Br. J. Pharmac. (1976), **58**, 201–209

BRADYKININ AND FUNCTIONAL VASODILATATION IN THE SALIVARY GLAND

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1 Blood flow through the submandibular gland of the dog was measured and the venous effluent monitored for potential mediators of the functional vasodilatation by passing it over a series of assay tissues.

2 On chorda lingual nerve stimulation there was salivation, an increased blood flow and the release of a bradykinin-like substance (kinin) into the venous effluent.

3 In about half of the preparations, increasing the frequency of stimulation from 2 to 10 Hz led to an increased output of kinin, whereas in the others successive stimulations led to a decreasing output of kinin in the face of normal secretory and vascular responses.

4 Following the administration of atropine, the vasodilatation persisted, salivation was abolished and after several stimulations kinin release could no longer be detected.

5 Release of prostaglandin did not appear to be responsible for the vasodilatation.

6 It is concluded that neither kallikrein nor kinin is the main mediator of parasympathetic vasodilatation in the salivary gland.

Introduction

Stimulation of the parasympathetic nerve supply to the submandibular gland in dogs and cats leads to salivation and an increase in the blood flow, but only the salivation is blocked by atropine (Heidenhain, 1872). This observation suggested that there were specific parasympathetic vasodilator nerve fibres resistant to atropine, but Ungar & Parrot (1936) suggested that kallikrein, present in the submandibular saliva may mediate the effects of the nervous stimulation. Hilton & Lewis (1955a; 1955b; 1956) provided experimental support for this thesis and extended it to functional vasodilatation in other organs. However, their views were subjected to criticism, (Bhoola, Morley, Schachter & Smaje, 1965; Schachter & Beilenson, 1967; Beilenson, Schachter & Smaje, 1968; Skinner & Webster, 1968; Darke & Smaje, 1973). A particular problem was that neither salivary kallikrein nor kinin could be found in the venous effluent of secreting glands. It was possible, however, that bradykinin released during activation of the gland, was not detected because of the very short half-life of the peptide in blood and tissues (Nobili, 1965; Ferreira & Vane, 1967b), or that any kallikrein or kinin released was eliminated in the lymph (Hilton & Lewis, 1955b).

A rapid method for the estimation of bradykinin in blood is thus required and in a preliminary report Ferreira, Corrado & Rocha e Silva (1969) have shown that the blood-bathed organ technique (Vane, 1964) could be used for the detection of kinin in the venous effluent of the salivary gland. In the present paper we have used the same technique and extended these observations. The destruction of kinins by the blood was prevented by the use of synthetic penta and nona bradykinin-potentiating peptides (BPP_{5a} and BPP_{9a}) which are two of the bradykinin-potentiating peptides originally described in the venom of *Bothrops jararaca* (Ferreira, 1965; Greene, Camargo, Kreiger, Stewart & Ferreira, 1972). Potentiation of the contractions of the assay tissues by BPP was taken as confirmation of bradykinin release. We have used additional isolated tissues in an attempt to detect substances other than kinins (e.g. prostaglandins) which could mediate the atropine-resistant vasodilatation caused by chorda tympani nerve stimulation.

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Methods

Seventeen dogs of various breeds and either sex, weighing between 17 and 35 kg were used. Anaesthesia was induced by intravenous pentobarbitone sodium (Nembutal, Abbot) 30 mg/kg given via a foreleg vein and supplemented as required via a cannula which was subsequently placed in the femoral vein. To facilitate respiration, a cuffed endotracheal tube was inserted after induction of anaesthesia. Blood pressure was monitored from the femoral artery with a S.E. 4–28 pressure transducer and registered on a Watanabe pen recorder.

Blood flow

The venous drainage of the gland was isolated and other veins draining into the external jugular vein were tied off. Thermocautery was used extensively in order to produce a bloodless field. The external jugular vein was cannulated and drops of blood passed through a closed drop counter. The outflow from the counter went to the stem of a Y tube. One limb of the tube joined a cannula placed in the external jugular vein facing towards the heart, and the other went to a roller pump which supplied the assay tissues. This arrangement ensured a constant supply of 10 ml per min of blood to the assay tissues. When salivary gland blood flow was less than 10 ml/min (e.g. during resting conditions) the balance required was pumped from the external jugular vein; if the glandular blood flow exceeded 10 ml/min (e.g. during maximal vasodilatation) any excess passed into the external jugular vein.

