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THE ROLE OF CYCLIC NUCLEOTIDES AND RELATED COMPOUNDS IN NERVE-MEDIATED VASODILATATION IN THE CAT SUBMANDIBULAR GLAND

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1 Intra-arterial administration of a number of purine compounds to the cat submandibular salivary gland led to an increased blood flow. The threshold concentration of the most potent vasodilators, adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP) was about 2 μmol/l. Adenosine and guanosine 5'-triphosphate (GTP) required about 25 μmol/l, adenosine 3',5'-cyclic monophosphate (cyclic AMP) 40 μmol/l, guanosine 5'-diphosphate (GDP) 125 μmol/l and dibutyryl guanosine 3',5' cyclic monophosphate (db cyclic GMP) 400 μmol/l. Dibutyryl cyclic AMP and cyclic GMP were ineffective.

2 The cyclic nucleotide phosphodiesterase inhibitors, theophylline, papaverine, quinine and 3-isobutyl-1-methylxanthine (IBMX), all acted as vasodilators.

3 When intra-arterial infusion of theophylline or IBMX was combined with sympathetic nerve stimulation, the vasodilatation observed after the stimulus ceased was significantly potentiated.

4 Theophylline and IBMX also potentiated the vasodilatation accompanying parasympathetic nerve stimulation and this response persisted after atropine.

5 These results are discussed in relation to the possible mediators of sympathetic and parasympathetic vasodilatation in the gland.

Introduction

The production of saliva by the cat submandibular gland can be induced either by sympathetic or parasympathetic nerve stimulation. In both cases, there is an accompanying change in blood flow. With sympathetic stimulation, vasoconstriction is observed during the stimulus period but on switching off the current this is replaced by a vasodilatation (the 'after dilatation'). Parasympathetic stimulation also produces a vasodilatation but this occurs during the stimulus period and is usually more marked than the sympathetic response. Atropine blocks the secretion produced by parasympathetic stimulation but the vasodilatation persists (see Burgen & Emmelin, 1961). This observation thus poses a problem as to the mediator of the vasodilatation.

Early studies of nerve-mediated responses in the cat submandibular gland led to the suggestion that metabolites might be responsible for the changes in blood flow (see Barcroft, 1914). A more specific interpretation of this thesis was put forward by Hilton & Lewis (1955a; 1955b; 1956), who suggested that kinins, released in the active gland, could explain both the sympathetic and parasympathetic vasodilator responses. However, others have criticised this view, maintaining, in the case of parasympathetic stimulation, that vasodilatation is secondary to activation of specific vasodilator fibres and, in the case of sympathetic stimulation, to triggering of β-adrenoceptors (Davey, Davies, Reinert & Scholfield, 1965; Bhoola, Morley, Schachter & Smaje, 1965; Morley, Schachter & Smaje, 1966). The middle ground between these extremes was adopted by Gautvik (1970a), who showed that while kallikrein seemed to be involved, direct vasodilator nerves also appeared to play a role. Ferreira & Smaje (1976) came to a similar conclusion.

Skinner & Webster (1968) considered that β-receptors were activated by parasympathetic stimulation, largely on the grounds that the vasodilatation remaining after atropine was found to be reduced or completely abolished by large doses of the β-adrenoceptor blocker, propranolol. This interpretation was questioned by Schachter & Beilenson (1968) and Gautvik

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(1970b) who demonstrated the non-specific action of propranolol by its reduction of the hyperaemia induced by close-arterial injection of acetylcholine. Furthermore, the former authors showed that when the animals were pretreated with the sympatholytic agent, reserpine, the chorda vasodilatation remained unaffected, thus agreeing with earlier findings where reserpine (Davey *et al.*, 1965) and guanethidine (Davey *et al.*, 1965; Bhoola *et al.*, 1965) had been used.

If β-receptor activation were involved in either sympathetic or parasympathetic stimulation, then one might expect adenosine 3',5' cyclic monophosphate (cyclic AMP) to act as the intracellular mediator (Robison, Butcher & Sutherland, 1971). Certainly β-receptor stimulation leads to an increase in adenylate cyclase activity (see Butcher, Goldman & Nemerovski, 1976) and cyclic AMP concentration (see Albano, Bhoola, Heap & Lemon, 1976) in the whole gland but experiments so far have been concerned with secretory activity, not the control of blood flow.

An intracellular mediator proposed for some parasympathetic responses is guanosine 3',5' cyclic monophosphate (cyclic GMP) but this is less well established. Cyclic nucleotides could thus be involved in the vasodilatation associated with both sympathetic and parasympathetic stimulation.

If cyclic nucleotides are involved, then administration of phosphodiesterase inhibitors, which prevent intracellular breakdown of cyclic nucleotides (Sutherland, Robison & Butcher, 1968), should potentiate nerve-mediated responses. Whatever the mediator, exogenous administration should be able to mimic the natural response.

An alternative explanation for some atropine-resistant phenomena and other non-cholinergic, non-adrenergic responses is the release of the non-cyclic nucleotide adenosine 5'-triphosphate (ATP) from 'purinergic' nerves (Burnstock, 1972; 1975). This view is not universally accepted and an alternative hypothesis implicating peptides has been proposed (Bloom & Polak, 1978).

The situation is thus complex, if not confused, for both sympathetic and parasympathetic vasodilatation. In the present study, attention has been directed towards the vascular effects of purine compounds and phosphodiesterase inhibitors and how these might explain some of the underlying mechanisms in nerve mediated vasodilatation in the cat submandibular gland. Preliminary accounts of part of this work have already been published (Jones & Mann, 1976; 1977).

Methods

Thirty-five cats of either sex, weighing between 1.45 kg and 4.65 kg, were used. Anaesthesia was induced by sodium pentobarbitone (35 mg/kg i.p. Sagatal, May & Baker Ltd.) and supplementary doses administered as required by means of a cannula inserted in the femoral vein. The trachea and femoral artery were also cannulated.

Recording techniques

Arterial blood pressure, salivation and blood flow through the right submandibular gland were monitored on a Devices 4-channel pen recorder.

Arterial blood pressure was determined by means of a Bell and Howell pressure transducer (Type 4-327-L221) connected to a cannula ('red' Portex nylon) inserted in the right femoral artery.

