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Tachykinin involvement in parasympathetic nerve-evoked salivation of the ferret

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1 The tachykinin antagonist (D-Arg[1], D-Cl_2Phe[5], Asn[6], D-Trp[7,9], Nle[11])-substance P, injected intravenously, blocked salivary secretion from the ferret parotid and submandibular glands in response to subsequent i.v. injections of the tachykinins, substance P and neurokinin A.

2 The tachykinin antagonist reduced the parasympathetic nerve-evoked secretion of parotid and submandibular saliva by 15–20% and 35–40%, respectively. Atropine abolished the remaining secretory response.

3 The 'atropine-resistant' parasympathetic nerve-evoked secretion of saliva from the parotid and submandibular glands (about 5 and 30%, respectively, of that before administration of atropine) was abolished by the tachykinin antagonist.

4 The tachykinin antagonist was without effect on the protein concentration of parotid and submandibular saliva secreted in response to parasympathetic nerve stimulation. Parotid and submandibular saliva lacked amylase.

5 Atropine reduced the protein concentration of the submandibular saliva secreted in response to parasympathetic nerve stimulation by 50%; this was the protein concentration of substance P-evoked saliva.

6 The secretory response to methacholine and to stimulation of preganglionic sympathetic nerve fibres, tested in rats, was unaffected by the tachykinin antagonist, contra-indicating an unspecific action of the antagonist.

7 The results suggest that the neuronal release of tachykinins is probably important in the nerve-evoked secretory response of the parotid and submandibular glands.

Introduction

In ferrets, intravenous injection of the tachykinins substance P (SP) or neurokinin A (NKA) causes a profuse flow of saliva from the parotid and submandibular glands (Ekström & Olgart, 1986). Salivary secretion from these glands evoked by electrical stimulation of the parasympathetic nerve is partially resistant to antimuscarinic agents. In the submandibular glands the 'atropine-resistant' response is 30% of the response in the absence of atropine, while in the parotid glands the corresponding figure is 5% (Ekström *et al.*, 1987a; 1988a).

In the present study we investigated the effect of a tachykinin antagonist on tachykinin- and parasympathetic nerve-evoked salivary secretion in the ferret. A preliminary account of some of the findings has been given elsewhere (Ekström *et al.*, 1987c).

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Methods

Thirty-two adult ferrets, of either sex, (1.02 ± 0.05 kg, mean ± s.e. mean) were anaesthetized with pentobarbitone (30–40 mg kg^{-1} i.p., further anaesthetic was injected i.v.). The animals were fitted with a tracheal cannula and a rectal thermometer; the body temperature was maintained at 38°C. The ducts of the parotid and submandibular glands were cannulated with glass cannulae (Ekström *et al.*, 1988a); only one gland of each type was tested in the same animal. Drops of saliva falling from the tip of the cannula were recorded on a smoked drum by means of electromagnetic pens. All saliva secreted in response to

the test procedure was collected in (pre-weighed) tubes and weighed. The density of saliva was regarded as 1.0. Samples of saliva were analysed for their protein content by the method of Lowry et al. (1951), using bovine serum albumin as standard, and for their amylase activity by an enzymatic colorimetric test (Boehringer Mannheim GmbH, Mannheim, F.R.G.) with α-4-nitrophenyl maltoheptaoside (4NP-G_7) as substrate (Hägele et al., 1982); one unit (u) of catalytic activity of α-amylase is defined as the hydrolysis of 1 μmol of 4NP-G_7 $min^{-1} ml^{-1}$, being equivalent to the definition of the International Unit. The auriculo-temporal nerve of the parotid gland and the chorda-lingual nerve of the submandibular gland were exposed and cut as far proximally as possible (the auriculo-temporal nerve where it emerged from the base of the skull). The peripheral stumps of the nerves were placed on bipolar electrodes and stimulated supramaximally (8 V, 2 ms) at variable frequencies (0.2–60 Hz) for periods, usually, of 1 or 2 min. The effect of the tachykinin antagonist was tested by adding the agent after the first and before the second frequency-response curve. In control experiments on four parotid glands and four submandibular glands the second frequency-response curve was constructed without addition of the antagonist. There was an interval of 30 min between the first and second frequency-response curve. At the end of the experiment the stimulated gland was removed and weighed.

The arterial blood pressure was monitored (pressure transducer) via a catheter placed in the femoral artery. The heart rate was counted from the ECG, which was recorded from needle electrodes placed under the skin. The respiratory rate was also monitored.

Observations were also made on five female rats (266 ± 10 g) anaesthetized with chloralose (80–100 $mg\,kg^{-1}$ i.v.) after induction with ether. Here, the cranial part of the sectioned sympathetic cervical (preganglionic) nerve trunk was stimulated supramaximally (4 V, 2 ms, 5 Hz, 1 min) and submandibular saliva was collected.