The output from the drop counter was either passed to a rate meter or the individual drops were integrated and the counter discharged at regular intervals with a clock (see Darke & Smaje, 1972).

After the dissection the preparation was left for 30–60 min before beginning blood flow recording, at which time heparin (1000 i.u./kg i.v., Boots) was given.

Intra-arterial injections were made via a cannula passed retrogradely into the lingual artery so that its tip lay at the junction of the lingual artery and the external carotid artery.

Nerve stimulation

The chorda lingual nerve was freed from the connective tissue dorsal to the mylohyoid muscle and separated from the hyoglossus muscle. The nerve was cut as far rostral as possible and stimulated with a fluid electrode at supramaximal voltage (10–20 V) with square wave pulses at a frequency of 2–20 Hz and of 1 ms duration.

The submandibular duct was cannulated distal to the point at which it was crossed by the lingual nerve, using polythene tubing of 2–3 mm external diameter.

Assay method

Variations in the concentration of bradykinin-like substance (kinin) or prostaglandins in the venous effluent were measured continuously by the blood-bathed organ technique (Vane, 1964) with 4 tissues in series: (1) cat jejunum (CJ) or terminal ileum (CTI), or kitten terminal ileum (KTI) (Ferreira & Vane, 1967a; Ferreira, Ng & Vane, 1973), (2) rat stomach strip, (3) rat colon and (4) chick rectum. The cat and kitten tissues contract in the presence of bradykinin; the terminal ileum responds to histamine as well but this was prevented by pretreatment with mepyramine (see below). The rat stomach strip (Vane, 1957), rat colon (Regoli & Vane, 1964) and chick rectum (Mann & West, 1950) respond to prostaglandins. The isolated tissues were pretreated with antagonists of adrenaline, 5-hydroxytryptamine, acetylcholine and histamine (Gillmore, Vane & Wyllie, 1968), so that the only known agonist which contracts the cat tissues was bradykinin (Ferreira & Vane, 1967a), and prostaglandins were the only substances known to contract all three of the other tissues (Gillmore *et al.*, 1968).

An increase in oxygen tension is known to lead to an increased tension in isolated tissues. The P_{O_2} in the venous effluent was higher during stimulation than at rest and the resulting organ contractions had to be distinguished from those due to release of potential mediators (see Results section). Oxygenation of the venous effluent before it superfused the tissues led to inconsistent results, so it was omitted. Contractions of the assay tissues were monitored with auxotonic levers, attached to 'Harvard' smooth muscle transducers and displayed on a multi-channel recorder (Watanabe). The overall amplification of the system was four to eightfold. The isolated assay tissues were suspended in polypropylene chambers and superfused in series, first with Kreb's solution, while the gland was being prepared and then with the venous effluent from the gland. Blood reached the tissues 30–50 s after leaving the gland.

Test substances (bradykinin, Sandoz; purified hog pancreatic kallikrein, Bayer; prostaglandin E_1 and E_2, Upjohn; histamine acid phosphate, Burroughs Wellcome; adenosine diphosphate, Sigma; bradykinin potentiating pentapeptide, BPP_{5a} and bradykinin potentiating nona peptide BPP_{9a} kindly supplied by L. Greene, Brookhaven National Laboratories, U.S.A.) were injected into the tubing leaving the gland.

