Submandibular responses to stimulation of the parasympathetic innervation in anesthetized sheep

C. M. B. Edwards,1 P. P. Corkery,2 and A. V. Edwards3

1Imperial College School of Medicine Endocrine Unit, Hammersmith Hospital, London W12 0NN, United Kingdom; 2Department of Veterinary Physiology and Biochemistry, Veterinary College of Ireland, Dublin 4, Ireland; and 3Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, United Kingdom

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Edwards, C. M. B., P. P. Corkery, and A. V. Edwards. Submandibular responses to stimulation of the parasympathetic innervation in anesthetized sheep. J Appl Physiol 95: 1598–1605, 2003. First published July 11, 2003; 10.1152/japplphysiol.00991.2002.—Submandibular secretory and vascular responses to stimulation of the parasympathetic innervation and the output of vasoactive intestinal peptide (VIP) were investigated in anesthetized sheep in the presence and absence of atropine (≥0.5 mg/kg). In the absence of atropine, parasympathetic stimulation caused an increase in the flow of saliva and a decrease in submandibular vascular resistance; the latter response persisted after the administration of atropine and was then significantly reduced at the lowest but not at the higher frequencies tested. The output of VIP from the gland was frequency dependent over the range of 10–20 Hz (continuously) and significantly increased after atropine (P < 0.02). Furthermore, the fall in vascular resistance was linearly related to log VIP output after total muscarinic blockade. Intracarotid infusions of synthetic VIP produced dose-dependent falls in submandibular vascular resistance, together with a corresponding increase in submandibular blood flow. It is concluded that the atropine-resistant vasodilatation that occurs in this gland during parasympathetic stimulation is likely to be due largely, if not entirely, to the release of VIP submandibular gland; blood flow; saliva; vasoactive intestinal peptide.

THE ATROPINE-RESISTANT VASODILATATION that occurs in response to stimulation of the parasympathetic innervation to the submandibular gland was first reported by Heidenhain (17) in a dog. Most of the subsequent work on this phenomenon was carried out in cats, and it is now generally accepted that the phenomenon depends on the release of vasoactive intestinal peptide (VIP) from the parasympathetic nerve terminals (5, 23). In the cat, submandibular secretion is completely blocked by atropine and cannot then be elicited by the administration of VIP, but this peptide nevertheless strongly potentiates the secretory response to exogenous acetylcholine (ACh) in the absence of atropine (22). Furthermore, both the release of the peptide from the nerve terminals and its subsequent actions in the gland on the vasculature and the secretion of protein depend on the production of nitric oxide (7, 11, 27), as does the vasodilator response to parasympathetic stimulation in the rat (1).

VIP acts as a parasympathetic neurotransmitter in salivary glands in a wide variety of species, including rabbits, ferrets, rats, dogs, and minks (14–16, 31, 37, 38), and probably sheep, cattle, and humans (8, 18, 30). However, attempts to demonstrate release of the peptide from the ovine parotid gland during stimulation of the parasympathetic innervation have been unsuccessful (2, 42), casting some doubt on its role as a parasympathetic neurotransmitter in that tissue.

In the submandibular gland of the sheep, it has been established that VIP is present in nerve terminals adjacent to both small blood vessels and acini (41). When injected into the carotid artery, VIP causes secretion of protein-rich submandibular saliva, even after atropinization (30), as in most species in which the effect has been studied (see Ref. 13 for review). The release of VIP from submandibular glands and effects attributable thereto have generally been found to be potentiated when the parasympathetic innervation is activated in bursts, so long as the total number of impulses delivered in unit time is not excessive (3, 7, 39). Accordingly, the present study was undertaken to explore further the role of VIP in mediating responses to parasympathetic stimulation in the submandibular gland of sheep. This was done by establishing the effects of different patterns of parasympathetic stimulation, quantifying the output of VIP during continuous high-frequency stimulation, and defining the submandibular responses to intra-arterial infusions of the peptide. Certain of these results have been published previously in a preliminary form (9, 12).

