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THE RELATIONSHIP BETWEEN GLANDULAR ACTIVITY, BRADYKININ FORMATION AND FUNCTIONAL VASODILATATION IN THE SUBMANDIBULAR SALIVARY GLAND

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As a result of previous experiments on the submandibular salivary gland, it was concluded that functional hyperaemia in this organ is due to the vasodilator action of a material indistinguishable from bradykinin. This is formed in the interstitial fluid by the action on the plasma proteins of an enzyme-like substance escaping from the gland cells during activity (Hilton & Lewis, 1955*a*, *b*).

In those experiments the enzyme was demonstrated in the effluent collected from the perfused gland activated by stimulation of the chorda tympani. In the present investigation we have examined the question whether this release is always obtained following glandular activity, no matter whether this is produced by chorda or sympathetic stimulation, or by injection of acetylcholine or sympathomimetic amines. The release of bradykinin-forming enzyme from the perfused gland has been followed quantitatively, and the results show a consistent relationship between glandular activity, local vasodilatation and release of bradykinin-forming enzyme.

METHODS

The experiments were performed on cats under pentobarbitone anaesthesia (40 mg/kg). The preparation of the submandibular salivary gland for the isolation of its arterial inflow and venous outflow, and for the stimulation of the chordo-lingual nerve, was carried out as previously described (Hilton & Lewis, 1955*a*). In some experiments the cervical sympathetic nerve trunk was separated out and divided in the neck, the peripheral end being fixed in a fluid electrode. The lingual artery was cannulated for injection and perfusion. The venous outflow was measured using a photoelectric drop recorder (Hilton & Lywood, 1954), drop-formation being registered with a Thorp impulse counter or a Gaddum drop-timer. The fluid used for perfusion was either oxygenated Locke's solution or an oxygenated dextran-saline solution (Plasmosan, May and Baker). The chordo-lingual and cervical sympathetic nerves were stimulated with square waves of supra-

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maximal voltage and 0·5 msec duration at 10/sec. One femoral vein was cannulated for intravenous injections, and the femoral artery of the other leg for recording the arterial blood pressure. Heparin (10 mg/kg) was injected into the vein cannula before any of the other vessels were cannulated.

Collection and assay of perfusate

Perfusion was carried out from a Marriotte bottle at a low head of pressure (60–70 mm Hg), maintaining a flow through the gland of 1–1·5 ml./min. After 2–3 min perfusion, when the outflow was almost clear of blood, perfusate was collected in successive 3 or 5 min samples for a total period of 30–35 min. The samples were collected in siliconed glass containers, which were kept in ice until the assay was completed. The assays were carried out on the isolated non-pregnant rat uterus. Each horn was mounted in a 15 ml. organ bath containing de Jalon's solution and maintained at a temperature of 29–30° C. When perfusate was tested for its content of bradykinin-forming enzyme, an appropriate volume was added to excess of dog's pseudoglobulin, and the mixture was incubated at 30° C for 1 min. It was then transferred to the organ bath, and assayed against a standard preparation of bradykinin.

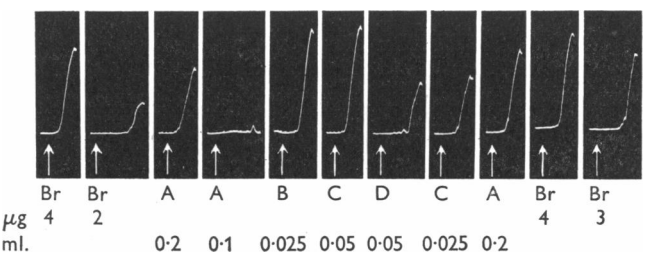

Fig. 1. Rat uterus suspended in 15 ml. de Jalon's solution. Assay against standard bradykinin (Br) of bradykinin-forming activity of samples of perfusate (A, B, C, D). Sample A taken from resting gland, and B during and in the 2 min following 1 min chorda stimulation. C and D were subsequent 5 min samples. The figures represent the volume in ml. taken for incubation with excess pseudoglobulin, each for 1 min at 30° C. B, C and D were diluted 1:10 with saline before incubation.