Salivation was monitored from a cannula present in the submandibular duct. Drops interrupted a light beam focused on a photodiode which triggered a square wave generator, and the square waves were then recorded directly.

Blood flow through the right submandibular gland was recorded from the cannulated external jugular vein by a venous outflow method. All tributaries entering this vessel were tied off except that coming from the gland itself. Flow was then determined in a manner analogous to salivation except that the drops were used to produce square waves which were integrated and the integrator discharged at regular intervals. Venous effluent was returned to the animal automatically using the reservoir system described by Bhoola *et al.* (1965). Before any recording began, the external circuit was filled with heparin-saline (10 iu heparin/ml of 0.9% w/v NaCl solution) and the animal itself was heparinised (1,000 iu/kg i.v.).

Nerve dissection and stimulation

Chorda-lingual nerve After dissecting the chorda-lingual nerve as described by Darke & Smaje (1972), the distal end was mounted in a shielded electrode which was then filled with 0.9% w/v NaCl solution (saline).

Sympathetic supply The sympathetic trunk was freed from the vagus and cut in the region 2 to 3 cm caudal to the superior cervical ganglion. The distal end was then mounted in a manner similar to that described above. Both nerves were stimulated at supramaximal voltage (5 to 12 V) with square waves of 2 ms duration. Stimulus frequencies were slected in the range 1 to 8 Hz for the parasympathetic and 2 to 20 Hz for the sympathetic.

Arterial injections

The main route of administration was retrogradely down the lingual artery via a nylon cannula ('blue' Portex) initially filled with heparin-saline. Other branches of the common carotid artery were not usually tied as this damages the sympathetic supply to the gland: injections performed in this way are referred to as 'intra-arterial'. In 5 experiments, the arterial supply was completely isolated by tying all branches of the carotid artery except the lingual and submandibular arteries: this type of injection is referred to as 'close-arterial'. In two of these experiments, the gland was perfused at constant flow via the carotid artery with autologous blood from the femoral artery using a Harvard model 1203 peristaltic pump (Harvard Apparatus, Millis, Mass., U.S.A.).

Compounds used

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine, guanosine 5'-triphosphate (GTP), guanosine 5'-diphosphate (GDP), adenosine 3',5' cyclic monophosphate (cyclic AMP), N^6–$O^{2'}$ dibutyryl cyclic AMP (db cyclic AMP), guanosine 3',5' cyclic monophosphate (cyclic GMP) and N^6–$O^{2'}$ dibutyryl cyclic GMP (db cyclic GMP) were purchased from Boehringer (Mannheim); theophylline, papaverine and quinine hydrochloride from Sigma, 3-isobutyl-1-methylxanthine (IBMX) from the Aldrich Chemical Co.; atropine sulphate from BDH, propranolol hydrochloride (Inderal, 0.1% solution) from ICI and guanethidine from CIBA. Dipyridamole (Persantin) was donated by Boehringer (Ingelheim). Heparin was supplied as a solution of 5000 iu/ml by Weddel Pharmaceuticals.

Microspheres 15 μm in diameter, obtained from Pharmacia U.K. Ltd., were labelled with ^{51}Cr (Radiochemical Centre, Amersham) and washed with saline to remove free isotope. The spheres were then suspended in blood before injection.

Results

Vasodilator activity of purine compounds and phosphodiesterase inhibitors

The vasodilator effects of the following substances were investigated on the vasculature of the submandibular salivary gland: ATP, ADP, adenosine, GTP, GDP, cyclic AMP, cyclic GMP, dibutyryl cyclic AMP (db cyclic AMP), dibutyryl cyclic GMP (db cyclic GMP), and several phosphodiesterase inhibitors. Compounds were administered either close-arterially or intra-arterially (see Methods) at 4 to 10 μl/s for 20 to 30 s in concentrations ranging from 1 μmol/l to 20 mmol/l at the site of injection. Changes in blood flow are expressed in terms of conductance (1/resistance, where resistance = mean arterial blood pressure (mmHg)/blood flow (ml/min)). The use of conductance rather than blood flow minimizes variations due to changes in blood pressure. When measuring the efficacy of drugs, the basal blood flow was taken as that following infusion of an equivalent volume of saline, so a relative conductance of 2.5 means a total flow 2.5 times that obtained during saline infusion, assuming no change in blood pressure. Normally, a 2 min period after the beginning of the injection was used for analysis. Typically during this period basal flow would be 16 to 20 drops, saline would add 2 to 4 drops, so a relative conductance of 2.5 would indicate 45 to 60 drops during the 2 min. The results of these experiments are described in detail below.

It is worth observing here that the concentration of compounds at the site of injection was greater than that in the gland vasculature. With close-arterial injection, the rate of infusion was approx. 40% of the blood flow, so the blood concentration was approx. 30% of that injected. With intra-arterial injections, the discrepancy was greater as branches of the carotid artery were not ligated (see above). Total carotid flow was calculated from the basal blood flow to the gland and the proportion of microspheres injected into the lingual artery that were trapped by the gland (see later). This information, together with the rate of injection, allows determination of the drug concentration in the gland vasculature, which was calculated to be approx. 6% of that injected.

Purine compounds

ATP, ADP, adenosine, GTP, GDP These compounds were administered by close-arterial injection at 4 μl/s for 30 s and dose-response curves determined in 2 animals. Figure 1 illustrates part of one of these. For each drug, 3 doses were used and log dose was plotted against conductance. The dose which produced a conductance just greater than that found during infusion of an equivalent volume of saline was termed the threshold. ATP and ADP were the most potent vasodilators with a threshold concentration in the blood vessels of about 2 μmol/l. The threshold concentrations of the other purine compounds were: GTP, 25 μmol/l; adenosine, 28 μmol/l; GDP 125 μmol/l.

