J. Physiol. (1980), **300**, *pp.* 41–53
With 8 text-figures
Printed in Great Britain

VASOACTIVE INTESTINAL PEPTIDE IN RELATION TO ATROPINE RESISTANT VASODILATATION IN THE SUBMAXILLARY GLAND OF THE CAT

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(*Received 17 July 1979*)

SUMMARY

1. Release of VIP from the submaxillary gland, in response to stimulation of the chorda tympani, and its vasodilator action at the site of release have been investigated in anaesthetised cats.

2. Chorda stimulation at 20 Hz produced an abrupt rise in the concentration of VIP in the submaxillary venous effluent plasma, accompanied by a substantial increase in submaxillary blood flow, in the presence or absence of atropine.

3. Intra-arterial infusions of VIP which reproduced the rise in submaxillary venous plasma concentration that occurred during chorda stimulation at 20 Hz, also produced a rise in submaxillary blood flow of the same order of magnitude.

4. Direct comparison of the responses of the submaxillary vasculature to intra-arterial infusions of VIP, ACh and bradykinin showed that the vasodilator potency of VIP far exceeded that of either of the other agonists.

5. Intra-arterial infusion of ACh, sufficient to elicit a maximal submaxillary vasodilator response, caused no detectable release of VIP from the gland.

6. The results are discussed in relation to the proposition that VIP is released from post-ganglionic parasympathetic neurones, in the submaxillary gland of the cat, and acts, *as a transmitter*, to cause vasodilatation, which is resistant to atropine.

INTRODUCTION

Stimulation of the chorda tympani produces an intense vasodilatation in the submaxillary gland (Bernard, 1858) which, unlike the secretory response, is resistant to atropine (Heidenhain, 1872). This phenomenon has been investigated by numerous workers during the intervening century and various explanations have been advanced. Barcroft (1914) found that atropine failed to abolish the rise in metabolic rate in the gland in response to chorda stimulation and attributed the vasodilator response to the accumulation of vasoactive metabolites. Bayliss (1923) challenged the view that such metabolites could account for the hyperaemia completely, in view of the poor correlation between the increase in submaxillary oxygen consumption and the extent of the vasodilation in the atropinised gland; this view is strongly supported by the more recent findings of Terroux, Sekely & Burgen (1959). Dale &

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Gaddum (1930) supposed that the response was mediated by special cholinergic vasodilator fibres whose terminals come into such an intimate relationship with the receptive mechanism 'that atropine cannot prevent its access thereto'. Barcroft's belief that the response was secondary to increased metabolic activity has been supported by Hilton & Lewis (1955*a*, *b*, 1956), who have advanced a substantial body of evidence to suggest that the vasodilatation is due to the formation of bradykinin, consequent upon release of salivary kallikrein. However, it is difficult to accept that this mechanism provides a complete explanation of the phenomenon in view of the finding that the salivary vasculature can be largely desensitised to bradykinin without blocking the vasodilator response to chorda stimulation (Bhoola, Morley, Schachter & Smaje, 1965).

More recent studies have shown that the potent vasodilator, vasoactive intestinal peptide (VIP), is released from the gastrointestinal tract in response to stimulation of the peripheral ends of the vagus nerves in various species, including pigs, cats and calves (Fahrenkrug, Galbo, Holst & Schaffalitzky de Muckadell, 1978*a*; Fahrenkrug, Haglund, Jodal, Lundgren & Schaffalitzky de Muckadell, 1978*b*; Bloom & Edwards, 1980) and that it might act as a transmitter, which could account for various atropine resistant responses to parasympathetic stimulation that occur in the gastrointestinal tract (Fahrenkrug *et al.* 1978*b*; Bloom & Edwards, 1980). VIP is present in nerve terminals in mammalian salivary glands (Bloom, Bryant, Polak, Van Noorden & Wharton, 1979) and the present experiments were undertaken to investigate the conditions under which it might be released within the gland.

Certain of these results have been published previously in a preliminary form (Bloom & Edwards, 1979).

METHODS

Animals

The experiments were carried out on adult cats (3·1–5·3 kg body weight) under chloralose anaesthesia (70 mg/kg) following induction with chloroform and ether. The animals had previously been kept in the laboratory animal house for at least a week, during which time they were maintained on Diet B (BP nutrition) *ad libitum*, supplemented with a tinned pet food (Bonus, Spillers). Food but not water was witheld for 12–18 h before each experiment.