Results

Kinin release

In 13 experiments the cat tissues used for bradykinin assay responded to concentrations of the peptide of 2–8 ng per ml of bathing blood. They were insensitive

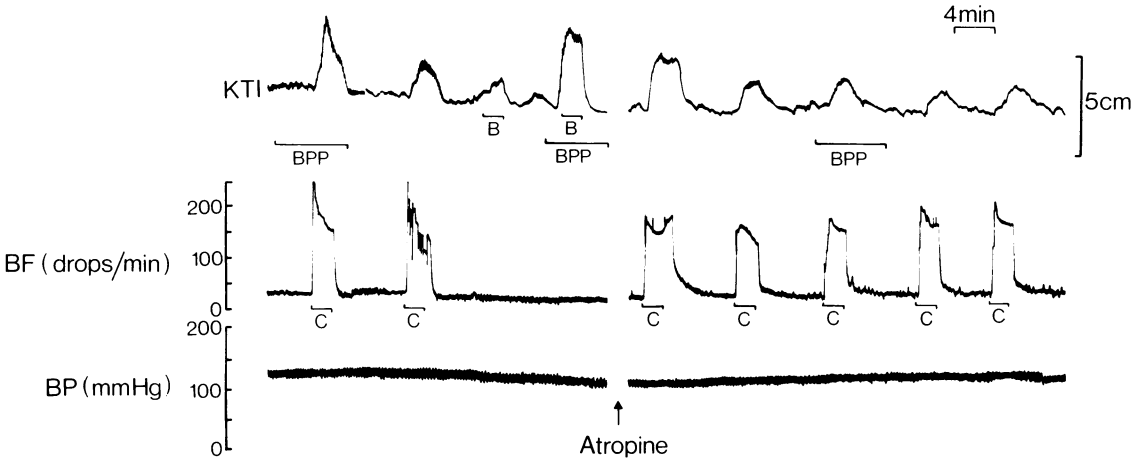

Figure 1 Vascular response and release of kinin following chorda lingual nerve stimulation before and after atropine. The tracings show contractions of kitten terminal ileum (KTI); submandibular gland blood flow (BF); and blood pressure, mmHg (BP). Before atropine, KTI contractions in response to chorda stimulation at a frequency of 5 Hz (C) and to bradykinin 1 ng/ml (B), were enhanced by bradykinin potentiating penta-peptide (BPP_{5a}), 2 μg/ml. BPP_{5a} and bradykinin were added to the blood leaving the gland on its way to superfusing the tissues. After atropine (250 μg/kg i.v.) BPP_{5a} did not potentiate the KTI response to chorda stimulation.

to bradykinin in 4 experiments and only the responses to chorda stimulation of the other assay organs (rat colon, chick rectum, rat stomach strip) were studied. In 10 of the 13 dogs, chorda lingual nerve stimulation for 2 min at frequencies of 2–10 Hz led to salivation and vasodilatation, and to contraction of the cat tissues (see left side of Figure 1 and Figure 3). These contractions were not due to a higher oxygen tension in the venous effluent during gland activity because this would have caused contraction of all the assay tissues, which was not observed. The rat colon (not shown in Figures 1 and 3) relaxed following each stimulation period, whereas it contracted when the Po_2 of the superfusing blood was raised by changing from the venous effluent of the gland to femoral arterial blood (see Figure 6).

Addition of bradykinin-potentiating peptide (BPP) to the blood leaving the gland potentiated the responses (Figure 1) of the cat tissues to bradykinin and to the substance released on chorda stimulation in 3 experiments with BPP_{5a} and 4 with BPP_{9a} (1–10 μg/ml). This suggests that chorda stimulation induced release of kallikrein or kinin from the gland.

In 5 of the 10 experiments in which kinin release was detected, the vasodilator response in the gland remained steady with successive chorda lingual nerve stimulation periods, although the output of kinin diminished. In the other 5 both the vasodilatation and the kinin release remained similar to the initial values for several stimulation periods: in these experiments raising the frequency of stimulation from 2 to 10 Hz increased both vasodilatation and kinin release (Figure 2). Thus in the range of stimulus frequencies used, both responses were graded and observed vasodilatation was accompanied by detectable amounts of kinin.

Intra-arterial infusion of bradykinin potentiating peptide

If the bradykinin released during activation of the gland were responsible for the vasodilatation, the latter should be potentiated by BPP. In two experiments, infusion of BPP_{9a} (10 μg/min) into the lingual artery did not affect the vascular responses to chorda lingual nerve stimulation at 2 and 10 Hz but did enhance the contractions of the kitten terminal ileum (KTI). Intra-arterial infusion of bradykinin also produced vasodilatation and subsequently KTI contraction and systemic hypotension. The vasodilatation was enhanced by BPP in one experiment and the systemic hypotension and tissue contractions were increased in both experiments.