METHODS

Animals. The experiments were carried out on adult ewes of various breeds (35–72 kg body wt) as provided for under the Animals Scientific Procedures Act (1986), Project Licence PPL 80/1316. Food but not water was withheld for 48 h before each experiment. Anesthesia was induced and maintained with pentobarbital sodium [Sagitale, Rhône Mérieux, Harlow, UK; 15–30 mg/kg iv and then 0.1–0.3 mg·min⁻¹·kg⁻¹ iv].

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Address for reprint requests and other correspondence: A. V. Edwards, Physiological Laboratory, Downing St., Cambridge CB2 3EG, UK (E-mail: ave1000@cam.ac.uk).
Surgical and experimental procedures. The trachea was intubated and then exposed via a midline incision low in the neck. The ipsilateral ascending cervical sympathetic nerve was identified and cut. An arterial catheter was introduced to the abdominal aorta via a femoral artery and later employed to monitor arterial blood pressure and heart rate; samples of arterial blood were also collected periodically for measurements of packed cell volume. The femoral vein was cannulated to provide a conduit for the continuous infusion of pentobarbitone sodium. The chorda-lingual nerve was exposed and cut, and the submandibular duct was cannulated with the widest bore nylon tubing practicable (0.58-mm inner diameter). The free end was then positioned above a photoelectric drop counter (constructed in the laboratory electronics workshop). A neighboring length of the hypoglossal nerve was excised to minimize spread of stimulus. Each of the tributaries of the ipsilateral linguofacial vein, except that draining the submandibular gland, was heparinized (Multiparin, CP Pharmaceuticals, Wrexham, UK; 1,000 IU/kg iv), and the linguofacial vein was cannulated with a short length of polythene tubing. The submandibular venous effluent was thereby diverted through a second photoelectric drop counter and returned to the animal by a pump, via the ipsilateral jugular vein, in a typical response from a submandibular postganglionic nerve terminal and the point arterialized (Trasylol, Bayer; 500 KIU/ml) for infusion at a dose of either 20 or 40 pmol/kg/min (2,500 KIU/ml blood). They were then centrifuged at +4°C as soon as possible, and the plasma was stored at −20°C.

At postmortem examination, both submandibular glands were removed and weighed. Small representative pieces were fixed in formal saline. Later they were processed to paraffin wax, sectioned (7-μm sections), stained with celestine blue, eosin, and alcian blue, and examined microscopically.

Estimations. Plasma VIP and substance P concentrations were measured by radioimmunoassay (6). Submandibular vascular resistance (SVR) was estimated by dividing the perfusion (arterial blood) pressure (in mmHg) by the submandibular blood flow (SBF; in μl · min⁻¹ · g gland⁻¹) and expressed as the percent changes from experimental time = 0. Results are expressed as means ± SE and were assessed statistically by means of paired or unpaired Student’s t-test as appropriate, with n representing the number of animals and significance assumed when P < 0.05. Statistical significance between gradients and analysis of covariance was calculated as described by Armitage et al. (4). All flows and outputs are expressed per unit weight of the contralateral gland, as is now customary, to eliminate any error arising from edema in the experimental gland.

RESULTS

Cardiovascular responses to stimulation of the parasympathetic innervation. Stimulation of the peripheral end of the C-T at 1, 2, and 4 Hz produced a frequency-dependent fall in SVR (Fig. 1) without affecting aortic blood pressure or heart rate. A typical response from a single animal (Fig. 2) best illustrates the rapidity of the

![Figure 1](http://jap.physiology.org/) Comparison of the mean percent decreases in submandibular vascular resistance (SVR) during chorda tympani (C-T) stimulation at 1, 2, and 4 Hz continuously over a 4-min period and at 10, 20, and 40 Hz for 1 s at 10-s intervals over the same period of time in 8 anesthetized sheep. Values are means ± SE. *P < 0.05 with respect to the value during continuous stimulation at 1 Hz.
onset of salivation and the associated vasodilatation in response to continuous stimulation of the C-T. Both responses were well maintained for the duration of stimulation and subsided gradually toward the initial levels over ~5 min when stimulation was discontinued.