Surgical and experimental procedures

The submaxillary duct and chorda tympani were exposed according to the classical description of Liddell & Sherrington (1929). In each experiment the duct was cannulated using a narrow bore glass cannula attached to a short length of polyethylene tubing. The lingual nerve was ligated and the central end, together with all the peripheral branches except for the chorda tympani, were cut in order to provide a convenient means of chorda stimulation. This was effected either by means of an appropriate fluid or manual electrode and in each case a standard 10 V square-wave stimulus (pulse width 0·5 msec) was applied for 10 min at 20 Hz. The animals were heparinised (800 u./kg) and the peripheral end of the ipsilateral jugular vein was then cannulated using a short length of polyethylene tubing, after each of the jugular tributaries, except that draining the submaxillary gland, had been ligated.

Permanent records of the rapid variations in salivary and salivary venous blood flow were obtained using photoelectric drop counters; whenever samples were collected for VIP assays

salivary blood flow was estimated gravimetrically. Salivary venous effluent blood in excess of that required for samples was returned to the animal through a cannula inserted into the right femoral vein, using a Harvard peristaltic pump, the speed of which was adjusted from time to time in order to match input to output. The ipsilateral ascending cervical sympathetic nerve was cut in the neck and narrow bore polyethylene catheters were inserted into both femoral arteries so that the tips lay in the abdominal aorta. These were used subsequently to monitor heart rate and aortic blood pressure, by means of a Devices L221 pressure transducer connected to a Devices M19 recorder, and for collection of arterial samples. The shaft of a 25 gauge needle, attached to tightly fitting narrow bore polyethylene tubing, was inserted into the ipsilateral carotid artery to provide a convenient conduit for close-arterial infusions using a Sage injection pump. When required atropine (atropine sulphate B.D.H.) was given by I.V. injection (0·9% (w/v) NaCl, 0·1 g/100 ml) at a dose of 1·0 mg/kg. Peptides for infusion (synthetic bradykinin triacetate (Sigma); pure porcine VIP (Professor V. Mutt); cyclic ovine somatostatin (Beckman)) were dissolved in a 5% solution of cat plasma in sterile saline to which aprotinin had previously been added (50 K.I.U./ml). Acetylcholine chloride (Koch–Light) was dissolved in a 4% solution of sodium dihydrogen phosphate in 0·9% NaCl to produce a final concentration of 0·1 g/100 ml (w/v).

Estimations

Samples of arterial and submaxillary venous effluent blood were collected into heparinised tubes containing aprotonin (Trasylol, Bayer; 1000 K.i.u./ml. blood) for VIP assays. These tubes were centrifuged at +4 °C immediately and the plasma then stored at −20 °C. VIP was measured by radioimmunoassay as described previously (Mitchell & Bloom, 1978). The arterial haematocrit was monitored at intervals.

Statistical analyses have been made according to the methods of Snedecor & Cochran (1967)

RESULTS

Responses to stimulation of the parasympathetic innervation

Stimulation of the peripheral end of the isolated chorda tympani at 20 Hz for 10 min produced an abrupt increase in the concentration of VIP in the ipsilateral submaxillary venous effluent plasma with no perceptible change in the concentration of the peptide in the arterial plasma (Fig. 1). Thus in a group of four cats mean submaxillary venous plasma VIP concentration had risen from 38 ± 11 p-mole/l., immediately prior to stimulation, to 288 ± 61 p-mole/l. at 5 min ($P < 0{\cdot}01$) and had then subsided to 58 ± 7 p-mole/l. 5 min after stimulation was discontinued. This response was accompanied by the expected rise in ipsilateral submaxillary blood flow, which rose from $0{\cdot}68 \pm 0{\cdot}33$ ml. g gland^{-1}.min^{-1}, before stimulation, to between 2·50 and 3·05 ml. g gland^{-1}.min^{-1} during stimulation. Chorda stimulation was associated with a slight, but statistically insignificant fall in mean aortic pressure (Fig. 1). As there was no rise in the effective perfusion pressure, the increase in submaxillary blood flow can be attributed entirely to dilatation of blood vessels within the gland.