Effect of atropine

Atropine (0.2–1 mg/kg, i.v.) blocked the secretory response to chorda stimulation, but not the vasodilator response. The first vasodilator response to chorda stimulation after atropine was usually greater and more prolonged than that found before atropine, and this was accompanied by a greater than normal

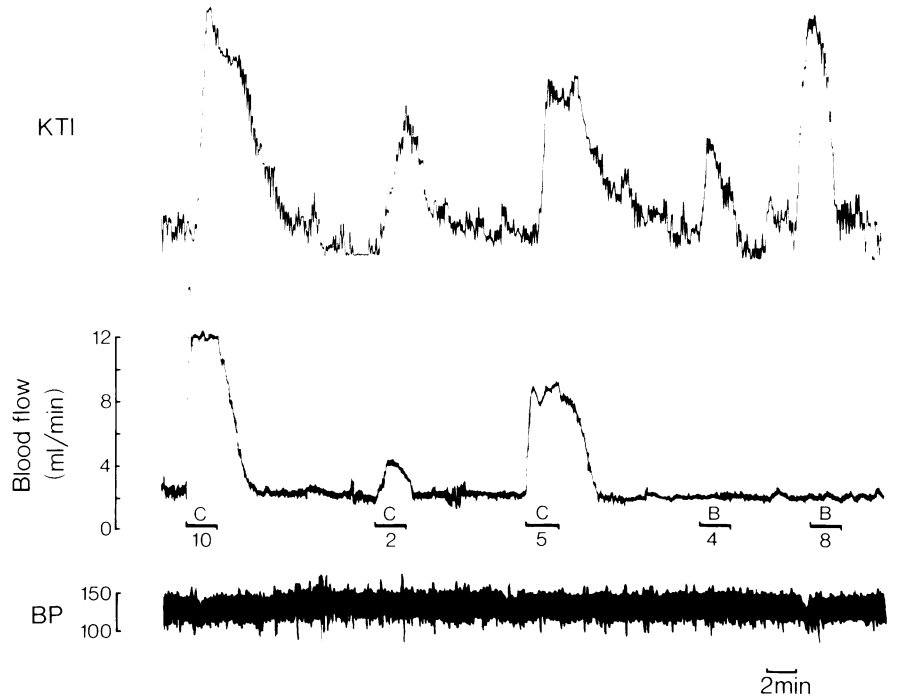

Figure 2 Effect of changing the frequency of chorda stimulation on submandibular blood flow and on the contractions of the kitten terminal ileum (KTI). C = chorda stimulation at 2, 5 or 10 Hz; B = bradykinin 4 or 8 ng/ml of blood superfusing the KTI.

