CONTROL OF SUBMANDIBULAR blood flow in the cat was first investigated by Claude Bernard (3). It has attracted great interest ever since it was shown that the vasodilatation that occurs in response to activation of the parasympathetic innervation is atropine resistant, which was first established in dogs by Heidenhain (16). After the discovery that kallikrein is present in saliva (33) and releases the vasodilator agonist bradykinin from a precursor in the plasma, the atropine-resistant vasodilatation was widely attributed to production of this peptide (17–19). However, the issue was highly controversial, and other groups variously attributed the phenomenon to increased metabolism (2), hyperosmolarity (23), accumulation of potassium ions (7, 8), histamine (32), or prostaglandins (12). The possibility that a peptide, or peptides, might be implicated was originally suggested by the observation that vasoactive intestinal peptide is present in nerve terminals in the submandibular gland of the dog (31). It is now generally accepted that the phenomenon of atropine-resistant vasodilatation during parasympathetic stimulation is attributable to the release of vasoactive intestinal peptide-like peptides in the submandibular gland of the cat (4, 22).

However, the question of the extent to which secretion depends on the associated increase in blood flow has attracted relatively little attention and is still controversial. There is evidence that secretory activity during sympathetic stimulation may be compromised by vasoconstriction because the flow of saliva is potenti-ated by intermittent patterns of stimulation that, unlike continuous patterns of stimulation, do not reduce the blood flow (5, 10). Stimulation of the parasympathetic innervation produces a far more copious secretion that is invariably accompanied by pronounced vasodilatation (3). However, relatively recent studies of the consequences of restricting the flow of blood through the submandibular glands of anaesthetized dogs during parasympathetic stimulation have shown that the secretory mechanism is remarkably resistant to this circumstance. Thus secretion continued unabated during parasympathetic stimulation at frequencies up to 8 Hz while the arterial inflow was occluded for at least 2 min (24). There are numerous arteriovenous anastomoses in this particular gland (25) that may serve to maintain capillary filtration during periods of reduced arterial inflow by raising the hilar venous pressure. This has been found to be related to the rate of secretion under these precise conditions (26) and to be relatively independent of the blood flow.

The effects of reducing submandibular blood flow on the production of parasympathetic saliva have also been investigated in anaesthetized cats. In these experiments, the animals were pretreated with propranolol and phentolamine to block any reflex sympathetic effects, and the arterial blood pressure was reduced (by ~50%) by withdrawing blood (20 ml/kg) from an ar-
rial catheter. This procedure resulted in a significant reduction in submandibular blood flow (77 ± 4% (SE)) and was associated with a significant reduction in the flow of saliva in response to parasympathetic stimulation at each of the frequencies tested (2–16 Hz) over the last 4 min of a 5-min test period (15). It seems most likely that the reduction in the secretory response was causally related to the reduction in blood flow through the gland. However, the possibility that it might have been due to some other consequence of severe hypotension in animals pretreated with large doses of adrenergic-blocking agents was not excluded. Accordingly, the present study was undertaken to investigate the effects of manipulating the submandibular blood flow by a completely different technique. This was achieved by infusing the potent vasoconstrictor peptide endothelin-1 (ET-1) via the ipsilateral carotid artery. Results similar to those reported previously were obtained, indicating that submandibular secretion, in response to parasympathetic stimulation, is significantly reduced when the blood flow is compromised by ET-1 administration. Certain results have been published previously in a preliminary form (30).

METHODS

Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Animals (Scientific Procedures) Act 1986 (UK), under project license PPL 80/1316.

Animals. The experiments were carried out on four adult male cats (2.6–3.5 kg body wt). Food, but not water, was withheld for 12–18 h before surgery. The animals were anesthetized with chloralose (80 mg/kg; a-chloralose, BDH Chemicals, Poole, UK) and, after induction with chloroform (Fisons Scientific Apparatus, Loughborough, UK) and insertion of the arterial catheter, were infused via the femoral artery. Supplementary doses of pentobarbital sodium (Sagatal; May & Baker, Dagenham, UK) were administered in bolus doses (1–2 mg/kg iv), in the event that spontaneous jerking movements occurred or a flexion reflex could be elicited. At the end of each experiment, the animal was given a lethal dose of pentobarbital sodium (5 ml, 20% wt/vol; Pentoject, Animal Care, York, UK).