Submaxillary blood flow was estimated gravimetrically and corrected for haematocrit. The output of VIP from the gland was then estimated from the plasma flow and the concentration of the peptide therein. At rest, the concentration of VIP in the arterial plasma was found to be slightly higher than that in the submaxillary venous effluent plasma, so producing a small positive arterio-venous difference of between 3 ± 9 and 8 ± 3 f-mole/min. This situation was reversed during stimulation of the chorda tympani, when the output of VIP rose rapidly to a peak of 1150 ± 400

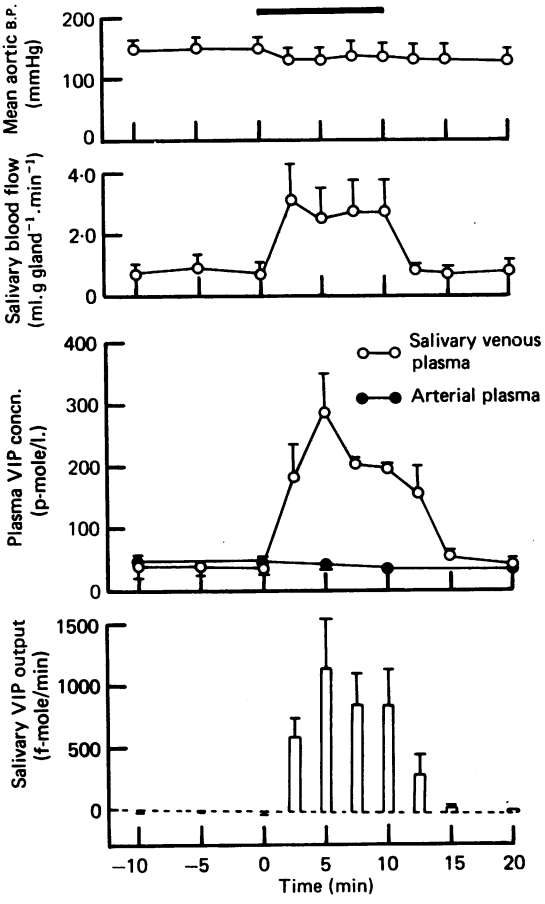

Fig. 1. Changes in mean aortic blood pressure, submaxillary blood flow, arterial (●) and submaxillary venous (○) plasma VIP concentration, and in the output of VIP from the submaxillary gland in anaesthetized cats in response to stimulation of the chorda tympani at 20 Hz for 10 min (n = 4). Horizontal bar: duration of stimulus. Vertical bars: S.E. of each mean value.

f-mole/min at 5 min and to plateau values of 861 ± 249 and 859 ± 294 f-mole/min at 7·5 and 10 min respectively. The output of VIP from the submaxillary gland fell abruptly when stimulation was discontinued. It should be noted that, whereas efforts were made to reduce the dead space in these experiments so far as was practicable the values presented here have not been adjusted in any way to correct for 'irreducible dead-space'; theoretical correction for this factor would increase the output at 2·5 min and reduce it at 12·5 min (Fig. 1), producing an even more abrupt change at onset and offset of stimulation.

The question whether muscarinic receptors might be implicated in the release of VIP from the submaxillary gland, in response to stimulation of the chorda tympani, was investigated in a group of five cats tested in precisely the same way except that they were each given atropine (1·0 mg/kg, I.V.) 10 min before stimulation. The results of these experiments are illustrated in Fig. 2, and show

that the changes in ipsilateral submaxillary blood flow and submaxillary venous plasma VIP concentration are of the same order of magnitude as those which occurred in response to chorda stimulation in the previous group, in the absence of atropine. Thus salivary blood flow rose from $1{\cdot}01 \pm 0{\cdot}30$ ml.g gland^{-1}.min^{-1}, immediately before stimulation, to a peak value of $3{\cdot}13 \pm 0{\cdot}87$ ml.g gland^{-1}.min^{-1} at 2·5 min and the concentrations of VIP in the submaxillary venous effluent plasma at these times were 48 ± 10 and 227 ± 56 respectively ($P < 0{\cdot}02$). The changes in mean aortic blood pressure in the two groups of animals were also closely similar (Figs. 1 and 2).