Cyclic AMP, cyclic GMP and their dibutyryl derivatives The cyclic nucleotides were less potent vasodilators than ATP or ADP. The threshold concentration of cyclic AMP was 40 μmol/l, 20 times greater than that for ATP. Intra-arterial infusion of 10 mmol/l cyclic AMP increased mean conductance

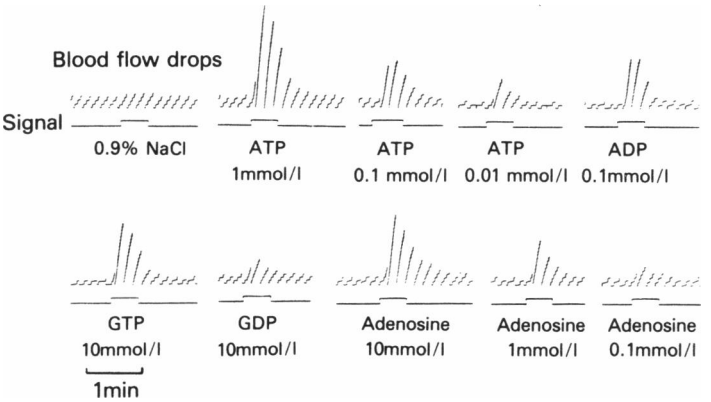

Figure 1 The effect of certain purine compounds on blood flow through the submandibular gland. Drops are registered cumulatively and the counter is reset every 10 s. Substances were infused close-arterially, at 4 μl/s in the concentrations shown. Full dose-response curves are given for ATP and adenosine and representative tracings shown for ADP, GTP, GDP and saline control. Blood pressure remained stable throughout.

through the gland 2.6 fold ($P < 0.005$, $n = 6$). By contrast, dibutyryl cyclic AMP in concentrations up to 10 mmol/l at the site of injection was without effect and neither nucleotide produced visible salivation. The effect of cyclic GMP was tested in 7 animals and concentrations up to 10 mmol/l failed to produce an increase in blood flow whereas dibutyryl cyclic GMP (10 mmol/l), tested in 4 cats, increased conductance by about 60%. The threshold concentration appeared to be about 400 μmol/l, 10 times that of cyclic AMP and 200 times that of ATP and ADP.

The pH of 10 mmol/l cyclic AMP in saline is 2.75, so the possibility that low pH was responsible for the vasodilator activity was tested. Infusion of cyclic AMP buffered to pH 6.6 was found to produce an equivalent response to that of unbuffered nucleotide while infusion of saline treated with dilute HCl to bring the pH to 2.75 had no significant effect.

Phosphodiesterase inhibitors Four different cyclic nucleotide phosphodiesterase inhibitors were used: the methylxanthines, theophylline and isobutylmethylxanthine (IBMX), and the chemically distinct quinine and papaverine. All increased blood flow through the submandibular gland but quinine seemed to sensitize the preparation in that the vascular response increased with repeated injections of the same dose. None of the compounds caused salivation. The results of these experiments are summarized in Table 1 and the effects of theophylline and IBMX are illustrated in several of the figures. The effect of combining phosphodiesterase inhibition with autonomic

Table 1 Summary of the effectiveness of certain agents as vasodilators in the submandibular gland

Agent	*Concentration injected (mmol/l)*	*Relative conductance (mean ± s.d.)*	n
Cyclic AMP	10	2.6 ± 0.25	6
db Cyclic GMP	10	1.6 ± 0.15	4
Theophylline	10	1.9 ± 0.7	5
IBMX	0.5	1.6 ± 0.18	3
Quinine	10	1.8	2
Papaverine	10	6.9	2
db Cyclic AMP	10	1.0	4
Cyclic GMP	10	1.0	7

Substances were given via the lingual artery without tying branches of the carotid artery. The concentration in the gland blood vessels would have been about 6% of that injected. Conductance (total flow/mean arterial blood pressure) is expressed relative to control values obtained following injection of an equivalent volume of saline at the same rate. See text for further details. IBMX = 3-isobutyl-1-methylxanthine.

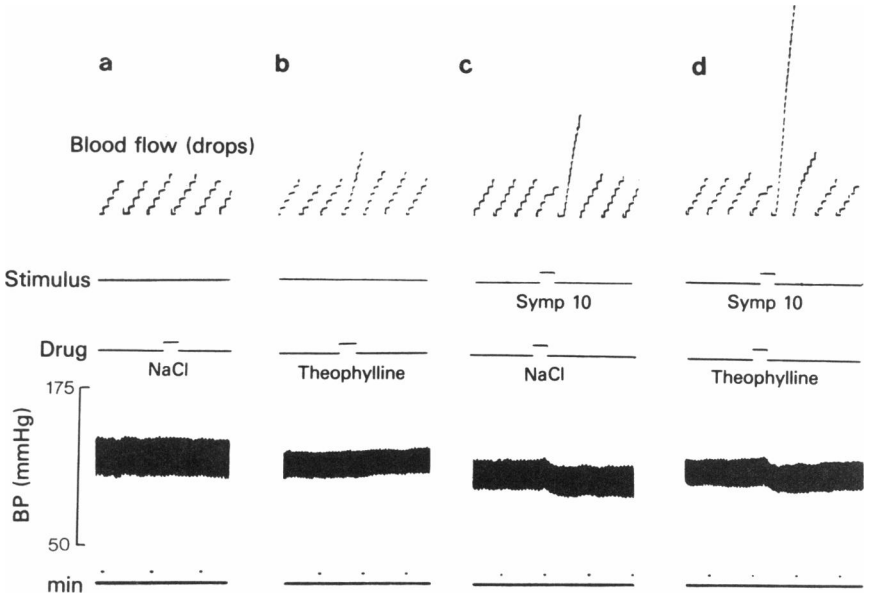

Figure 2 The vascular response to intra-arterial infusions of saline and 20 mmol/l theophylline alone ((a) and (b) respectively) and when accompanied by sympathetic stimulation (symp) at 10 Hz ((c) and (d) respectively). Compounds were infused at 10 µl/s from 0 to 20 s and sympathetic stimulation superimposed from 10 to 30 s. In this and subsequent figures, all times are quoted from an arbitrary 0.

stimulation was then investigated with theophylline, IBMX and quinine.