Some of the results obtained in a typical assay are shown in Fig. 1. Using the rat uterus as a test object, the tests can only be carried out at 5 min intervals in most preparations. Therefore, since it was necessary to test the samples of perfusate as soon as possible after collection, the procedure adopted was to establish the dose-response curve before samples were collected, to test the samples themselves and finally to repeat the dose-response curve. In many experiments it was possible to interpolate doses of bradykinin during the testing of samples. The enzyme activity of the perfusate has been expressed in terms of equi-active amounts of standard bradykinin, the appropriate calculation being made for the volume and time of collection in order to derive the amount of activity per minute of perfusion for each sample.

RESULTS

Release of bradykinin-forming enzyme from the resting submandibular salivary gland

In previous experiments the salivary gland was perfused with Locke's solution and the perfusate examined for the presence of bradykinin-forming enzyme. Samples of perfusate, after incubation with dog plasma, were tested for bradykinin on the guinea-pig ileum. It was found that, whereas incubated

perfusate from the resting gland was inactive, incubated perfusate collected during chorda stimulation possessed bradykinin-like activity (Hilton & Lewis, 1955*b*). In the present investigation perfusate has been incubated with pseudoglobulin instead of plasma and the subsequent assay for bradykinin carried out on the rat uterus, which is 20 times more sensitive than the guinea-pig ileum. With this sensitive preparation even perfusate collected from the resting gland showed bradykinin-like activity after incubation. Thus small amounts of the enzyme do escape from the perfused gland even at rest.

TABLE 1. Activity of perfusate (expressed as μg bradykinin per min perfusion) from the resting gland, in 32 experiments

1–9 (μg/min)	10–19 (μg/min)	20–250 (μg/min)
1	10	23
1·5	11	36
2	11·5	37
2	13	50
4	13	51
4	14	60
4·5	15	76
5	17	78
5	17	150
6		235
7		
8·5		
9		

The enzyme activity of perfusate from the resting gland was assayed in thirty-two experiments and the results are shown in Table 1. The figures express in μg bradykinin the activity for 1 min perfusion. In two-thirds of the experiments the activity was less than 20 μg/min, and in most of these it was even less than 10 μg/min; but in a few experiments much higher values were obtained. When perfusion of the resting gland was continued for 30–35 min, the enzyme activity of the perfusate remained fairly steady throughout, as illustrated in Fig. 2*a*.

Release of bradykinin-forming enzyme on activation of the salivary gland by chorda stimulation or by acetylcholine

In these experiments one or more 5 min resting samples of perfusate were first collected. Then either the chorda was stimulated or ACh was infused arterially for 1 min, and the next sample was collected during this period and the following 2 min. Subsequent samples were again collected in 5 min periods.

Chorda stimulation increased the enzyme activity of the perfusate, no matter whether the resting value was low or high: the activity was increased between 2·5 and 8·5 times. A fourfold increase is shown in the experiment of Fig. 2*b*. The sample collected during and immediately after the 1 min chorda stimulation invariably contained the highest enzyme activity, though increased

activity was always found also in the following 5 min sample, sometimes in the second, and occasionally even in the third.

All the samples of perfusate, when tested on the rat uterus without previous incubation with pseudoglobulin, were without effect, with the exception of the sample collected during and immediately after chorda stimulation, which did, on occasion, cause the rat uterus to contract. Since the sample was rendered inactive on incubation with chymotrypsin, the contraction was probably due to bradykinin. This pre-formed bradykinin never accounted for more than 10 % of the activity obtained after incubation of the sample with pseudoglobulin.