Intermittent stimulation at the corresponding frequencies that were required to deliver precisely the same number of impulses to the C-T fibers over the 5-min test period (10, 20, and 40 Hz) also produced a frequency-dependent fall in SVR (Fig. 1). The response during stimulation at 10 Hz intermittently was significantly greater than that during stimulation at 1 Hz continuously ($P < 0.05$; paired $t$-test), but no such difference was recorded between the effects of the two patterns of stimulation at higher frequencies (Fig. 1).

Secretory responses to stimulation of the parasympathetic innervation. C-T stimulation at 1, 2, and 4 Hz also produced a frequency-dependent increase in the flow of submandibular saliva, as did intermittent stimulation at the corresponding frequencies of 10, 20, and 40 Hz (Fig. 3). The response during stimulation at 10 Hz intermittently was greater than that during stimulation at 1 Hz continuously ($P < 0.05$; paired $t$-test), but no such difference was achieved statistically significant. The latency of the secretory response was also invariably reduced when the intermittent pattern of stimulation was employed at this range (Fig. 4). However, at the higher frequencies, the salivary response was less during intermittent stimulation than during continuous stimulation at the corresponding frequency, and this effect was significant at the highest frequencies compared in this way (4 and 40 Hz, $P < 0.02$; Fig. 3).

Responses after administration of atropine. Pretreatment with atropine ($\geq 0.5$ mg/kg iv) effectively abolished the secretion of saliva at these relatively low frequencies (Fig. 5), but an atropine-resistant response was observed during C-T stimulation at higher frequencies of stimulation. Thus continuous stimulation at 10 Hz elicited secretion, which persisted for up to 15 min. This response was characterized by a long latency (at least 60 s; Fig. 6), and the flow of saliva persisted for several minutes after stimulation had been discontinued. The response was invariably associated with an intense vasodilatation in the gland, and the rise in blood flow preceded the onset of salivation (Fig. 6).

In contrast, a fall in SVR was always observed during C-T stimulation after atropine. It was significantly reduced at the lowest frequencies employed. Thus the fall in SVR during C-T stimulation at 1 Hz continuously was $27 \pm 5\%$ at 3–5 min in the absence of atropine but only $11 \pm 4\%$ after atropine ($P < 0.02$; Fig. 7). The corresponding values during C-T stimulation at 10 Hz in bursts were $35 \pm 4\%$ without atropine and $18 \pm 5\%$ with atropine ($P < 0.02$). After atropine, the fall in SVR during intermittent stimulation at 10 Hz still exceeded that during continuous stimulation at 1 Hz, but this difference did not achieve statistical significance ($P > 0.3$).

The administration of atropine also reduced the fall in SVR in response to C-T stimulation at both 2 Hz continuously and 20 Hz in bursts, but neither difference achieved statistical significance (data not shown). At the higher frequencies, atropine had no significant effect on the fall in SVR in response to C-T stimulation, regardless of the pattern of stimulation employed. The responses to continuous C-T stimulation for 5 min at 4 Hz are presented in Fig. 8 and are closely similar.

Output of VIP from the submandibular gland during C-T stimulation. The effect of stimulation of the peripheral end of the C-T on the release of VIP from the submandibular gland was investigated by stimulating the nerve at either 10 or 20 Hz before and after administration of atropine ($\geq 0.5$ mg/kg iv). C-T stimulation at these high frequencies invariably produced a profuse flow of saliva, which was substantially reduced by

![Fig. 2. Changes in salivary (top) and submandibular blood (middle) flow (drops) and in aortic blood pressure (ABP; bottom) in a single anesthetized sheep in which the C-T was stimulated at 4 Hz continuously for 5 min.](jap.org)