Effect of phosphodiesterase inhibitors on the vascular responses to sympathetic nerve stimulation

Theophylline When intra-arterial infusion of theophylline (5 to 20 mmol/l, 10 µl/s for 20 or 45 s) was combined with sympathetic nerve stimulation for 15 or 20 s (5, 10 and 20 Hz, used in randomized order), the after-dilatation was significantly potentiated. The results from a typical experiment are illustrated in Figure 2. Panel (a) shows the few extra drops above baseline produced by the infusion of saline while panel (b) illustrates the increase in blood flow following infusion of an equivalent volume of 20 mmol/l theophylline. The effect of superimposing sympathetic stimulation on either saline or theophylline infusion is shown respectively in panels (c) and (d).

In every experiment each stimulus frequency was superimposed on saline infusion (10 µl/s for 20 or 45 s) and a frequency-response relationship established. Theophylline was then infused in the absence of stimulation at a rate of 10 µl/s for 20 or 45 s and a dose-response curve determined. A dose that produced a submaximal vasodilator response was then combined with sympathetic stimulation. Thus, frequency-response curves to sympathetic stimulation were established during infusion of saline and theophylline.

For each frequency used, the sympathetic response in the presence of theophylline (Figure 2d) was compared, using paired t tests, with the sum of the conductance value for sympathetic stimulation (in the presence of saline) plus the value for theophylline alone minus the response to saline alone (c + b − a, respectively). The variation in background flow and response in different animals led to large standard errors which were minimized by expressing the conductances relative to the maximum value obtained in the preparation. Figure 3 summarizes the data for 6 animals plotted in this way. Simultaneous administration of theophylline with sympathetic stimulation gives a mean conductance greater than the sum of the individual responses at each frequency used and this difference is significant at 10 and 20 Hz ($P < 0.0005$ and $P < 0.005$ respectively). Such responses are considered to be potentiated. At 5 Hz, the mean value was increased, but here the difference was not statistically significant ($0.1 < P < 0.15$).

3-Isobutyl-1-methylxanthine and quinine Because the use of theophylline as a phosphodiesterase inhibitor is open to question (Berridge, 1975, and H.P. Bär, personal communication), the effect of IBMX was also tested in 5 animals. When IBMX infusion was super-

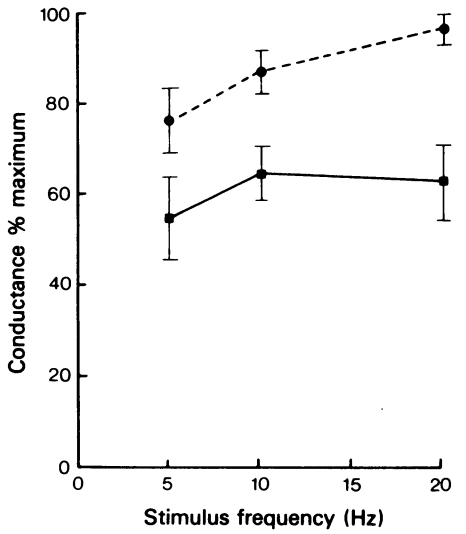

Figure 3 The effect of sympathetic stimulation on conductance responses. Data from 6 cats are combined and in each cat the responses are expressed as % of the peak vasodilatation. The solid line (■——■) joins the mean conductances obtained by summing the values for sympathetic vasodilatation in the presence of saline together with those for theophylline alone and then substracting the values for saline alone (c + b − a in Figure 1). The dotted line (●···●) joins the mean conductances during infusion of theophylline combined with sympathetic stimulation (d in Figure 1). Error bars show ±s.e. mean. For further details, see text.

imposed on sympathetic stimulation (10 Hz), the vasoconstriction occurring during the stimulus period became less pronounced while the after-dilatation was significantly potentiated (paired t test, $P < 0.05$).

In one experiment the chemically distinct phosphodiesterase inhibitor quinine was used. When quinine administration was superimposed on sympathetic stimulation (10 Hz), a potentiated response was seen.

Effect of phosphodiesterase inhibitors on the vascular responses to parasympathetic nerve stimulation

An analogous series of experiments to those described in the previous section was performed with parasympathetic nerve stimulation by combining chorda stimulation with phosphodiesterase inhibitor infusion. The vascular response to chorda stimulation is confined to a vasodilatation observed during the stimulus period, and, at low frequencies of stimulation, the initial increase in blood flow is greater than the response obtained towards the end of continuous stimulation for 2 min (the maintained response, see Darke & Smaje, 1972). It is possible that the two phases involve different mediators, and so phosphodiesterase inhibitors were infused during the first 30 s of stimulation and also during the maintained phase, to see if there were any differences in the responses obtained.

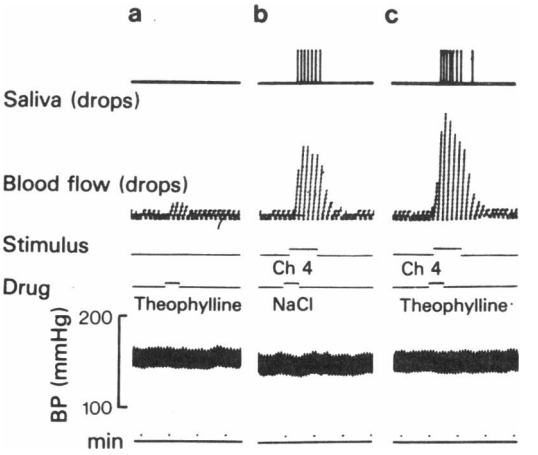

Figure 4 Potentiation of the chorda vasodilatation by theophylline. The response to 20 mmol/l theophylline (10 µl/s for 0 to 30 s) is shown in (a) and the effects of chorda stimulation (Ch, 4 Hz, 15 to 75 s) are shown superimposed on either saline (b) or theophylline (c).

Theophylline Figure 4 shows that theophylline alone has a slight effect but when infused during the initial phase of parasympathetic stimulation, there was a pronounced potentiation of the vascular response. Similar potentiation of the maintained phase of vasodilatation was also observed (Figure 5).

Figure 6 summarizes the frequency-response relationship for the early (6a) and maintained (6b) phases of parasympathetic stimulation plotted in the same way as that used for sympathetic stimulation. Plots of conductance versus stimulus frequency are presented and the data have been analyzed using paired t tests at the different frequencies used. As with sympathetic stimulation, the combination of parasympathetic stimulation with theophylline led to potentiated increases in mean blood flow at each of the frequencies used. The P values are given in the legend to Figure 6.