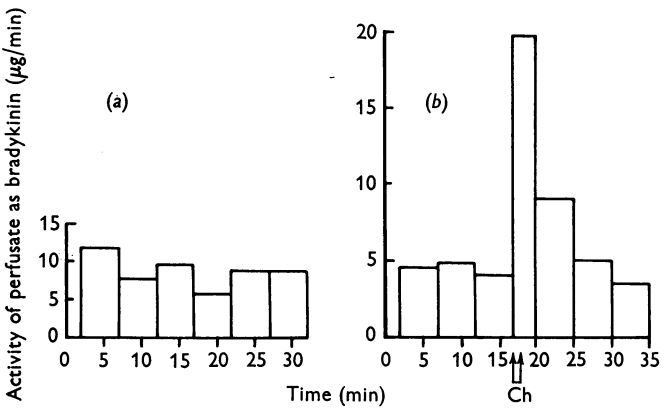

Fig. 2. Output of bradykinin-forming enzyme from the submandibular salivary glands of two cats, perfused for 35 min. *a*, resting gland; *b*, gland activated by 1 min chorda stimulation (at Ch). In each experiment gland was perfused for 2 min, then perfusate was collected in 5 min periods, except for the 3 min samples collected during and in the 2 min following chorda stimulation.

In these experiments with Locke's solution the perfusion pressure must be kept low, in order to avoid gross oedema, and under these conditions vasodilatation is not observed, either from chorda stimulation or injections of acetylcholine or histamine. On the other hand, when the gland is perfused with a dextran-saline solution, higher perfusion pressures can be used, which are closer to normal, and all the usual vasodilator responses can be obtained. In a few experiments the output of bradykinin-forming enzyme was examined from the gland perfused with dextran-saline, and the results were essentially the same as those obtained when Locke's solution was used. There was a small output from the resting gland, and after chorda stimulation the enzyme activity increased up to 20 times.

Arterial infusion of acetylcholine. Previous experiments showed that the vasodilatation produced on injection of ACh into the arterial blood supply to the gland cannot be fully explained by a direct effect of ACh on the blood vessels of the gland, but must be attributed, at least in part, to the formation

within the gland of a relatively stable vasodilator substance (Hilton & Lewis, 1955*a*). The present perfusion experiments have shown that activation of the gland by ACh, like activation by chorda stimulation, releases the bradykinin-forming enzyme.

In preliminary experiments, it was found that 5–7·5 μg ACh infused over 1 min gave secretory and vasodilator responses comparable with those of 1 min maximal chorda stimulation. These doses of ACh were therefore used in the perfusion experiments; they caused an increase in the enzyme activity of the perfusate of the same order as that resulting from 1 min chorda stimulation (Figs. 3*a*, *b*).