![Fig. 3. Comparison of the changes in mean submandibular salivary secretion during C-T stimulation at 1, 2, and 4 Hz continuously over a 4-min period and at 10, 20, and 40 Hz for 1 s at 10-s intervals over the same period of time in 8 anesthetized sheep. Values are means ± SE. **$P < 0.02$ with respect to the value during continuous stimulation at 4 Hz.](jap.org)
atropine, but had no effect on aortic blood pressure, heart rate, or packed cell volume in these animals. There were substantial rises in SBF, which increased from $0.24 \pm 0.04$ to $0.98 \pm 0.12$ ml/min/g gland at 8–10 min during C-T stimulation at 10 Hz ($P < 0.01$) and from $0.32 \pm 0.04$ to $1.61 \pm 0.45$ ml/min/g gland during stimulation at 20 Hz ($P < 0.05$; Fig. 8). There was also a steady rise in the concentration of VIP in the submandibular venous effluent plasma during C-T stimulation at both frequencies. This effect was frequency dependent with the concentration of VIP rising from $8 \pm 3$ to $20 \pm 4$ pmol/l during stimulation at 10 Hz ($P < 0.05$) and from $11 \pm 4$ to $58 \pm 16$ pmol/l at 20 Hz ($P < 0.05$; Fig. 8). There was no significant change in the concentration of the peptide in the arterial plasma, and, when the arterial concentration was allowed for, the outputs of VIP were found to have risen by 25 and 47 pmol/l in response to C-T stimulation at 10 and 20 Hz, respectively.

After atropine, closely similar changes in SBF were found to occur in response to C-T stimulation at both 10 and 20 Hz. However, the rises in the concentration of VIP in the submandibular venous effluent plasma were increased, as was VIP output, although there was no change in the basal values of either variable. During stimulation at 10 Hz, the mean of the average values for VIP output during C-T stimulation was $10 \pm 3$ pmol/l without atropine and $35 \pm 10$ pmol/l with atropine ($P < 0.05$); during stimulation at 20 Hz, the corresponding values were $46 \pm 7$ and $92 \pm 24$ pmol/l (Fig. 9; $P =$ not significant).

Neither stimulation of the peripheral end of the C-T nor of the ascending cervical sympathetic nerve had any reproducible effect on the output of substance P from the gland, nor did ascending cervical sympathetic stimulation have any effect on the output of VIP (data not shown).
Responses to intracarotid infusions of exogenous VIP. Intracarotid infusions of exogenous VIP at 10 and 20 pmol/min/g gland produced a dose-dependent fall in SVR without significantly affecting either the arterial blood pressure or heart rate. Thus the average mean SVR was reduced by 42.4 ± 0.8% during the infusion of VIP at the lower dose (Fig. 10) and by 54.9 ± 2.3% at the higher dose (P < 0.001). These changes were associated with increases in SBF from 0.26 ± 0.03 and 0.29 ± 0.06 ml/g gland to mean average values of 0.47 ± 0.01 and 0.62 ± 0.02 ml/g gland, respectively (P < 0.001). The average mean VIP concentrations in the submandibular venous effluent plasma during these infusions of VIP were 262 ± 1.2 pmol/l at the lower and 302 ± 23 pmol/l at the higher dose.

Histological examination postmortem failed to reveal any gross differences between glands that had been tested and those on the contralateral side; both appeared normal.

DISCUSSION

The results of this study show that VIP is released from the submandibular gland of sheep during stimulation of the parasympathetic but not of the sympathetic innervation, whereas substance P is not. Furthermore, the output of VIP is linearly related to the fall in SVR that occurs during C-T stimulation, when muscarinic activation of the vascular smooth muscle is suppressed with atropine (r² = 0.84; Fig. 11). The relation holds over the whole of the response range of 0 to ~80% and VIP output of 1 to 100 fmol/min/g gland.