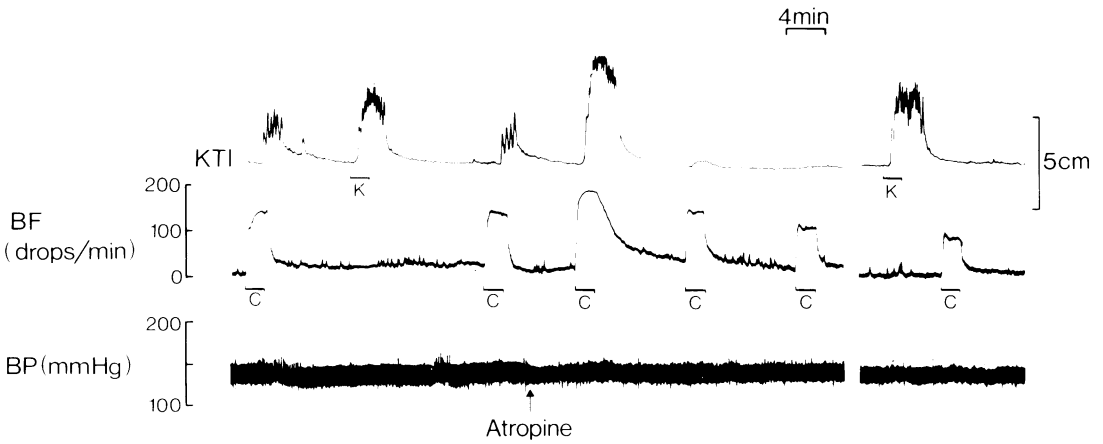

Figure 3 Vascular response and release of kinin following chorda lingual nerve stimulation. Abbreviations as in Figure 1. The output of kinin and the vascular response to chorda stimulation, 10 Hz (C) were both increased immediately after atropine, (200 μg/kg i.v.). With repeated stimulation, vasodilatation returned to the control level but kinin release diminished to zero. Atropine, if anything, increased the sensitivity of the KTI as the response to kallikrein (K) 60 μu/ml was greater than to K 100 μu/ml given before atropine.

release of kinin-like material (Figures 1 and 3). In some cases the delayed return to the baseline was the most obvious effect.

In 4 experiments the cat tissue contractions diminished with successive stimulation periods and the vascular responses returned towards or were even below the control values (Figure 3). In two of these experiments BPP was administered during the stimulation period but still no kinin-like material was detected. In six other animals chorda stimulation led to contraction of the cat assay tissues even after four or five stimulation periods, but the tissue contractions were not significantly potentiated by BPP (Figures 1 and 4). Figure 4 also shows that atropine did not diminish the contractions of the cat tissue in response to bradykinin, nor affect the increased contraction found with simultaneous infusion of BPP and kinin. The cat tissue contractions remaining after atropine were unlikely to be due to a change in Po_2 as the other assay tissues did not contract at the same time.

A second dose of atropine did not lead to a further increase in kinin release or an increased vasodilator response to stimulation of the chorda lingual nerve.

Effect of increasing interstitial kinin concentration

Kallikrein concentration in the interstitium may be raised by stimulation of the chorda with the excretory duct clamped, or by retrograde infusion of kallikrein (e.g. in saliva) into the salivary duct. These manoeuvres should increase blood flow above normal if kallikrein mediates the vasodilatation.

In three experiments the salivary duct was clamped during stimulation of the chorda lingual nerve before the administration of atropine. Clamping the duct invariably enhanced the effect of chorda stimulation on the kinin released into the blood but not on the vasodilator response.

Retrograde infusion of saliva into the submandibular gland duct was performed in 4 experiments when there was no release of kinin on chorda stimulation after atropine. On stimulation of the chorda lingual nerve substantial amounts of kinin were released in all cases (Figures 5a and b). The vasodilator response was increased in one experiment (Figure 5a), reduced in two (e.g. Figure 5b) and unchanged in one.

Release of other mediators

In an attempt to explain the atropine-resistant vasodilatation, which was not accompanied by kinin release, alternative mediators were considered by parallel assays on the rat stomach strip, chick rectum and rat colon. In 14 out of the 17 experiments chorda lingual nerve stimulation led to the following responses: the rat stomach strip relaxed or relaxed and contracted. The response usually persisted after