3-Isobutyl-1-methylxanthine and quinine In view of the uncertainties surrounding the use of theophylline as a phosphodiesterase inhibitor, the effect of IBMX on the maintained phase of parasympathetic vasodilatation was also studied and a typical result is shown in Figure 5. As can be seen, the blood flow response was potentiated when IBMX infusion was combined with the stimulation.

The effects of quinine (10 mmol/l, 10 µl/s for 30 s) were studied on the initial phase of chorda stimulation at 1 and 2 Hz in one cat and at 8 Hz in

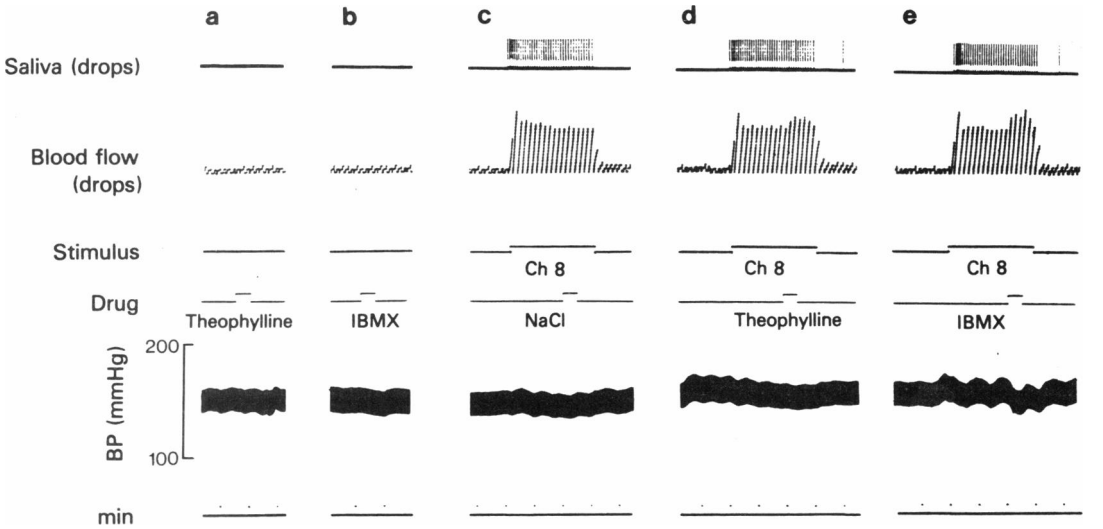

Figure 5 The secretory and vascular effects of theophylline and 3-isobutyl-1-methylxanthine (IBMX) alone and combined with the maintained phase of chorda lingual nerve stimulation. Panels (a) and (b) show the effect of 10 mmol/l theophylline and 1 mmol/l IBMX when infused at 10 µl/s for 30 s. Panels (c) to (e) illustrate the effect of superimposing chorda lingual nerve stimulation (Ch 8 Hz, 180 s) on the responses to saline and the phosphodiesterase inhibitors.

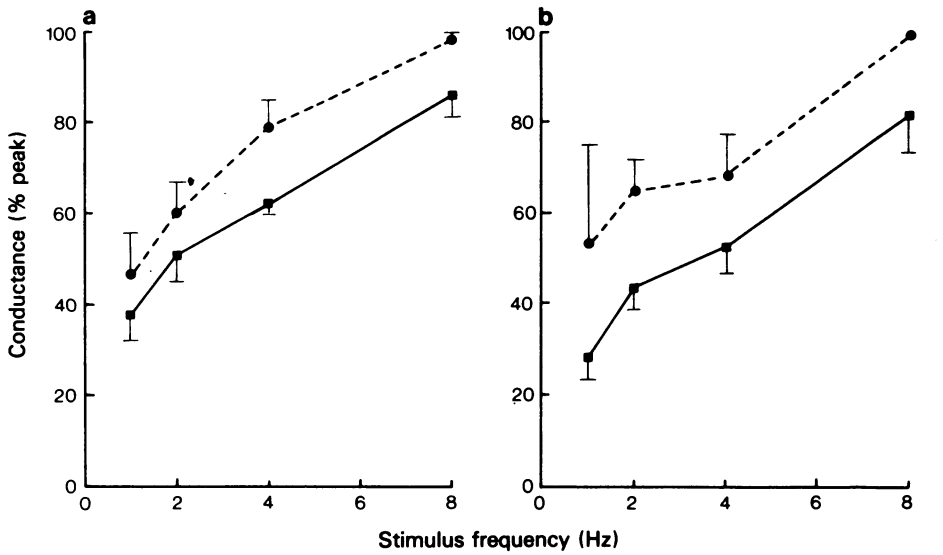

Figure 6 The effect of combining chorda stimulation with administration of theophylline (a) early in the vasodilatation and (b) during the maintained phase of vasodilatation, compared with the sum of the responses to chorda stimulation and theophylline alone. Data from 6 animals are combined and in each cat, the responses are expressed as % of the peak vasodilatation. Dotted lines join the mean conductances during the combination of chorda stimulation and theophylline (●···●) while the solid lines (■——■) join the mean conductances obtained by summing the responses to chorda stimulation in the presence of saline together with those for theophylline alone and then subtracting the values for saline alone. Error bars show ±s.e. mean. Paired *t* tests give the following *P* values: 1 Hz (a) $0.15 < P < 0.2$ (b) $P = 0.15$; 2 *Hz* (a) $0.05 < P < 0.1$ (b) $0.005 < P < 0.01$; 4 Hz (a) $P = 0.025$ (b) $0.0025 < P < 0.005$; 8 Hz (a) $0.025 < P < 0.05$ (b) $P = 0.05$.