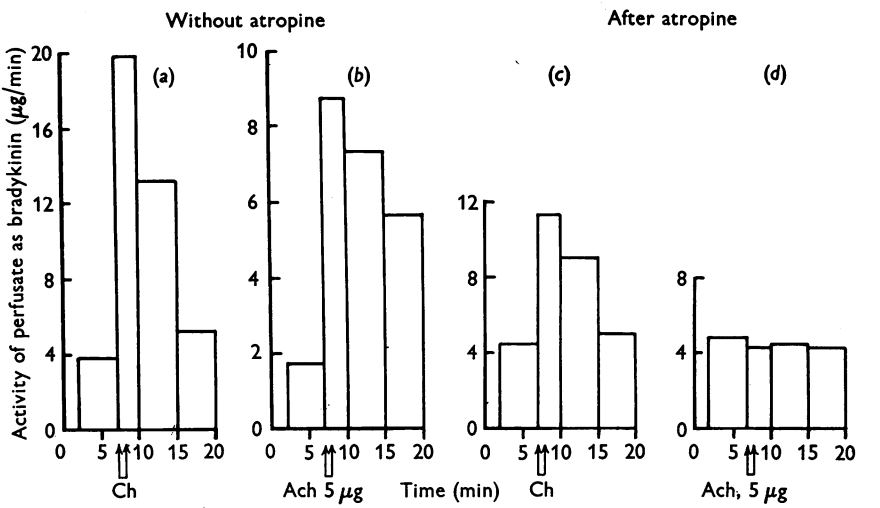

Fig. 3. Output of bradykinin-forming enzyme from the submandibular salivary glands of four cats, perfused for 20 min, and activated either by 1 min chorda stimulation (*a* and *c*) or by infusion of 5 μg ACh (*b* and *d*). Perfusion experiments (*a*) and (*b*) were performed without atropine, (*c*) and (*d*) after an intravenous injection of atropine, 0·5 mg/kg. In each experiment, gland was perfused for 2 min, then perfusate was collected in 5 min periods, except for the 3 min samples collected during and in the 2 min following activation.

This increase in enzyme activity results from a direct effect of ACh on the gland cells, and does not depend on stimulation of post-ganglionic neurones, for the increase also occurs when synaptic transmission has been abolished by hexamethonium. For instance, in one experiment, the gland was perfused with Locke's solution containing hexamethonium bromide (300 μg/ml.), which abolished the effects of chorda stimulation. 5 μg ACh, infused over a period of 1 min, actively stimulated salivary secretion, and caused a fourfold increase of enzyme activity in the perfusate. At the end of the experiment the continued effectiveness of the hexamethonium was shown by the fact that chorda stimulation still had no effect, whereas 5 μg ACh caused vigorous secretion.

Effect of atropine. It is known that in the submandibular salivary gland atropine abolishes both the secretory and vasodilator effects of injected ACh, whereas only the secretory effect of chorda stimulation is abolished, the vasodilatation being merely reduced. When the enzyme release was studied in perfused atropinized glands, it was found that chorda stimulation still produced an increase in enzyme output, the stimulation sample containing 2–2·5 times the resting level of activity. On the other hand, ACh no longer caused an increase in the enzyme output of the gland. The results are illustrated by the experiments of Fig. 3*c*, *d*. In each case the atropine (0·5 mg/kg) was injected intravenously some time before perfusion was started. The increased enzyme output of the atropinized gland after chorda stimulation, although reduced, recalls Barcroft's finding (1914) that the oxygen consumption of the atropinized gland is also increased after chorda stimulation, and that this increase is smaller than that of the normal gland.

The role of the bradykinin-forming enzyme in the hyperaemia following sympathetic stimulation

Experiments with normal blood circulation. Short periods of sympathetic stimulation produce in the submandibular salivary gland of the cat a small amount of secretion accompanied by vasoconstriction. When stimulation is ended, and occasionally even before, a vasodilatation develops, which is frequently large and prolonged. In Fig. 4A is seen a typical response to stimulation of the cervical sympathetic trunk for 20 sec, compared with that of chorda stimulation for 10 sec. Arterial injections of noradrenaline and adrenaline produce the same type of secretory and vascular response as sympathetic stimulation. The vascular response to 1 μg noradrenaline is shown in Fig. 5A. The after-dilatation, which is such a prominent feature of these responses, cannot be attributed to reactive hyperaemia following the vasoconstriction because the vasodilator effect of a 20 sec period of circulatory arrest is so small as to be insignificant by comparison (Hilton & Lewis, 1955*a*).

Anti-adrenaline drugs, which abolish the secretory effect of cervical sympathetic stimulation, adrenaline and noradrenaline, reverse the immediate vasoconstrictor effect and abolish the after-dilatation. As no anti-adrenaline drug has been found to antagonize the inhibitory vascular responses of sympathomimetic amines, this finding is consistent with the view that the prolonged after-dilatation is due to activation of the gland.

In Fig. 4 the effect of 20 sec cervical sympathetic stimulation is seen, before and after an arterial injection of phentolamine. After the injection sympathetic stimulation no longer caused a secretion, and instead of an immediate vasoconstriction there was an immediate vasodilatation which, however, began to wear off even during the period of stimulation. When stimulation was maintained for as long as 1 min, the blood flow had returned to normal before the

end of the period of stimulation. In Fig. 5 is seen the similar effect of the β-haloethylamine, SY 28, on the vascular response to noradrenaline. There is no longer any sign of an after-dilatation. On the other hand, the secretory and vasodilator responses to chorda stimulation were not abolished by the injections.