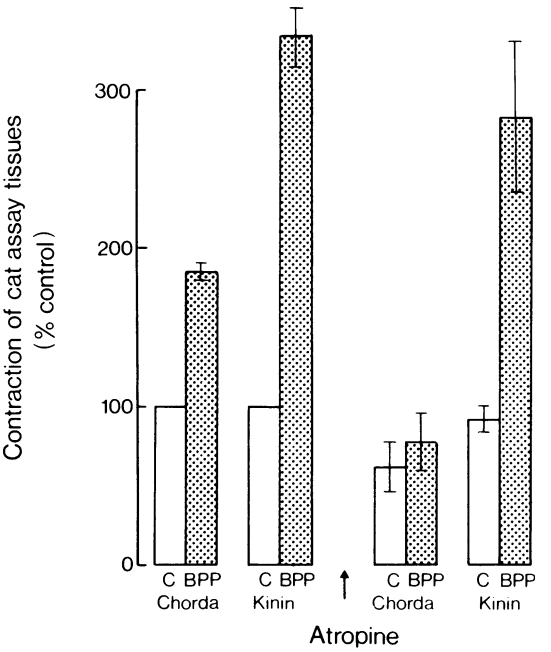

Figure 4 Effect of bradykinin potentiating peptide (BPP) on contractions of cat or kitten terminal ileum or cat jejunum in response to chorda stimulation or to the addition of bradykinin or kallikrein to the superfusing fluid. The six experiments are shown in which atropine did not abolish contractile responses to chorda stimulation. Control responses (C) were elicited by standard stimuli (5 or 10 Hz, supramaximal voltage) and standard kinin doses, and the effects of BPP expressed as a % of these responses. Mean values are shown; Vertical lines show s.e. mean. Paired *t*-tests indicated that before atropine BPP significantly increased tissue contractions induced by chorda stimulation and by bradykinin ($P<0.01$). After atropine, BPP significantly increased the effect of kinin ($P<0.01$), but not of chorda stimulation. There was no significant difference between the BPP-enhanced responses to kinin before and after atropine, but the effects of chorda stimulation were significantly reduced ($P<0.01$).

atropine, but often only relaxation developed. The responses of the chick rectum were inconsistent. The rat colon consistently relaxed, although a small contraction was occasionally seen afterwards. The response was usually unaffected by atropine (Figure 6). This relaxation excluded prostaglandins as mediators of the chorda vasodilatation since they contract the colon. Stimulation of the vago-sympathetic trunk and massage of the gland also did not lead to tissue responses typical of prostaglandins. Furthermore, the administration of aspirin (100 mg/kg i.v.) to 2 dogs and indomethacin (1–2 mg/kg i.v.) to 3

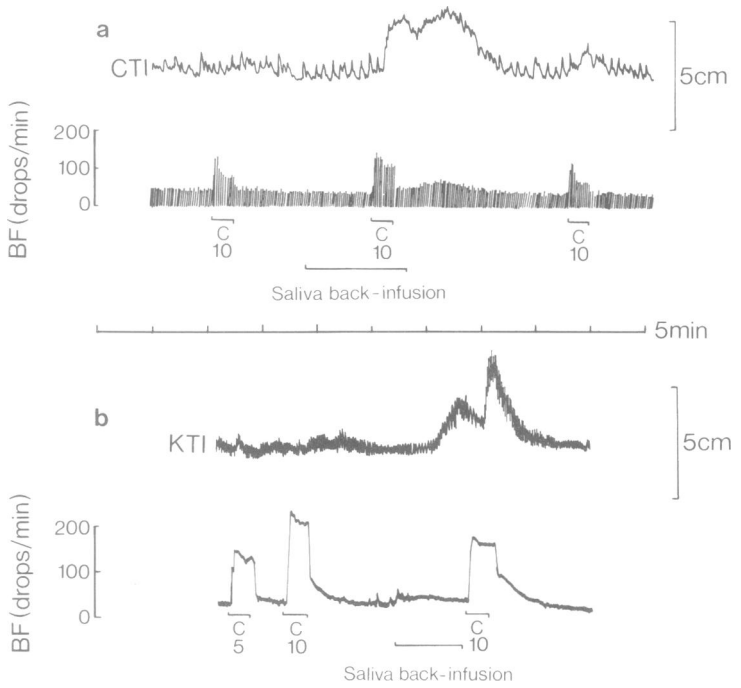

Figure 5 Blood flow (BF) and appearance of kinin on chorda lingual nerve stimulation at frequencies of 5 or 10 Hz (C5, C10) after infusion of saliva 0.1 ml/min retrogradely into the submandibular duct in dogs treated with atropine (1 mg/kg i.v.). (a) CTI, contractions of cat terminal ileum. (b) KTI, contractions of kitten terminal ileum. Note that in (a) kinin only appears following stimulation of the nerve, whereas in (b) there is an increase in basal blood flow and appearance of kinin before chorda stimulation.

dogs had no effect on the responses to parasympathetic nerve stimulation.

The responses of the isolated tissues induced by chorda stimulation or by bradykinin either before or after atropine were not due to a higher Po_2 in the superfusate; in 3 experiments tissues superfused with blood from the femoral artery produced a different pattern of responses (Figure 6).