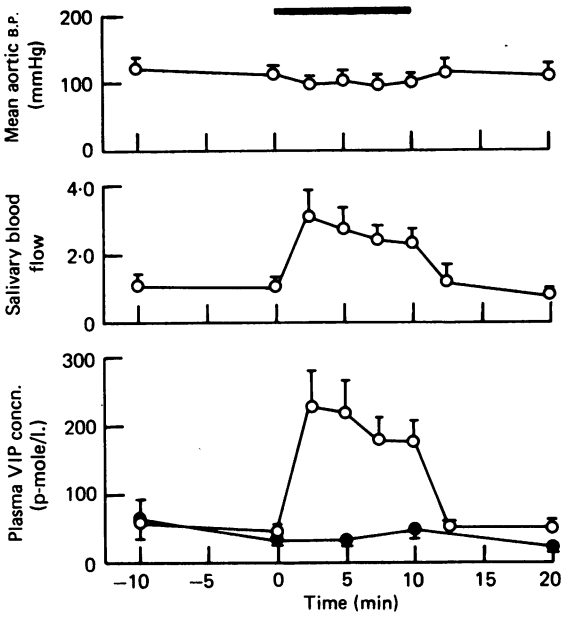

Fig. 2. Changes in mean aortic blood pressure, submaxillary blood flow, arterial (●) and submaxillary venous (○) plasma VIP concentration, in anaesthetized cats given atropine (1·0 mg/kg), in response to stimulation of the chorda tympani at 20 Hz for 10 min ($n = 5$). Horizontal bar: duration of stimulus. Vertical bars: S.E. of each mean value.

The possibility that one or more of the responses to chorda stimulation might be blocked by somatostatin was examined in a further group of three cats. Each of these was tested, first by stimulating the peripheral end of the right chorda tympani at 20 Hz for 10 min in the absence of somatostatin, and then by repeating the stimulus during a continuous I.V. infusion of the peptide at a high dose (500 ng.kg^{-1}.min^{-1}). None of the responses to chorda stimulation, namely secretion of submaxillary saliva, submaxillary vasodilatation or release of VIP from the gland were significantly affected by the infusion of somatostatin and the record of salivary secretion and cardiovascular responses from one of these experiments is shown in Fig. 3.

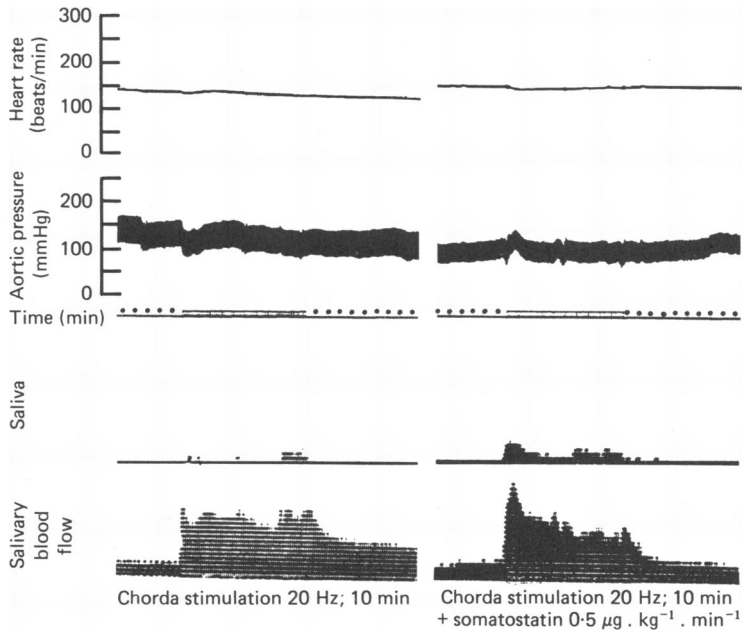

Fig. 3. Comparison of the changes in heart rate, aortic blood pressure, production of saliva from the right submaxillary gland and right submaxillary blood flow in an anaesthetized cat in response to stimulation of the ipsilateral chorda tympani at 20 Hz for 10 min in the presence and absence of somatostatin (infused I.V.; 500 ng.kg^{-1}. min^{-1}). Time in min and the two events are indicated immediately below the aortic blood pressure record.

Effects of intra-arterial infusions

The further question whether VIP released within the submaxillary gland, in response to chorda stimulation, might account for, or contribute to, the vasodilatation, which persists in the presence of atropine, was examined by infusing exogenous VIP directly into the carotid artery of five cats for 10 min. Each of these animals was atropinised (1·0 mg/kg body wt.) 10 min prior to infusion. The dose of VIP chosen (20 ng/min) for these intra-carotid infusions raised the concentration of the peptide in the submaxillary venous effluent plasma by approximately the same amount as that previously encountered in response to chorda stimulation at 20 Hz. Thus, the concentration of VIP in the venous plasma rose from 42 ± 6 p-mole/l. initially, to between 240 and 323 p-mole/l. during the course of the infusion. The results of these infusion experiments differ from those of the nerve stimulation studies in that there was also a significant rise in the concentration of VIP in the arterial plasma (Fig. 4). This is accounted for by the fact that the release of VIP in response to nerve stimulation was confined to those tissues innervated by the chorda tympani, whereas in these experiments the peptide was, of necessity, infused intra-arterially throughout the ipsilateral carotid vascular bed.