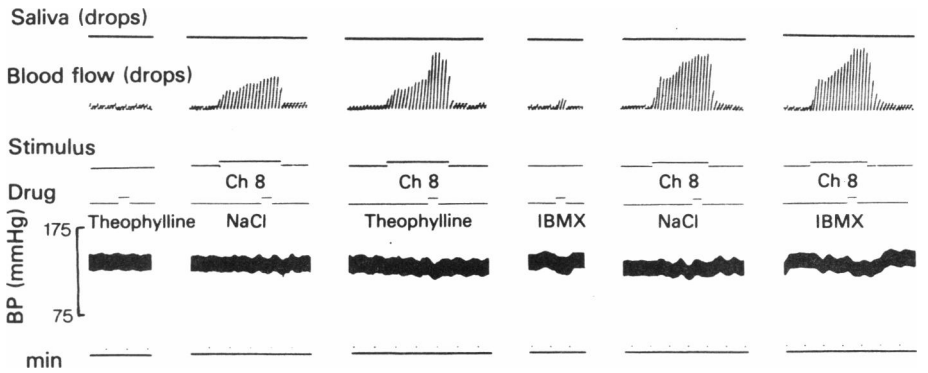

Figure 7 The persistence after atropine of the chorda vasodilatation and its potentiation by theophylline and 3-isobutyl-1-methylxanthine (IBMX). The Figure is a continuation of Figure 5 and between them atropine (100 μg/kg) was administered intravenously.

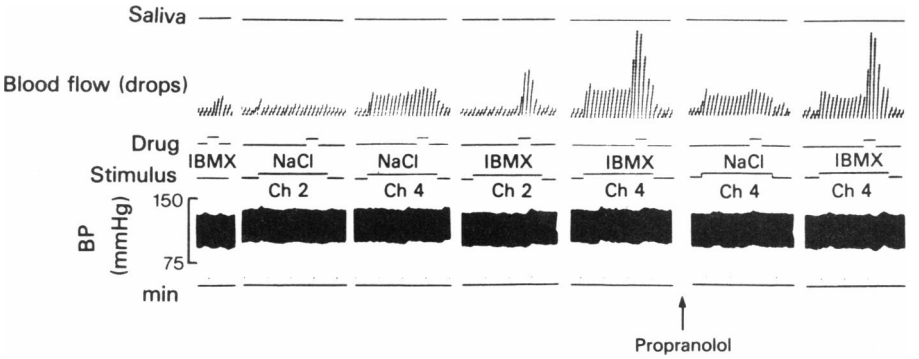

Figure 8 The persistence after atropine and propranolol of the chorda vasodilatation, potentiated by 3-isobutyl-1-methylxanthine (IBMX). Atropine was given before the beginning of the figure and propranolol (1.6 mg/kg i.v.) at the arrow. Abbreviations are as in previous figures.

another. A potentiated response was obtained in each case.

Effect of atropine on the parasympathetic response

In 4 cats, atropine (100 μg/kg i.v.) was administered after demonstrating the potentiating effects of theophylline and IBMX. In each case, salivation was blocked but the vasodilator response persisted and was potentiated to a similar or greater extent by both phosphodiesterase inhibitors. Before atropine, there was an increase in conductance of 2.8 ± 0.8 fold on combining the phosphodiesterase inhibitor with chorda stimulation, while after atropine the flow increased by a factor of 5.0 ± 0.8. This apparent enhancement of the potentiation is not significant ($0.05 < P < 0.1$, 7 replications in 4 animals). Examples of the type of response obtained are shown in Figures 7 and 8.

Effect of dipyridamole on the parasympathetic response

Dipyridamole prevents the uptake of adenosine (Kollassa, Pfleger & Rummel, 1970; Hopkins, 1973b), so if adenosine or ATP, which is hydrolyzed to adenosine (Hopkins, 1973a), were released following parasympathetic stimulation, then dipyridamole should potentiate this response (see Burnstock, 1972). In 2 experiments, dipyridamole was administered alone or during parasympathetic stimulation. In both cases it was observed that doses of drug sufficient to produce a small or negligible vasodilatation (1 mmol/l, 10 μl/s for 30 s) were able to produce a potentiated response when coupled with parasympathetic stimulation. This effect, which was more marked at lower stimulus frequencies (Figure 9), persisted after atropine.

Analysis of the potentiating effect of phosphodiesterase inhibitors on the response to parasympathetic nerve stimulation

Interpretation of the effects of phosphodiesterase inhibitors on the vasodilatation produced by parasympathetic nerve stimulation is not simple. Firstly, it is possible that by giving a drug during a period of local vasodilatation, more of the substance may be

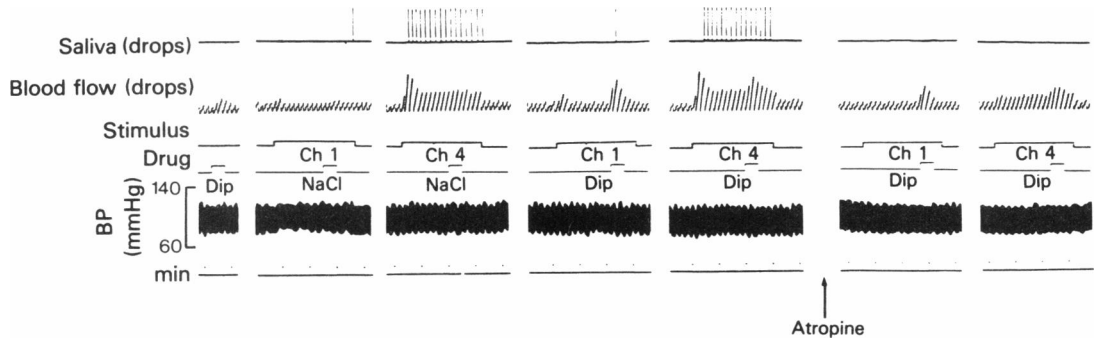

Figure 9 Potentiation of chorda vasodilatation by dipyridamole (Dip). The effect of infusion of dipyridamole (1 mmol/l, 10 μl/s) alone and during chorda stimulation (Ch) at 1 Hz and 4 Hz both before and after atropine (100 μg/kg i.v.)).

diverted through the gland and in this way account for the increased blood flow. Secondly, it has been suggested that parasympathetic stimulation may activate β-receptors present on the blood vessels (Skinner & Webster, 1968) and such a mechanism might also explain the enhanced response to phosphodiesterase inhibitors. Thirdly, the enhanced vasodilator response to phosphodiesterase inhibitors observed during nerve stimulation could be secondary to a non-specific increase in responsiveness during stimulation.