Discussion

Perhaps the most intriguing feature of this investigation was the evidence that kallikrein or kinin was released into the venous outflow from the submandibular gland during chorda lingual nerve stimulation before atropine; yet, kallikrein did not appear to be responsible for the vasodilatation.

Stimulation of the chorda led to contractions of the cat assay tissues, and their enhancement by BPP suggested that stimulation of the chorda lingual nerve before atropine, could induce the release of bradykinin or kallikrein into the venous effluent. If kallikrein were released from the gland, the kinin detected would be generated in the venous blood between the gland and the assay tissues. In the perfusion experiments of Hilton & Lewis (1956) kallikrein and not kinin was detected after chorda stimulation. The present experiments do not distinguish which of the two factors was released.

Several factors argue against a role for kinins in mediating the vasodilator response to chorda stimulation. Increasing the interstitial kallikrein concentration by back infusion of saliva or by chorda stimulation with the excretory duct clamped, did not lead to significantly increased vasodilator responses. In some animals, repeated chorda stimulation led to a diminishing kinin output without a corresponding diminution in the vasodilator response. Following atropine, the output of kinin always declined to undetectable levels while the vasodilatation persisted. The contractions of the cat assay tissues seen in six experiments after atropine were not potentiated by BPP, and therefore not due to kinin. It seems unlikely that the amount of kinin required to cause vasodilatation was below the threshold of our assay system

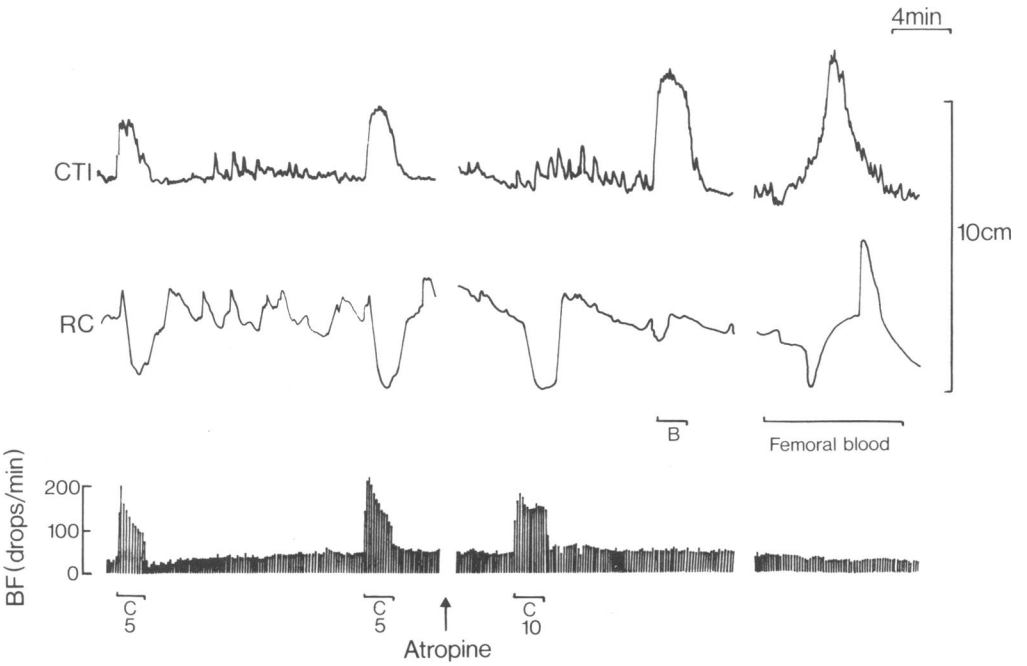

Figure 6 Chorda lingual nerve stimulation and appearance of rat colon (RC) relaxing and cat terminal ileum (CTI) contracting material; C5, C10=chorda lingual nerve stimulation at 5 or 10 Hz. After atropine the contractile response of the CTI disappeared although the tissue remained sensitive to bradykinin (B 4 ng/ml), whereas relaxation of the rat colon persisted. During the break in the record after atropine, there were six periods of chorda stimulation. Superfusion with femoral arterial blood induced a different pattern of responses from that caused by chorda stimulation.