The increase in mean submaxillary blood flow which occurred in response to

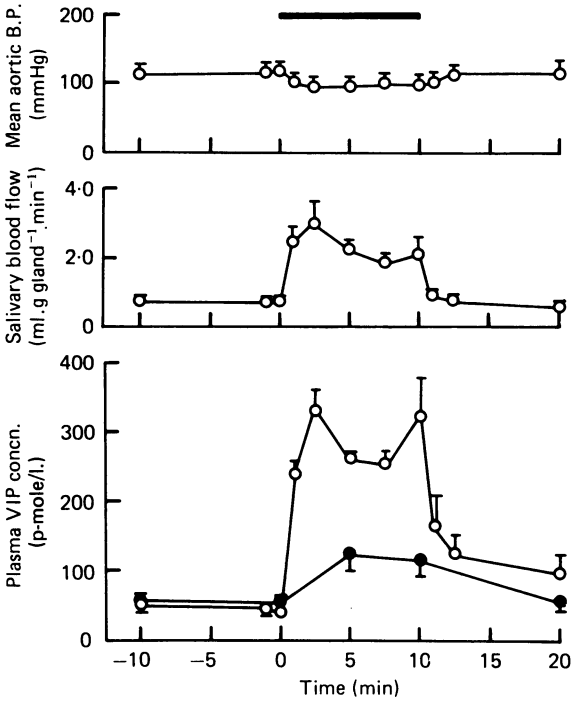

Fig. 4. Changes in mean aortic blood pressure, submaxillary blood flow, and in arterial (●) and submaxillary venous (○) plasma VIP concentration in anaesthetized cats (n = 5) in response to intra-carotid infusions of exogenous VIP (20 ng.min^{-1} for 10 min). Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value.

intra-carotid infusions of VIP in these cats was found to correspond closely with that observed in response to chorda stimulation at 20 Hz. This can best be seen by comparing the responses illustrated in Fig. 4 with those in Figs. 1 and 2. The absolute values for ipsilateral submaxillary blood flow were 0·74 ± 0·14 ml.g gland^{-1}.min^{-1}, before infusion, rising to between 1·84 and 2·99 ml.g gland^{-1}.min^{-1} during infusion.

As with the nerve stimulation experiments, there was no statistically significant change in mean aortic blood pressure, but the perceptible fall which occurred provides convincing evidence that the rise in submaxillary blood flow is wholly attributable to vasodilatation of submaxillary blood vessels. In no case did intra-carotid infusions of VIP stimulate secretion of submaxillary saliva, either at this or higher doses.

An attempt was made to match the submaxillary vasodilator response to intra-carotid infusions of VIP with ACh, delivered to the gland in the same way. It was found that the two agonists produced about the same effect on submaxillary blood flow and mean aortic pressure blood when infused intra-arterially on a weight to weight basis in the ratio 1·0 VIP : 10·0 ACh (Fig. 5). Given the disparity in molecular weight (VIP about 3200; ACh about 175) we concude that VIP is a more potent vasodilator agent than ACh, in respect of these particular blood vessels, by at least one, and probably two, orders of magnitude. A further point which emerges from this