Surgical and experimental procedures. The preparatory surgical techniques have been described previously (5). Briefly, the lingual nerve was cut proximal to the point at which the chorda tympani becomes separated, and the peripheral end was ligated for subsequent electrical stimulation; this standard procedure enables the parasympathetic nerve fibers that pass to the submandibular gland in the chorda tympani to be stimulated within the chorda-lingual nerve and minimizes mechanical damage. The ipsilateral ascending cervical sympathetic nerve in the neck was cut. The animals were heparinized (700 U/kg iv; Multiparin, CP Pharmaceuticals, Wrexham, UK), and each of the tributaries of the external jugular vein, except that draining the submandibular gland, was ligated before the external jugular vein was cannulated with a short length of polythene tubing. The submandibular venous effluent blood was diverted through a photoelectric drop counter and returned to the animal by an electronically controlled pump, via a cannula inserted into a femoral vein. The rate was adjusted automatically to match input to output. The submandibular duct was cannulated with a fine-bore nylon catheter attached to polythene tubing, the free end of which was positioned above a photoelectric drop counter to record the flow of saliva (mean dead space = 0.17 ± 0.01 ml).

The protocols involved continuously comparing submandibular vascular and secretory responses to chorda-lingual stimulation at 2, 4, 8, and 16 Hz for 5 min (10–20 V square-wave; 2.0-ms pulse width) before, during, and after the administration of ET-1 (Bachem). Episodes of chorda-lingual stimulation were carried out at intervals of ~10 min, and the order in which the various frequencies of stimulation were tested was varied from animal to animal. Thus each complete sequence of tests occupied between 60 and 70 min before, during, and after recovery from submandibular vasoconstriction. Aortic blood pressure and heart rate were monitored continuously by means of an Elcomatic pressure transducer connected to an amplifier that was designed and constructed in the laboratory electronics workshop and were recorded on a polygraph recorder. During stimulation, samples of saliva for analysis were collected in preweighed tubes over minute 1 at 2 and minute 3–5, and the flow was measured gravimetrically. Saliva produced during the first minute was discarded, thus ensuring complete evacuation of the dead space and equilibration of the components of the saliva.

After animals were killed, both submandibular glands were removed, weighed, and fixed in formalin sucrose. Later, they were processed to paraffin wax, sectioned, and stained with haematoxylin and eosin.

Estimations. Sodium and potassium concentrations in arterial plasma and saliva were measured using a Corning 435 Flame photometer. Salivary protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Results are the average of the 1–3 and 3–5 min samples, expressed as means ± SE. Statistical significance was determined by Student’s unpaired t-test. Outputs of electrolytes and protein in the saliva are expressed per unit weight of the contralateral gland (398 ± 24 mg/kg).

RESULTS

Cardiovascular consequences of ET-1. Mean arterial blood pressure during chorda-lingual nerve stimulation before infusion of ET-1 was relatively stable at all frequencies and averaged 116 ± 4 mmHg. During the infusion of ET-1, there was a significant rise in blood pressure to an average mean value of 137 ± 2 mmHg. In each case, recovery to within the normal range occurred by the time chorda-lingual stimulation was retested after ET-1 infusion (Fig. 1A). Mean heart rate under the same conditions was relatively stable at ~200 beats/min and was affected by neither the frequency of stimulation nor the administration of ET-1 (Fig. 1B).