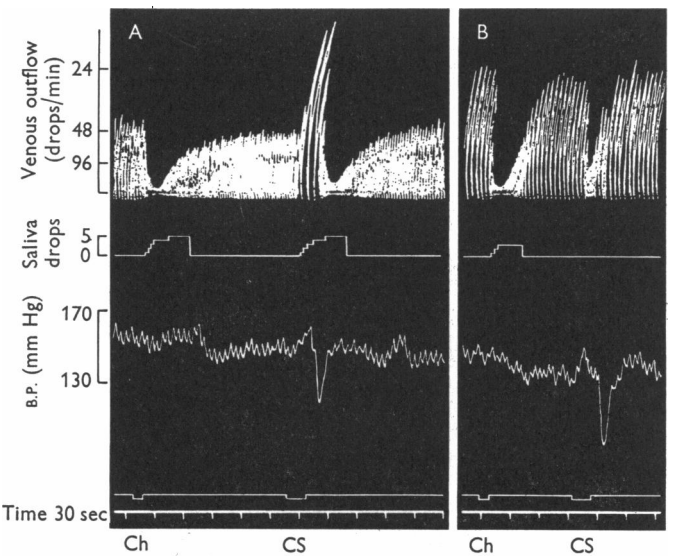

Fig. 4. Cat, 3·4 kg. Records of venous outflow (Gaddum drop-timer) from the submandibular salivary gland, of salivary secretion and arterial blood pressure. Effect of 10 sec chorda stimulation (Ch) and of 20 sec cervical sympathetic stimulation (CS) (A) before and (B) after 1 mg phentolamine injected arterially into the gland.

In 1912 Barcroft & Piper arrived at the same conclusion, namely, that the prolonged after-dilatation is due to activation of the gland. They had found that following an injection of ergotamine all the actions of intravenously injected adrenaline were abolished, including the increase in oxygen-consumption which was normally obtained during the after-dilatation.

Perfusion experiments. The following results show that the after-dilatation resulting from noradrenaline and adrenaline must be attributed to activation of the gland and to the release of bradykinin-forming enzyme. Preliminary experiments were first carried out which showed that 3–5 μg noradrenaline or adrenaline gave rise to secretory and vasodilator effects comparable with those of 1 min chorda stimulation. In the perfusion experiments these doses were infused over a period of 1 min, after resting samples of perfusate had been collected. The next sample was collected during and in the 2 min following the infusion, and further 5 min samples were collected subsequently. These infusions usually led to a large increase in the output of bradykinin-forming enzyme. However, unlike the experiments in which the gland was activated by chorda

stimulation, the greatest amount of enzyme activity was found in the third sample collected after the infusion. Since the first two samples were collected during the initial phase of vasoconstriction, the volumes were consequently very small and it was probably only during the third collection period after activation that the enzyme escaping from the gland cells was washed out by