This is in agreement with the relation between these variables reported previously in cats (3). However, estimates of the amounts released by each impulse show them to be lower than those estimated in either cats, ferrets, or rabbits. In the present study, the mean VIP output during continuous C-T stimulation at 10 Hz amounted to ~0.02 fmol/impulse•g gland•min and at 20 Hz to ~0.04 fmol/impulse•g gland•min. In comparison, these values in the submandibular gland of the anesthetized cat are 0.6 fmol/impulse•g gland•min during continuous stimulation at 20 Hz (5) and 1.0 fmol/impulse•g gland•min at 10 Hz (24); in the anesthetized rabbit the value is ~1.0 fmol/impulse•g gland•min during continuous C-T stimulation at 15 Hz (16); and, in the anesthetized ferret, a peak value of ~0.16 fmol/impulse•g gland•min is reached (39). However, the output was substantially increased by pretreatment with atropine (from 0.02 to ~0.06
during C-T stimulation at 10 Hz and from 0.04 to 0.08 fmol·impulse⁻¹·g gland⁻¹ at 20 Hz). This corresponds with findings in cats and ferrets (24, 39), which provide further evidence to suggest that there is a presynaptic muscarinic mechanism that normally inhibits release of the peptide in this gland in some species (25), and the release of ACh itself in the myenteric plexus of the guinea pig (19, 20). However, this is not the case in all species; in the rabbit, atropine substantially and significantly reduces the output of VIP during continuous high-frequency C-T stimulation (16). It has yet to be established which particular type of muscarinic receptor is responsible for presynaptic inhibition of VIP release, but presynaptic inhibition of ACh release has generally been ascribed to activation of the M₂ subpopulation (32, 34, 35). This has also been demonstrated in the parotid gland of the rat (36) in which the release of VIP during parasympathetic stimulation is also enhanced after atropine (40).

As in the ferret (39), the flow of submandibular saliva was significantly increased by the intermittent pattern of C-T stimulation at low frequency (10 Hz at 1-s intervals, compared with 1 Hz continuously), although the difference was not significant in the present study. In contrast, the secretory response to C-T stimulation at 40 Hz in bursts was significantly less than that to stimulation at 4 Hz continuously. It is inevitable that the efficiency of ganglionic transmission must fail as the frequency of preganglionic stimulation is increased. In the celiac ganglion of the rabbit, this has been shown to occur during continuous stimulation at 15 Hz after ~15 s, and the phenomenon becomes more marked both with increasing stimulus frequency and the passage of time (28). This fits rather well with the
finding that the enhancement obtained by stimulation in 1-s bursts fails, in the case of salivary secretion, at frequencies of >20 Hz in the submandibular gland of the ferret and >10 Hz in sheep. The phenomenon also provides for the observation that autonomic responses to direct stimulation of the postganglionic innervation can be enhanced by stimulating in bursts at even higher frequencies (40 Hz), as with the fall in SVR in the parotid gland of the sheep during stimulation of the parotid nerve (2).

The enhancement of responses such as vasodilatation of salivary blood vessels is most likely due to an increased release of VIP from the nerve terminals because it is well established that release of neuropeptides is potentiated by high-frequency stimulation (3, 24), possibly by favoring the opening of L-type Ca\(^{2+}\) channels in the nerve terminals (33). VIP exerts a nitric oxide-dependent effect directly on the vasculature (10) and, in the cat, potentiates the secretory response to ACh. This could be due to enhanced muscarinic ligand binding (26) or to increased mobilization of cAMP in the acinar cells because the effect can be mimicked by forskolin, which exerts this action, in the parotid gland of the rat (21). The precise mechanism for the direct atropine-resistant secretory effect, which VIP exerts in the sheep as well as in the rat and mink (14, 37), has yet to be elucidated.

The evidence that ovine submandibular responses to parasympathetic stimulation depend, in part, on the release of VIP as well as ACh can now be summarized as follows. 1) The peptide is present in nerve terminals within the gland closely adjacent to both small blood vessels and acini (41). 2) Stimulation of the parasympathetic (C-T) innervation at relatively high frequencies produces an atropine-resistant secretion that cannot be accounted for by the release of substance P. 3) Intra-arterial infusions of the peptide elicit a protein-rich secretion (30) together with a pronounced vasodilatation (present study). 4) Stimulation of the parasympathetic (C-T) innervation produces a frequency-dependent increase in the output of VIP from the gland over the range of 10–20 Hz. 5) The fall in SVR that occurs under these conditions is linearly related to log VIP output after the administration of atropine. 6) Both secretory and vascular responses to C-T stimulation at low frequencies are enhanced by stimulation in bursts, which significantly increase the output of VIP from the submandibular salivary gland in the cat (3).

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