Before administration of ET-1, chorda-lingual stimulation produced a frequency-dependent increase in submandibular blood flow over the range of 2–8 Hz; the mean rate of flow during stimulation was 1.8 ± 0.5 ml/min·g gland⁻¹ at 2 Hz, 4.3 ± 0.7 ml/min·g gland⁻¹ at 4 Hz, and 5.8 ± 1.0 ml/min·g gland⁻¹ at 8 Hz (Fig. 2). Intracarotid infusions of ET-1 (2.5–7.5 pmol·min⁻¹·kg⁻¹) produced a significant fall in the vasodilator response at all frequencies, with the corresponding values being 1.0 ± 0.3 ml/min·g gland⁻¹ at both 2 and 4 Hz and 2.0 ± 0.6 ml/min·g gland⁻¹ at 8 Hz. The values during chorda-lingual stimulation at
a higher frequency (16 Hz), both in the presence and absence of ET-1, were similar to those during stimulation at 8 Hz (Fig. 2B). The average reduction in the blood flow in the presence of ET-1 during chorda-lingual stimulation at all frequencies amounted to 64 ± 7%. Because the reduction in submandibular blood flow during the infusions of ET-1 was invariably associated with a significant rise in the perfusion pressure, it was clearly attributable to an increase in submandibular vascular resistance. This amounted to an increase of ≈110% during chorda-lingual stimulation at 2 Hz, and somewhat higher values during stimulation at the higher frequencies. When stimulation was repeated after the infusion of ET-1 had been discontinued, the vasodilator responses were found to have returned toward the initial values. With the exception of the response during stimulation at 4 Hz, the values after ET-1 did not differ significantly from those obtained before the peptide was infused (Fig. 2B); the average reduction in the blood flow after ET-1 was 23 ± 12%.

Secretory consequences of ET-1. When submandibular blood flow was reduced by the infusion of ET-1, the flow of saliva in response to chorda-lingual stimulation was also reduced at all frequencies tested (Fig. 2A). Thus, during stimulation at 2 Hz before ET-1 administration, the rate of flow was 146 ± 46 μl·min⁻¹·g gland⁻¹ compared with a value of 69 ± 30 μl·min⁻¹·g gland⁻¹ in the presence of the peptide (not significant). At the higher frequencies of stimulation, the corresponding values before and during the infusion of ET-1 were as follows: 354 ± 42 and 85 ± 23 μl·min⁻¹·g gland⁻¹ at 4 Hz, P < 0.01; 381 ± 36 and 168 ± 41 μl·min⁻¹·g gland⁻¹ at 8 Hz, P < 0.01; and 313 ± 35 and 156 ± 58 μl·min⁻¹·g gland⁻¹ at 16 Hz, not significant. The average reduction in the flow of saliva during the infusion of ET-1 was 59 ± 6%. After the infusion of ET-1 had been discontinued, the flow of saliva in response to chorda-lingual stimulation was generally higher than it had been during the infusion but was still much lower than it had been before ET-1. The difference between the corresponding values was statistically significant at 4, 8, and 16 Hz (Fig. 2A), with the average reduction in the blood flow after ET-1 administration amounting to 34 ± 6% (P < 0.05). Responses from a single animal during chorda-lingual stimulation at 8 Hz are shown in Fig. 3. This illustrates how, provided that blood pressure is maintained reasonably constant, submandibular blood flow rises to a maximum plateau value that persists until stimulation is discontinued. In contrast, the flow of saliva reaches an initial peak within the first minute and then declines to attain a lower plateau value that is usually well-maintained for the duration of the stimulation period. This pattern is preserved when the blood flow is reduced, but the overall rate of salivary flow is also reduced. This particular set of responses was slightly unusual in that the vascular response had completely recovered by the time the chorda-lingual nerve was tested after the ET-1 infusion.
There was a linear relation between the flow of blood through the gland and the secretion of saliva before, during, and after the infusion of ET-1. The slope of the pooled data from the values obtained before and after ET-1 was such that the flow of saliva amounted to $\sim 6\%$ of the blood flow. During the infusion of ET-1, when the blood flow was reduced, the slope was steeper, so that the flow of saliva was $\sim 9\%$ of the blood flow.