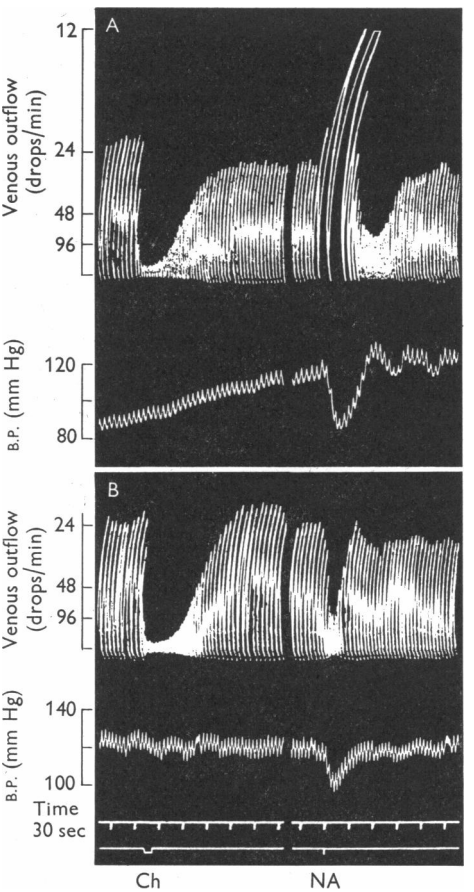

Fig. 5. Cat, 2·8 kg. Records of venous outflow (Gaddum drop-timer) from the submandibular salivary gland and of arterial blood pressure. Effect of 10 sec chorda stimulation (Ch) and arterial injection of 1 μg noradrenaline (NA), (A) before and (B) after 100 μg N-1 naphthylmethyl-2-bromoethylamine HBr (SY 28) injected arterially into the gland.

the perfusion fluid. A typical experiment is illustrated in Fig. 6 in which the increase in enzyme activity caused by an infusion of 5 μg noradrenaline is compared with that caused by 1 min chorda stimulation.

The active sample of perfusate contained not only the bradykinin-forming enzyme but also a large amount of bradykinin itself, as judged by the stimulating action of the perfusate on the rat uterus without incubation. This effect

was not due to the noradrenaline in the perfusate for it was not antagonized by the anti-adrenaline β-haloethylamine, SY 28; but, like that of bradykinin, the activity was destroyed by incubation with chymotrypsin.

In the experiments with adrenaline a difficulty arose because of the inhibition of the contractions of the rat uterus which this amine causes, even in high dilution. As little as 0·01 μg adrenaline reduces the contractions of the rat uterus to bradykinin. Samples of perfusate collected after an infusion of adrenaline contained sufficient of the amine to prevent the estimation of enzyme activity in several experiments. When, however, the increase in enzyme release was very great, the active perfusate could be diluted about 100 times, which made it possible to work below the threshold of the inhibitory action of the adrenaline in the sample.

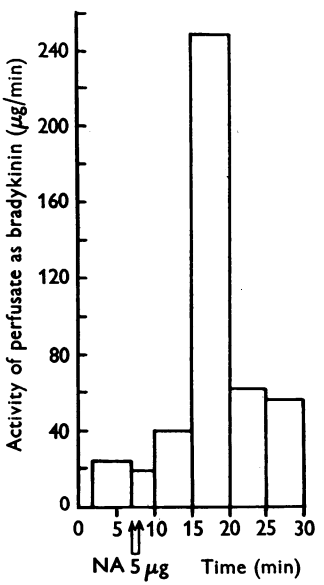

Fig. 6

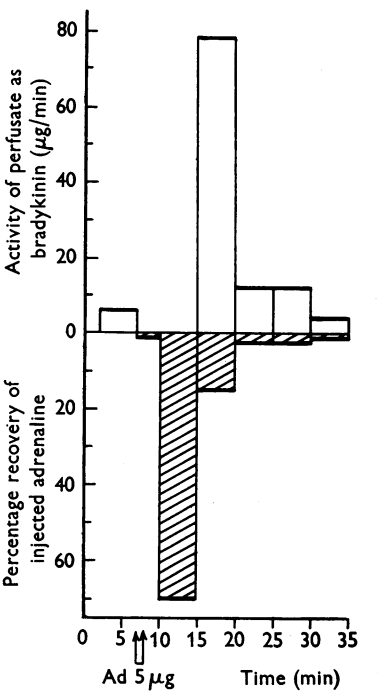

Fig. 7

Fig. 6. Output of bradykinin-forming enzyme from cat's submandibular salivary gland activated (at NA) by infusion of 5 μg noradrenaline. The gland was perfused for 2 min, then perfusate collected in 5 min periods except for the 3 min sample collected during and in the 2 min following activation.