Changes in blood flow to the gland The fact that an increased delivery of phosphodiesterase inhibitor probably does occur during parasympathetic stimulation was confirmed in an experiment using radioactive microspheres. Spheres, 15 μm in diameter and labelled with ^{51}Cr, were infused through the lingual arteries to the right and left submandibular glands. While this was in progress, the chorda-lingual nerve on the right-hand side was stimulated supramaximally at 8 Hz. Both glands were subsequently removed and radioactivity determined by counting in a Panax 160 Gamma Spectrometer. The proportion of blood flow reaching each gland was calculated from the percentage of injected spheres trapped. Because of the size of the spheres, all of those directed to each gland became lodged in the tissue microcirculation (Fraser & Smaje, 1977). The findings revealed that the stimulated gland trapped 10% of the spheres injected, and the control gland, 4%. Thus, the proportion of blood reaching the gland did increase on stimulation but as this was a small proportion of the total flow to the region, the change in flow would barely influence the concentration of phosphodiesterase inhibitor reaching the gland vasculature. Nevertheless, it was considered worthwhile to investigate this problem further and 3 different approaches were adopted: (1) Close-arterial preparations were established in which all branches of the common and external carotid arteries were ligated except for the lingual and submandibular vessels. In these situations, the gland would receive all of the compound infused. (2) In another experiment, after isolating the arterial supply, the gland was perfused at constant flow with blood drawn from the femoral artery. Changes in perfusion pressure were determined both before and after the addition of IBMX, either alone, or in the presence of chorda stimulation. (3) Experiments were performed in which low doses of theophylline and IBMX, producing little or no vasodilatation on their own, were combined with very low frequency chorda stimulation which also had little vasodilator effect. Under these circumstances, any vasodilatation resulting from the combination of the two treatments could not be attributed to an increased delivery of phosphodiesterase inhibitor.

In each case, the results failed to support the notion that potentiation was caused by an increased delivery of drug to the stimulated gland. In 4 experiments, potentiation of flow was still produced when IBMX was infused close-arterially while in the two preparations perfused at constant flow, combination of parasympathetic stimulation with IBMX infusion led to a greater fall in perfusion pressure than that produced by summing the individual responses. Finally, in experiments combining small doses of theophylline or IBMX with very low frequency stimulation, potentiated increases in flow were regularly obtained and these persisted after intravenous administration of atropine (100 μg/kg. Figure 8.)

β-Receptor activation by parasympathetic stimulation In two cats, the effect of guanethidine (2.5 to 5.0 mg/kg i.v.) on the potentiation of the early phase of parasympathetic vasodilatation by theophylline was investigated. Following a dose of guanethidine sufficient to abolish the responses to sympathetic stimulation at 10 Hz, the chorda vasodilatation remained unaffected and was still potentiated to the same degree by theophylline. This argues against the pres-

ence of sympathetic fibres in the parasympathetic supply explaining the potentiation, but does not exclude the possibility that β-receptors present on the blood vessels might be activated in some other way. To test for this, experiments were performed with the β-adrenoceptor blocker, propranolol. Here, a dose sufficient to abolish sympathetic after-dilatation (0.3 to 0.9 mg/kg i.v.) and its potentiation by IBMX, failed to have any effect upon the potentiation of the chorda response (Figure 8).

Non-specific increase in responsiveness during stimulation This possibility was tested in 2 cats by measuring the vasodilator responses to butyric acid and K^+ at rest and during chorda stimulation. There is no evidence to suggest that these vasodilators act by increasing the intracellular concentration of cyclic nucleotides and, indeed, the increases in blood flow they produced were identical both before and during chorda stimulation. In the same animals, infusion of IBMX potentiated the chorda vasodilatation.

Discussion

Interpretation of the present results depends critically on whether or not the increases in flow observed when autonomic stimulation is combined with phosphodiesterase inhibition can be termed potentiation. Potentiation is usually taken to mean a response greater than the sum of the constituent responses and the inference drawn is that the two effects are interacting via a common pathway.

If this can be applied to blood flow, it would appear that the vascular responses to both sympathetic and parasympathetic stimulation were potentiated by the simultaneous administration of phosphodiesterase inhibitors. It now becomes necessary to explain this in terms of a molecular mechanism.

Role of cyclic nucleotides

Cyclic AMP There is now good evidence to suggest an involvement of β-receptors in sympathetic vasodilatation, both in the submandibular gland of the cat (Davey *et al.*, 1965; Bhoola *et al.*, 1965; Gautvik, Kriz, Lund-Larsen & Waaler, 1974) and also of the rat (Thulin, 1976), while a similar involvement in parasympathetic vasodilatation seems unlikely (Schachter & Beilenson, 1968; Gautvik, 1970b; this paper). It is widely throught that β-adrenergic mechanisms are mediated, in part, by cyclic AMP, and cholinergic mechanisms by cyclic GMP and, in this context, it has been shown in salivary glands from a number of species that β-adrenergic stimulation leads to marked increases in adenylate cyclase activity and cyclic AMP concentration (see Introduction and Jones, 1977).

In order to be satisfied that cyclic AMP is functioning as a second messenger in a particular physiological process, Sutherland *et al.* (1968) proposed four criteria which should be fulfilled. These may be summarized as follows: (1) The activity of adenylate cyclase, the generative enzyme for cyclic AMP, should be stimulated by the hormone in broken cell preparations. (2) Hormonal stimulation should induce an appropriate change in the level of cyclic AMP in the intact target tissue. (3) Drugs which inhibit the activity of cyclic AMP-dependent phosphodiesterase, the enzyme catalyzing the breakdown of cyclic AMP, should potentiate the action of hormones stimulating adenylate cyclase. (4) Cyclic AMP or an appropriate derivative, administered exogenously, should mimic the effects of the hormone in the target tissue.