The concentration of sodium ions in the submandibular saliva increased steadily with the frequency of chorda-lingual stimulation over the range of 2–8 Hz (Fig. 4A). The effect of ET-1 was variable, increasing sodium concentration during stimulation at 2, 8, and 16 Hz and decreasing it at 4 Hz. Overall, ET-1 increased the average mean sodium concentration of the submandibular saliva by $21 \pm 17\%$, which failed to achieve statistical significance in this small group of animals. At the lowest frequency of chorda-lingual stimulation (2 Hz), the rise in salivary sodium concentration was sufficient to mitigate the reduction in flow and thus maintain the output of sodium from the gland. However, at higher frequencies, the reduction in flow was clearly reflected by a reduction in sodium output (Fig. 4B). The concentration of potassium ions was not affected by the frequency of chorda-lingual stimulation but was generally higher in saliva produced in the presence of ET-1 (Fig. 5A). Overall, there was an increase in salivary potassium concentration during the infusion of ET-1 ($33 \pm 6\%; P < 0.01$). Nevertheless, the output of potassium ions in the submandibular saliva was generally reduced in the presence of ET-1, reflecting the reduction in the flow of saliva (Fig. 5B). None of the experimental procedures had any significant effect on the concentrations of sodium and potassium in the circulating plasma, which varied between 135 and 145 mmol/l and between 3.0 and 4.5 mmol/l, respectively. Packed red blood cell volume was maintained between 41 and 43% throughout each experiment, demonstrating that the volumes of saliva collected during the course of these experiments had no significant effects on hydration.

The output of protein in submandibular saliva increased steadily with the frequency of chorda-lingual stimulation over the range of 2–8 Hz (Fig. 6). ET-1 significantly reduced the output of protein in the sub-
mandibular saliva in response to chorda-lingual stimulation at all frequencies, with no sign of recovery when the nerve was tested after the infusion had been discontinued. The average mean protein output during chorda-lingual stimulation at all frequencies was reduced by 64 ± 7% during the infusion of ET-1 and by 59 ± 15% after the infusion had been discontinued. The mean concentration of protein in the saliva reflected the amount secreted and the available volume for transportation. Thus, because the reduced output during the infusion of ET-1 was the same as the reduction in flow of saliva (59 ± 6%), protein was found to be present in very similar concentrations (0.56 ± 0.08 mg/ml before ET-1 and 0.47 ± 0.08 mg/ml during the infusion). The mean value was lower after the infusion (0.30 ± 0.05 mg/ml), reflecting the fact that the flow of saliva had at least partly recovered to preinfusion levels, whereas protein output had not (Figs. 2 and 6).

Postmortem histological examination failed to reveal any gross differences between glands that had been tested and those on the contralateral side, with both appearing quite normal.

**DISCUSSION**

The strategy this study was based on depends on the supposition that the very-low-dosage infusion of ET-1 we used would cause partial constriction of the submandibular vasculature, as it does throughout the body (1, 36), without evoking other effects in the gland that might influence parasympathetic secretion. ET-1 has been shown to be present in salivary glands and saliva (20, 35), but, as yet, there are no reports that it exerts any effect on salivary glands other than vasocstriction.