Fig. 7. Output of bradykinin-forming enzyme (upper histogram) and percentage of injected adrenaline recovered (lower histogram) from cat's submandibular salivary gland activated (at Ad) by infusion of 5 μg adrenaline. The gland was perfused for 2 min then perfusate collected in 5 min periods except for the 3 min sample collected during and in the 2 min following activation.

The experiment illustrated in Fig. 7 shows the relationship between the output of enzyme caused by an infusion of 5 μg adrenaline and the distribution of the injected adrenaline in the samples of perfusate. The latter was estimated by its inhibitory action on the responses of the rat uterus to standard bradykinin. The sample of perfusate collected during the infusion and in the 2 min following was of very small volume. It contained little adrenaline and only a small amount of enzyme. The next sample was still small but contained over 50% of the injected adrenaline; its enzyme activity therefore could not be assayed. In the subsequent sample about 1 μg adrenaline was present; the sample was diluted 100 times before incubation with pseudoglobulin, and the enzyme activity could be estimated fairly accurately. The adrenaline content of later samples was small enough not to interfere with the estimation of their enzyme activity.

DISCUSSION

The present investigation shows that in whatever manner the perfused salivary gland is activated, whether by chorda stimulation or by infusion of acetylcholine or sympathomimetic amines, a large output of bradykinin-forming enzyme occurs. In addition, small amounts of the enzyme escape from the gland when it is at rest.

Escape of bradykinin-forming enzyme from the resting perfused gland does not necessarily mean that this leakage occurs under normal conditions of circulation. When capillaries are perfused with isotonic saline solutions, they become abnormally permeable. Recently, Wilhelm, Miles & Mackay (1955) have separated from serum a permeability factor (Miles & Wilhelm, 1955) which is activated when serum is diluted with saline solutions. It follows that this permeability factor will be formed in the vessels of the gland, and perhaps in the interstitial fluid, in the course of perfusion experiments; and this may be the main reason why some of the enzyme leaks from the gland into the capillaries. On the other hand, under normal conditions of blood circulation even when the gland is stimulated, the active vasodilator material is apparently eliminated entirely via the lymph (Hilton & Lewis, 1955*a*, *b*).

The increase in enzyme output resulting from activation of the perfused gland was found to persist for some time. In the gland with normal blood circulation the vasodilator effect of 1 min chorda stimulation lasts no more than 2 min at the outside, whereas in the perfusion experiments the output of enzyme was sometimes raised for 15 min. The prolonged time-course of enzyme release is probably another abnormal phenomenon resulting from the conditions prevailing during perfusion with salt solutions.

The finding that atropine only reduces but does not abolish the increase in enzyme output following chorda stimulation also provides support for the view that bradykinin formation is responsible for functional vasodilatation in the gland; for atropine does not abolish the vasodilatation caused by chorda

stimulation. Bradykinin formation and the resulting vasodilatation must be attributed to increased glandular activity, since it is known that the atropinized gland is activated by chorda stimulation, although no secretion is produced. This was shown by Barcroft (1914) who measured oxygen consumption, and by Anrep & Cannan (1922) who studied glucose utilization.

The effect of atropine on the response to ACh also shows interesting parallels with the results of earlier investigations. The vasodilatation produced by ACh as well as the increase in oxygen consumption of slices of the gland have long been known to be abolished by atropine (Deutsch & Raper, 1936; Brock, Druckrey & Herken, 1939). The present experiments show that the increase in enzyme output of the gland activated by ACh is likewise abolished by atropine. Vasodilatation, metabolic changes in the gland and output of bradykinin-forming enzyme all go hand in hand. These observations do not imply that ACh cannot relax the blood vessels of the gland by direct action, but that both the direct vasodilator effect of the injected drug and its stimulating action on the gland cells are abolished together.