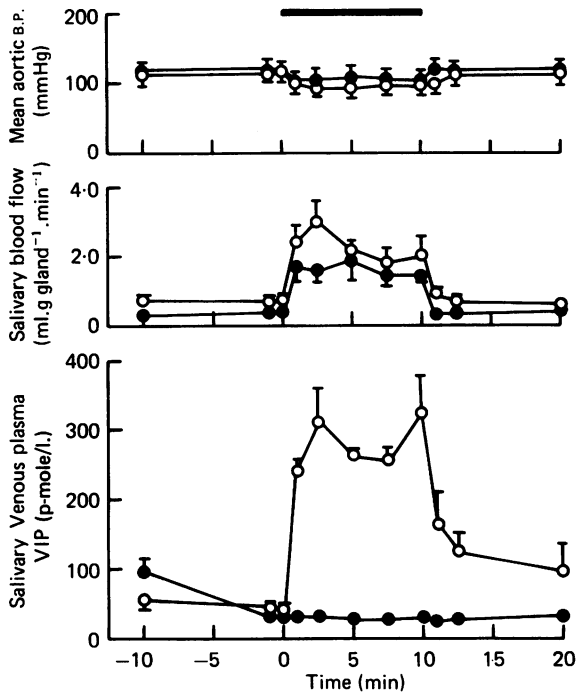

Fig. 5. Changes in mean aortic blood pressure, salivary blood flow and salivary venous plasma VIP concentration in anaesthetised cats in response to intra-carotid infusions of either acetyl choline (●); 200 ng.min^{-1}; n = 5) or VIP (○; 20 ng.min^{-1}; n = 5). Horizontal bar: duration of infusion. Vertical bars: S.E. of each mean value.

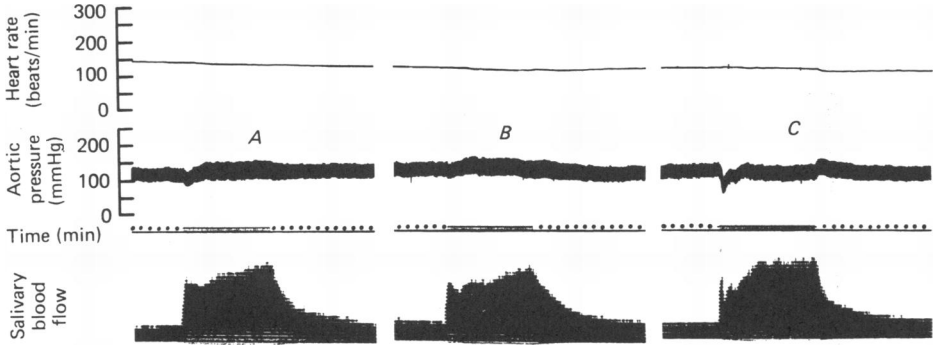

Fig. 6. Comparison of the changes in heart rate, aortic blood pressure and right submaxillary blood flow in an anaesthetized cat in response to (*A*) an infusion of VIP via the ipsilateral carotid artery (40 ng/min for 10 min). (*B*) stimulation of the ipsilateral chorda tympani (20 Hz for 10 min), and (*C*) an infusion of ACh via the ipsilateral carotid artery (200 ng/min for 10 min). Time in min and the three events are indicated immediately below the aortic blood pressure record.

direct comparison of the effects of two agonists is that this high dose of ACh produced no measurable release of VIP from the gland (Fig. 5).

The relative sensitivity of the submaxillary vasculature to VIP and ACh is illustrated in Fig. 6 in which the responses to intra-carotid infusions of the two agonists for 10 min are compared with the submaxillary vasodilatory response to chorda stimulation at 20 Hz for the same period. These tests were carried out in the same animal at intervals of 30 min. It can be seen that, in this particular

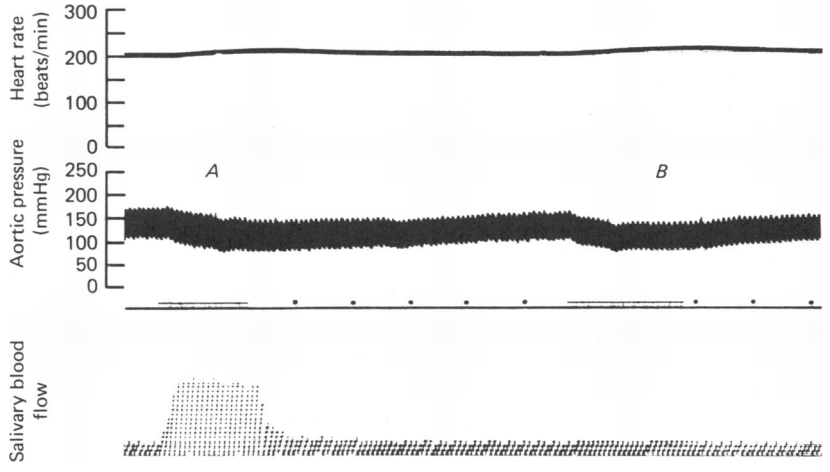

Fig. 7. Comparison of the changes in heart rate, aortic blood pressure and right submaxillary blood flow in an anaesthetized cat infused with VIP, first via the ipsilateral carotid artery (*A*) and again, 6 min later, intravenously (*B*) at the same dose (100 ng/min). Time in min and the two events are indicated immediately below the aortic blood pressure record.

experiment, ipsilateral submaxillary blood flow increases by roughly the same amount in response to an intracarotid infusion of VIP at a dose of 40 ng/min as it does to ACh at 200 ng/min and chorda stimulation at 20 Hz. The rapidity of on-set of all three responses were closely similar but salivary blood flow declined to the resting level more slowly following chorda stimulation than it did after intra-carotid infusions of either VIP or ACh.