because the relationship between stimulus frequency and kinin-output (Figure 2) was such that the vasodilator responses seen after atropine would be associated with detectable amounts of kinin. There was no change in tissue response to kinin as atropine did not reduce the sensitivity to injected bradykinin. Furthermore, BPP_{9a} given intra-arterially failed to enhance the vasodilator response to chorda stimulation although it did increase the contractions of the cat tissues and the vasodilatation produced by bradykinin. Similar observations were made in the cat by Schachter, Barton & Karpinski (1973), who also used BPP_{9a}, and by Smaje (1967) who employed mercaptoethanol in the rabbit to inhibit the kinin-destroying enzymes. Evidence for an atropine-resistant vasodilatation in glands perfused with media lacking substrate for kallikrein also casts doubt on claims that kinin mediates the vasodilatation (Bhoola *et al.*, 1965; Gautvik, 1970a; Poulsen, 1975).

If kinin does not produce the vasodilatation, could vasodilatation partly determine the amount of kinin released into the venous effluent? This could explain the relationship between stimulus frequency and kinin release (Figure 2) and the parallel increase in kinin output and vasodilatation following the first stimulus after atropine. Increased glandular blood flow could have increased the washout rate of the interstitial space, possibly by increased convective mixing in the interstitium (Mellander & Lundgren, 1968), thus carrying more kallikrein into the venous effluent. However, as atropine blocks the release of kallikrein from the gland successive chorda stimulations lead to less and less kinin in the venous output due to exhaustion of the kallikrein remaining in the ducts.

A reduction in kallikrein output after atropine had been noted by Hilton & Lewis (1956) but reduction to zero was not seen. This might have been because these authors apparently included in their collection glandular perfusate from the first stimulus after atropine. Atropine also prevents the consumption of kininogen by salivary kallikrein, which is specifically increased by chorda stimulation (Gautvik, Hilton & Torres, 1970). The inference from these experiments and those of the present paper is that the release of kallikrein by chorda stimulation, like the rest of the components of saliva, is prevented by atropine. Thus

kallikrein can be released by chorda stimulation and can contribute to the accompanying functional vasodilatation, but it is not the main mediator and it is not involved in the vasodilator response remaining after atropine.

In the present experiments the blood bathing the assay tissues came from two sources; the gland and the external jugular vein. During vasodilatation the proportion of gland blood increased and this could have contributed to the observed effects. However, chorda stimulation induced kinin release, before but not after atropine, whereas the blood flow increase was similar in both instances (Figures 1, 2 and 4). This implies that the superfusate contained similar proportions of blood to the gland and that the lack of kinin release after atropine was not an artefact.

The vasodilator substance released from the atropine-treated gland was not a prostaglandin as the responses of the assay tissues to prostaglandins differed from those induced by chorda stimulation. Also the effects of chorda stimulation were not affected by aspirin and indomethacin which block prostaglandin synthesis (Vane, 1971; Ferreira, Moncada & Vane, 1971). Histamine and potassium ions have been eliminated by previous investigations (Hilton & Lewis, 1955a, Darke & Smaje, 1972). Preliminary experiments showed that after atropine the vasodilator adenosine produced a relaxation of the isolated tissues similar to that found on chorda stimulation.

Since no single agent seems to mediate functional vasodilatation in the submandibular gland, could a combination of substances be responsible? Gautvik (1970a,b) suggested that the vasodilator response consists of an early phase due to activation of atropine-resistant parasympathetic vasodilator nerves and a phase in which kallikrein maintains the vasodilatation. Darke & Smaje (1972) also demonstrated a two-phase vasodilatation; atropine affected the first phase more than the second, and in glands depleted of kallikrein parasympathetic vasodilatation was smaller than in controls (Darke & Smaje, 1973). The available information suggests that kallikrein is not the sole mediator of vasodilatation in the submandibular gland but it may modulate a vasodilatation produced by another agent whose nature and mode of action remains unknown.

We are grateful to Dr J.R. Vane for his support during this work, and for his helpful criticism of the manuscript.

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(Received January 9, 1976.
Revised April 22, 1976.)