There is, therefore, no need to postulate the existence of special vasodilator nerve fibres in the chorda tympani. Even the remote possibility that there are nerve fibres which release bradykinin-forming enzyme can be discounted, since an infusion of ACh leads to increased release of enzyme equally well before or after ganglionic transmission has been abolished by hexamethonium. We conclude that the enzyme arises from the gland cells themselves. The present findings, however, do not explain why, after atropine, chorda stimulation still causes activation of the gland leading to an increase in enzyme output and vasodilatation.

The results obtained with sympathomimetic amines readily explain the after-dilatation following sympathetic stimulation. The origin of this after-dilatation has hitherto been obscure. Carlson (1907) suggested that it resulted from the action of sympathetic vasodilator nerve fibres, because he could obtain this effect in some animals without the preceding vasoconstriction which usually accompanies stimulation itself, and vasodilatation was seen, though rarely, without secretion. However, Barcroft & Piper (1912) showed that the oxygen-consumption of the gland stimulated by adrenaline increased in parallel with the increase in blood flow, and Emmelin's experiments with anti-adrenaline substances (1955) showed that the secretion and vasodilatation always diminished or disappeared together. In our experiments also secretion and vasoconstriction followed by prolonged vasodilatation disappeared after phentolamine or the β-haloethylamine, SY 28. When the perfused gland was activated by infusion of sympathomimetic amines, a large output of bradykinin-forming enzyme was found to occur. The effect of cervical sympathetic stimulation itself on the release of the enzyme could not be examined, because the sympathetic nerve supply to the gland was inevitably damaged in the

course of the preparation for isolation of its arterial inflow. However, it would be difficult to accept that such an increase in enzyme output does not occur when the sympathomimetic amines are released as transmitters by the sympathetic nerve endings instead of being injected, and we conclude therefore that the after-dilatation following sympathetic stimulation is also the outcome of bradykinin formation.

It is not possible to say for certain whether the bradykinin sometimes found in the perfusate after activation of the gland was formed in the interstitial spaces or in the perfusate. The largest amounts were found in those experiments in which the gland was activated by noradrenaline or adrenaline. The vasoconstriction produced by these amines brought the flow of perfusate almost to a standstill for several minutes, and it was when the perfusing fluid flowed freely again that the effluent was found to contain the large quantities of bradykinin. It therefore seems that whereas the released enzyme can usually pass freely into the perfusion stream, when it is held up in the gland it reacts with the substrate which is still retained; and it is also possible that the sympathomimetic amines assist this reaction by partly overcoming the increased capillary permeability, in this way allowing longer contact between the released enzyme and the substrate in the interstitial fluid.

SUMMARY

1. The release of bradykinin-forming enzyme from the submandibular salivary gland perfused with Locke's solution has been studied in the cat. The enzyme activity of the perfusate has been assayed, after incubation with pseudoglobulin, against standard bradykinin on the isolated rat uterus.

2. Small amounts of the enzyme escape from the perfused gland at rest.

3. Activation of the perfused gland by chorda stimulation or infusion of acetylcholine leads to a 2·5–8·5 times increase in the output of bradykinin-forming enzyme.

4. When the perfused gland is atropinized chorda stimulation still produces an increase in enzyme of 2–2·5 times, but an infusion of acetylcholine no longer has any effect. This accords well with the effects of atropine on the vasodilator and metabolic responses of the gland to chorda stimulation and acetylcholine.

5. Activation of the perfused gland by noradrenaline and adrenaline also increases the output of bradykinin-forming enzyme. The after-dilatation seen in the gland with normal blood circulation, following sympathetic stimulation or injection of the sympathomimetic amines, is explained by the formation of bradykinin.

6. It is concluded that however the gland is activated, by chorda or sympathetic stimulation or by infusions of acetylcholine or sympathomimetic amines, bradykinin-forming enzyme is released from the gland cells; and

further that the vasodilatation accompanying the glandular activity is produced by the bradykinin which is formed. There is no need to postulate a separate vasodilator innervation.

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