The effects of the peptide on submandibular responses to parasympathetic stimulation resembled those of induced hypotension (15) in a number of ways. The flow of saliva and blood through the gland were both substantially reduced during chorda-lingual stimulation, and this difference generally achieved statistical significance, despite the small number of animals used in the study. Furthermore, the mean values for salivary flow before, during, and after the infusion of ET-1 were linearly related to the existing blood flows, and the slope of this relation was shifted to the left. Except during chorda-lingual stimulation at 4 Hz, the concentration of sodium in the saliva was generally higher in the presence of ET-1, and the difference was statistically significant at 16 Hz (Fig. 4). This is noteworthy because the concentration of sodium in saliva normally rises with an increase in flow rate (14, 21), due to the fact that there is less time for reabsorption as the primary secretion passes down the ducts (27). The reduction in flow during the infusion of ET-1 was associated with an increase in salivary sodium concentration, rather than the expected decrease, as was previously reported during hypotension (15). It therefore appears that both procedures diminish the efficacy of the reabsorptive process in the ducts and do so at least to the extent that primary secretion is also inhibited. As during hypotension, the output of sodium during stimulation at the higher frequencies (4–16 Hz) was reduced in the presence of ET-1, reflecting the reduced volume of saliva being produced. Likewise, the effects of ET-1 on salivary potassium concentration and output during chorda-lingual stimulation were very similar to those of hypotension. The output of potassium was reduced at the lower frequencies (2 and 4 Hz, P < 0.05 and 0.02, respectively) but not at the higher frequencies (8 and 16 Hz; Fig. 5), whereas the concentration of potassium was invariably elevated, and the effect was statistically significant at the higher frequencies of stimulation (8 and 16 Hz, P < 0.05 and 0.01, respectively). Finally, both procedures generally produced a similar inhibition of protein output. Chorda-lingual stimulation produced the expected frequency-dependent increase in protein output. The inhibitory effects of ET-1 and hypotension were most pronounced at the higher frequencies, with no sign of recovery after the infusion of ET-1 had been discontinued or after replacement of blood in the case of hypotension (15). One difference that emerged relates to the protein response to low-frequency stimulation, which was unaffected by hypotension but was significantly reduced in the present study. It was suggested previously (15) that high-frequency stimulation was likely to stimulate secretion mainly by exocytosis and that the slow rate at which mature vesicles are replaced could account for the observed failure of recovery. At low frequencies of stimulation, secretion may occur mainly via the constitutive vesicular route; it seems possible that ET-1 might inhibit this process, in addition to the vasoconstrictor effect it exerts, but this has yet to be established.

The effect of ET-1 also differed from that of hypotension in that the flow of saliva, in response to chorda-lingual stimulation at the higher frequencies (4, 8, and 16 Hz), had not recovered after the infusion was discontinued to quite the extent that it had at the corresponding time after hypotension. The mean values were still significantly lower than the initial rates of flow after stimulation at each frequency (P < 0.05, P < 0.02, and P < 0.02, respectively). However, mean blood flow through the gland was also less than it had been before administration of ET-1, and this difference did achieve statistical significance during stimulation at 4 Hz (P < 0.05). There-
Therefore, it seems likely that the diminution of the secretory response was due to the persistence of ET-1-induced vasoconstriction. When this possibility was tested by repeating chorda-lingual stimulation at the frequency first tested after the infusion had been discontinued and after completion of the normal protocol, both the vascular and secretory responses were found to be greater. Accordingly, all available evidence suggests that the reduction in the flow of parasympathetically mediated submandibular saliva, which occurs when the perfusion pressure is reduced [as in our previous study (~50%) (15)], can be ascribed to the resultant fall in the blood flow through the gland.

Although the production of submandibular saliva is compromised when blood flow is substantially reduced for 5 min, it may be virtually immune to deprivation of blood over shorter periods, as Lung found in the dog (24). This may be due, at least in part, to the ability of the acinar cells to withstand a fluid debt, since this capability has been demonstrated in vitro in response to direct application of muscarinic agonists (13, 28, 29) and so necessarily entirely independent of any blood supply. The effect of such fluid debt is large and can amount to 25% of cell volume within a few seconds in both parotid and submandibular cells. If any such fluid debt, which involves both the cellular components and the interstitial fluid, was complete within the first minute of stimulation, then the volume of saliva produced must have depleted the available plasma by the same amount. When the volume of saliva is expressed as a percentage of the incremental increase in plasma flow during chorda-lingual stimulation, it is found to increase in the presence of ET-1. Thus the mean value for “plasma extraction” before the infusion of ET-1 was 13.8 ± 1.4% and rose to 21.3 ± 2.5% during the infusion \((P < 0.05)\). After the infusion, plasma extraction had fallen to 12.5 ± 2.0% \((P < 0.05, \text{with respect to the value during infusion})\). This may represent the physiological reserve available in terms of the plasma flow required to maintain the production of saliva in this gland.

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