Comparisons of the effects of exogenous VIP, infused directly into the carotid artery, with the effects of I.V. infusions of the peptide, at the same dose, showed that, whereas I.V. infusions produced a greater fall in aortic blood pressure, the submaxillary vasodilatory response was barely detectable (Fig. 7). This result is entirely consistent with the potent vasodilator activity of the peptide together with its short half-life in circulating blood. They also show that the peptide could not produce any *hormonal* vasodilator response in the submaxillary gland of the cat, at the concentrations which have been found to occur in the arterial blood in the present experiments.

Finally, we report the results of experiments in which we have compared the submaxillary vasodilatory responses to VIP and bradykinin infused for the same period via the ipsilateral carotid artery. In each experiment it was necessary to use

amounts of bradykinin one or more orders of magnitude higher than VIP in order to match the initial responses. We also found that, whereas the vasodilator response to intra-arterial infusions of VIP were maintained throughout the period of the infusion (so long as the aortic blood pressure was maintained within certain limits), the response to bradykinin invariably faded after 1–2 min. The result of a typical experiment, in which the two agonists were tested in the same cat for only 5 min are illustrated in Fig. 8.

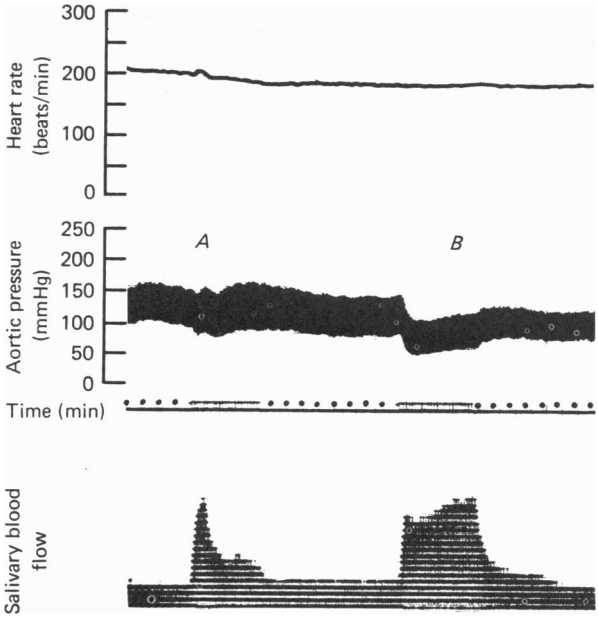

Fig. 8. Comparison of the changes in heart rate, aortic blood pressure and right submaxillary blood flow in an anaesthetised cat in response to ipsilateral intra-carotid infusions of (*A*) bradykinin (10 μg/min for 5 min) and (*B*) VIP (40 ng/min for 5 min). Time (min) and the two events are indicated immediately below the aortic blood pressure record.

DISCUSSION

It has been proposed that the atropine-resistant vasodilatation that occurs in the submaxillary gland, in response to stimulation of the chorda tympani can be accounted for by release of kallikrein from the salivary secretory cells, which promotes the production of a vasoactive kallidin (bradykinin) from an α-2 globulin in the tissue fluid (Ungar & Parrot, 1936; Hilton & Lewis, 1955*a*, *b*; 1956; Lewis, 1959; Hilton, 1960). However, the validity of this conclusion is questionable for various reasons. Atropine completely blocks the production of saliva from the submaxillary gland in response to chorda stimulation whereas the vasodilator response is resistant to atropine. In the dog, atropine produces a progressive reduction in the release of kinin in the venous effluent blood, in response to successive periods of chorda stimulation while the vasodilator response persists (Ferreira & Smaje,

1976). Bhoola *et al.* (1965) showed that the vasodilator response of the submaxillary vasculature to bradykinin could be substantially reduced, following the administration of relatively massive doses of the peptide, without significantly reducing that to chorda stimulation in atropinised cats. They also found that stimulation of the chorda tympani still produced marked vasodilatation in cats' submaxillary glands that had been perfused for prolonged periods with horse serum, from which *cat* salivary kallikrein releases no bradykinin (Bhoola *et al.* 1965). These workers concluded 'that kallikrein (and consequently bradykinin) does not play a significant role in the mediation of chorda-lingual induced vasodilatation in the submaxillary gland of the cat'.