In the present experiments, 2 of these criteria have been met (3 and 4) for both sympathetic and parasympathetic vasodilatation. With current techniques it is not feasible to satisfy all 4 criteria in the microvasculature following nerve stimulation, since quantitative histochemical methods are not available for estimating adenylate cyclase and cyclic nucleotides in small arterioles. Whilst there are innumerable studies on whole glands, these provide no information on events within the resistance vessels of the gland as the results will be dominated by the major tissue present, the glandular tissue. In large blood vessels, however, the relaxation of smooth muscle brought about by β-agonists does appear to be mediated by cyclic AMP (see Andersson, 1972; Bär, 1974) and since sympathetic vasodilatation in the salivary gland involves β-receptor activation (see Introduction and Discussion above), the case for cyclic AMP as an intracellular mediator during sympathetic vasodilatation becomes more compelling. However, the parasympathetic response is more difficult to interpret.

Cyclic GMP Phosphodiesterase inhibitors prevent the destruction of both cyclic AMP and cyclic GMP so the potentiation of parasympathetic vasodilatation observed in the presence of phosphodiesterase inhibitors could be a consequence of an increased cyclic GMP level in the gland. In this respect, it has been demonstrated in a number of tissues that cholinergic stimulation leads to increases in cyclic GMP level while cyclic AMP, when measured, shows little or no change (see Jones, 1977). However, Diamond & Hartle (1976) have shown that cyclic GMP levels in guinea-pig taenia coli increase after the contractile response to cholinergic stimulation and not before, arguing against an involvement of the nucleotide. In the salivary gland, too, the role of cyclic GMP in secretion is not very clear (Albano *et al.*, 1976; Durham, Butcher, Muir & Templeton, 1977) and is even less certain for its vasculature.

Before accepting cyclic GMP as a likely mediator, a number of alternative explanations need to be considered. From what has been said above, cyclic AMP seems to be an unlikely candidate and the persistence of the potentiated parasympathetic response after guanethidine and propranolol indicate that neither sympathetic fibres in the chorda lingual nerve nor β-receptors are responsible. Cyclic AMP could be involved indirectly, however, as the vasodilator activity of phosphodiesterase inhibitor alone is presumably explained by prevention of the destruction of cyclic AMP generated in the smooth muscle or gland cells by some process independent of the autonomic nervous system. If both cyclic AMP and autonomic neurotransmitters then acted on a further stage (e.g. intracellular Ca^{2+} levels) then potentiation could be observed even though the autonomic response was not mediated via the nucleotide. Such an explanation was favoured by Jenkinson & Koller (1977) to explain the potentiation of an α-adrenergic response by phosphodiesterase inhibitors in guinea-pig liver slices. However, it is worth pointing out that in the present work 3 chemically distinct phosphodiesterase inhibitors have been used and while they could produce potentiation of parasympathetic vasodilatation via cyclic AMP in the manner suggested above, it seems less likely that other non-specific actions of the inhibitors are responsible.

Role of purinergic nerves

Because chorda vasodilatation persists after both atropine and β-adrenoceptor antagonists, the presence of a non-cholinergic, non-adrenergic type of neurone has to be considered. A purinergic vasodilator nerve system activated by chorda stimulation could well explain some of our findings. ATP is rapidly hydrolyzed to adenosine (Hopkins, 1973a), which can activate the adenylate cyclase system (Blume & Foster, 1975), so the effects of the phosphodiesterase inhibitors could be explained in this way. Moreover the vasculature of the gland is extremely sensitive to ATP and ADP, much more so than to the cyclic nucleotides, and the order of potency of the purine compounds used is similar to that found in other systems thought to be purinergic (see Burnstock, 1975). In addition, Ferreira & Smaje (1976) showed that a substance was released by the stimulated salivary gland after atropine which in some ways resembled adenosine and the present results with dipyridamole are also consistent with a purinergic hypothesis.

Role of peptidergic nerves

The existence of a purinergic nervous system is not universally accepted and a system of peptidergic nerves has been suggested as an alternative possibility (Bloom & Polak, 1978). Nerves containing peptides, including vasoactive intestinal peptide (VIP), have been described in the brain and gastrointestinal tract and have been proposed as the mediators of non-cholinergic, non-adrenergic responses in the gut (Fahrenkrug, Haglund, Jodal, Lundgren, Olbe & Schaffalitzky De Muckadell, 1978). Recently varicose fibres containing VIP immunoreactivity have been found around blood vessels in certain salivary glands (Bloom, Bryant, Polak, Van Noorden & Wharton, 1979) and vasoactive intestinal peptide is known to activate cyclic AMP production in a number of tissues (see Fransden, Krishna & Said, 1978). If VIP were released into the vasculature by parasympathetic nerve stimulation and then activated adenylate cyclase, raising the endogenous levels of cyclic AMP, the potentiated vasodilatation produced by phosphodiesterase inhibitors could be readily explained. More work is needed to test this intriguing possibility.

Role of kallikrein

As mentioned in the Introduction, Gautvik's work (Gautvik, 1970a, b; Gautvik *et al.*, 1974) suggests that kallikrein is involved in both parasympathetic and sympathetic vasodilatation but that other mechanism(s) also play a role. The present experiments shed little light on this aspect of the problem except that even though atropine prevents the release of kallikrein (Ferreira & Smaje, 1976), the parasympathetic response after atropine was still potentiated by phosphodiesterase inhibitors. This emphasizes that kallikrein is not the sole mediator and suggests that the phosphodiesterase inhibitors are acting on the non-kallikrein component.

Conclusions

The control of nerve-mediated vasodilatation in the submandibular gland is evidently complex but the present paper provides some pointers. Although the drugs used in the present experiments are not entirely specific in their actions, the consistent results obtained with several phosphodiesterase inhibitors and the weight of evidence from the literature suggests that sympathetic after-dilatation is mediated, at least in part, by pathways involving cyclic AMP. The case for involvement of cyclic GMP in parasympathetic vasodilatation is less secure. Although 2 of Sutherland's criteria appear to have been satisfied, this is insufficient to establish cyclic GMP involvement without more support from the literature, particularly in view of the poor vasodilator activity of db cyclic GMP. A number of factors is almost certainly involved in controlling blood flow through the salivary gland but the present experiments suggest that both

sympathetic and parasympathetic vasodilatation include pathways involving cyclic nucleotides.

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