four experiments with guinea-pig lungs 97, 98, 98 and 99% of the infused bradykinin was inactivated. In lobes of lungs from three dogs, similar figures were obtained (92, 96 and 98%). Infusions of aprotinin (1, 10 and 40 KIU/ml) into the pulmonary arterial inflow did not alter the capacity of the guinea-pig or dog isolated lung preparations to destroy bradykinin (Fig. 6).

In three other experiments, aprotinin (2,000–10,000 KIU/kg) was injected intravenously into anaesthetized guinea-pigs. The lungs were removed 30, 60 or 90 min later. The isolated lung preparations from the guinea-pigs inactivated similar amounts of bradykinin (97, 98 and 99%) as did those from untreated animals.

Discussion

Kallikrein itself had no direct action on cat jejunum strips bathed in Krebs solution but when it was infused into blood in the external circuit the cat jejunum strips contracted, showing generation of kinins. The equilibrium between kallikrein inactivation, kinin generation and kinin destruction was reached so rapidly that the same amount of kinin was detected after 15 s as after 120 s incubation.

A concentration of kallikrein of 1 mU/ml in the blood in the extracorporeal circuit led to kinin concentrations equivalent to bradykinin at 1–4 ng/ml. A similar kinin concentration in the blood was achieved by intravenous infusions of kallikrein at (25 mU/kg)/minute. The intravenous infusions were accompanied by falls in blood pressure proportional to the dose. Recently Berry, Collier & Vane (1970) have shown that kinins are present in the circulation during haemorrhagic shock in the dog in concentrations of 1–5 ng/ml. These kinins were probably generated by a circulating enzyme such as kallikrein, released from the intestine. It is evident from our results that the release of kallikrein to produce these concentrations of kinins leads to substantial cardiovascular effects.

The commercial kallikrein preparation used also contains carboxypeptidase B and elastin (Haberland, personal communication), so the presence of these other enzymes may have influenced the time needed to reach an equilibrium between

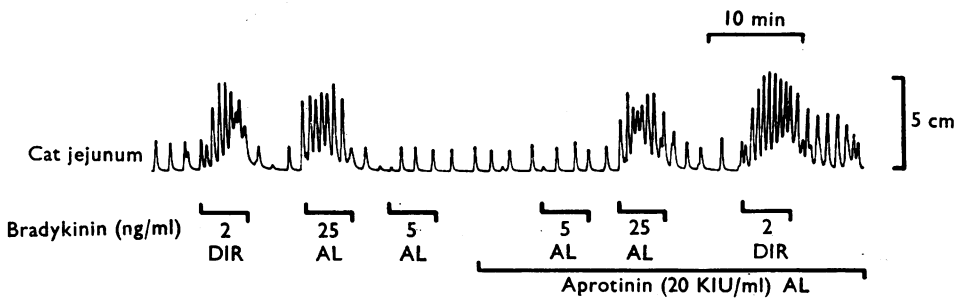

FIG. 6. The tracing is of the movements of a strip of cat jejunum bathed in the effluent from isolated lungs of a guinea-pig perfused with Krebs solution via the pulmonary artery. Bradykinin was either infused into the pulmonary artery (AL) or directly to the assay tissue (DIR). The inactivation of bradykinin in the lungs was unaffected by an infusion of aprotinin (20 KIU/ml). Time 10 min; vertical scale 5 cm.

generation and destruction of kinin in blood. However, a preparation of kallikrein free of the other enzymes gave qualitatively similar results (J. G. Collier, unpublished results).

In high concentrations (50 KIU/ml or 70 μg/ml) aprotinin inhibited the contractions of the cat jejunum strips induced by bradykinin. This effect was seen with tissues bathed in either Krebs solution or blood. We did not attempt to find whether or not the inhibition was specific for the effects of bradykinin. It is possible, however, that such an inhibition of the actions of bradykinin contributes to the clinical actions of aprotinin, for this concentration is exceeded in human blood after the recommended initial dose (Kaller, 1966).

The blood-bathed organ technique allows estimates of enzyme action or inhibition to be made in periods equivalent to one or two circulation times. The inhibition of kallikrein activity by aprotinin was so rapid as to be maximal in blood within 15 seconds. However, the inhibition was much less than we expected from the stated definition of the unit which is based on results *in vitro*. Aprotinin at 10 KIU/ml had to be used to inhibit by 50% the activity of kallikrein at a concentration of 1 mU/ml. From the definition of aprotinin units, the ratio between aprotinin and kallikrein concentrations should have been 1 to 2 rather than 10,000 to 1. We cannot explain this difference, unless it is due to rapid binding or inactivation of aprotinin in blood. Certainly aprotinin can be expected to bind to many other enzyme proteins apart from kallikrein. High doses of aprotinin also had to be given intravenously to the dog to inhibit the generation of kinins by intravenous kallikrein. Furthermore, the inhibiting effect was not long lasting and passed off in 20–60 minutes. This is in agreement with blood concentrations found in rats after intravenous injection (Werle, 1970). Only 20% of the injected aprotinin remained in the blood after 3 min, 9% after 15 min and 4% after 30 minutes. The gradual reduction in kininase activity leading to an increase in amounts of kinin surviving in the circulation might also have contributed to the apparent shortness of duration. This reduction in kininase activity was greatest 1–3 h after injection of aprotinin and declined slowly thereafter, although some potentiation of bradykinin half life was still seen 4 h after aprotinin injection.

The slow time course of the changes in kininase activity suggests that aprotinin may be metabolized to another substance which is responsible for kininase inhibition. There is evidence for structural alteration of aprotinin in the liver and in renal tissue (see Trautschold *et al.*, 1967 ; Werle, 1970). Another possible explanation of the results is that aprotinin prevents replacement of kininase in the circulation.

This is not the first report of aprotinin causing an inhibition of kininase. Although Erdös, Renfrew, Sloane & Wohler (1963) found that aprotinin had little or no effect in preventing destruction of bradykinin in human plasma *in vitro*, Camargo & Graeff (1969) found that it inhibited inactivation of bradykinin by a partially purified enzyme from rabbit brain. However, lung kininase is more important than plasma kininase for the inactivation of circulating kinins (Ferreira & Vane, 1967b) and the kininase in perfused lungs from guinea-pigs and dogs was not inhibited by aprotinin. This lack of inhibition may have been a reflection of the slowness in onset of action of aprotinin (or a metabolite), or it may have been because the lung enzymes have a different specificity for inhibitors. For these reasons, it is difficult to know how much the possible inhibition of kininase would influence the clinical effectiveness

of aprotinin. Certainly such an inhibition would, by potentiating the effects of kinins, tend to limit the usefulness of aprotinin as a kallikrein inhibitor.

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