The results of the present experiments provide further evidence in support of this view and for the alternative explanation that the atropine-resistant vasodilatation can be accounted for largely by the release and subsequent action of VIP. Direct comparison of the vasodilator response to intra-arterial infusions of VIP and bradykinin (Fig. 8) shows that the vasoactive potency of VIP is at least 500 times greater than that of bradykinin. Similar comparison of VIP with ACh (Fig. 6) shows VIP to be about 100 times as potent as ACh, in precise agreement with recent findings by others in the perfused submaxillary gland of the dog (Shimizu & Taira, 1979). In addition, chorda stimulation invariably produces an abrupt rise in the output of VIP from the gland via the venous effluent blood, in the presence or absence of atropine (Figs. 1 and 2), and the concentration of VIP in saliva has been found to be consistently lower than that of the arterial plasma (S. R. Bloom & A. V. Edwards, unpublished observations). In direct contrast, kallikrein is normally expelled from the gland in the saliva. Furthermore, intra-arterial infusions of VIP produce a maintained vasodilator response in the submaxillary gland for at least 10 min, whereas the response to bradykinin 'fades' within 1–2 min.

The present experiments were not designed to exclude the possibility that the kallikrein or kallidin (bradykinin) system contributes to the vasodilator response of the submaxillary vasculature to stimulation of the parasympathetic innervation. In the absence of atropine it is logical to suppose that release of ACh, which is clearly a pre-requisite for salivation, contributes to the vascular response. This view is strongly supported by the observation that whenever ACh is infused intra-arterially at a sufficiently high dose to elicit secretion of saliva, the secretory response is always accompanied by a substantial increase in submaxillary blood flow. We have found that, even after administration of atropine, submaxillary blood flow subsides more rapidly to normal following intra-arterial infusions of VIP than after chorda stimulation, which could well be accounted for by accumulation of bradykinin within the gland in the latter but not the former situation. Occlusion of Wharton's duct during chorda stimulation produces prolonged vasodilatation in the gland (Hilton & Lewis, 1955*b*) under conditions which presumably maximise the rate at which bradykinin is produced within the tissue, by blocking the salivary route for expulsion of kallikrein. However, it is not immediately apparent how such a finding could be extrapolated to evaluate the role of the kallikrein/kallidin system in the normal gland. The results presented here indicate that if bradykinin contributes to submaxillary vasodilatation in response to chorda stimulation, it is subsidiary to the effect of VIP and, in the absence of atropine, that of ACh.

The principal aim of these experiments was to investigate the question whether VIP might act as a transmitter to produce vasodilatation in the submaxillary gland, as has been suggested in respect of other actions of VIP, less susceptible to direct analysis, elsewhere in the gastro-intestinal tract (Fahrenkrug *et al.* 1978*a*, *b*; Bloom & Edwards, 1980). The evidence at present available may be summarized as follows.

1. VIP has been identified by immunocytochemical techniques within nerve terminals in the submaxillary gland (Bloom *et al.* 1979).

2. It is released from the gland in response to electrical stimulation of the parasympathetic innervation, in the presence or absence of atropine.

3. Intra-arterial infusions of ACh, at a dose which produces a maximal vasodilator response has no effect on submaxillary VIP release.

4. VIP is a far more potent vasodilator agent than the established transmitter, ACh.

5. Intra-arterial infusions of VIP, which mimic the rise in the concentration of the peptide in submaxillary venous effluent plasma during chorda stimulation, also produce an increase in submaxillary blood flow of the same order of magnitude as that observed during chorda stimulation at the same frequency.

We conclude that VIP is released from post-ganglionic parasympathetic neurones in the submaxillary gland of the cat and acts, as a transmitter, to cause vasodilatation and that this response is resistant to atropine.

This work has been supported by the Medical Research Council. We are also indebted to Dr D. M. Burley (Ciba) for his continued support. It is a particular pleasure to record our appreciation of the skilled technical assistance provided by Messrs P. M. M. Bircham and G. P. McGregor.

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