The following drugs were used: atropine sulphate (Sigma Chemical Co., St Louis, U.S.A.), dihydroergotamine methansulphonate (Sandoz AG, Basel, Switzerland), methacholine chloride (Sigma), propranolol hydrochloride (ICI Pharmaceuticals, Macclesfield, UK), neurokinin A (NKA; substance K; Bachem, Basel, Switzerland), substance P (SP; Sigma) and a tachykinin antagonist (D-Arg^1, D-Cl_2Phe^5, Asn^6, D-$Trp^{7,9}$, Nle^{11})-SP (SP 150:II, Ekström et al., 1987c; a kind gift from Ferring Pharmaceuticals, Malmö, Sweden). This antagonist was selected from a series of novel SP analogues with antagonistic properties because of its potency in blocking (a) effects of SP on the isolated taenia coli of the guinea-pig and (b) nerve stimulation-evoked, SP-mediated responses in the isolated iris sphincter from the rabbit (Håkanson et al., 1988). The peptides were dissolved in saline containing 0.5% bovine serum albumin. They were stored in stock solutions at −20°C. All drugs were injected into the cannulated femoral vein via a polyethylene catheter. Each injection was immediately followed by an injection of 0.4 (ferret) or 0.2 (rat) ml of saline that considerably exceeded the cannula dead-space. The tachykinin antagonist was injected at least 10 min before the test procedure. Further details are given below. Values are means ± s.e. mean. Student's t test for paired data was used to test for statistically significant differences and $P < 0.05$ was considered statistically significant.

Results

Observations on the ferret

The experiments were performed in the presence of α-(dihydroergotamine 0.5–1 $mg\,kg^{-1}$ i.v.) and β-(propranolol 0.5–1 $mg\,kg^{-1}$ i.v.) adrenoceptor blockers. There was no resting secretion from the glands. The mean wet weights of the parotid and submandibular glands were 176.5 ± 10.4 ($n = 29$) and 427.5 ± 19.0 ($n = 31$) mg, respectively. In four animals, the mean arterial blood pressure was 117 ± 13 and 120 ± 11 mmHg before and 10 min after the injection of the tachykinin antagonist (0.75 $mg\,kg^{-1}$ i.v.), respectively; the immediate response to the injection was a prompt and short-lasting decline in pressure (down to 99 ± 12 mmHg). The heart rate was 210 ± 12 beats min^{-1} (before) and 207 ± 13 beats min^{-1} (after 10 min); there was no change in heart rate immediately after the injection. The rate of respiration was 32 ± 1 (before) and 33 ± 2 (after 10 min) breaths min^{-1}. These basal values of blood pressure, heart rate and respiratory rate remained unchanged throughout the experiment (up to 90 min).

Substance P-, neurokinin A- and methacholine-evoked secretion

The tachykinin antagonist (0.75 $mg\,kg^{-1}$ i.v.) caused no salivary secretion. In response to a standard dose of SP (0.2 $\mu g\,kg^{-1}$ i.v.), the parotid and submandibular glands secreted 53.2 ± 7.6 ($n = 18$) and 51.8 ± 5.0 ($n = 17$) μl saliva, respectively; SP at a dose of 0.5 $\mu g\,kg^{-1}$ i.v. caused these glands to secrete 132.2 ± 8.9 ($n = 5$) and 191.2 ± 31.3 ($n = 6$) μl saliva. Administration of the antagonist (0.75 $mg\,kg^{-1}$ i.v.) before the injection of SP completely blocked the response to these doses of SP in the two glands for at least 2–3 h (Figure 1). Under

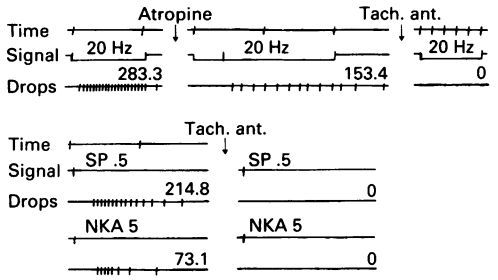

Figure 1 Secretion of submandibular saliva in a ferret. In the upper row, the responses to stimulation of the parasympathetic nerve at 20 Hz before (1 min, left) and after atropine, 2 mg kg^{-1} i.v., (2 min, middle) or atropine and the tachykinin antagonist (D-Arg^{1}, D-Cl$_2$Phe^{5}, Asn^{6}, D-Trp^{7,9}, Nle^{11})-substance P, 0.75 mg kg^{-1} i.v., (5 min, right). In the middle and lower rows, the responses to i.v. injection of substance P (SP, 0.5 μg kg^{-1}) and neurokinin A (NKA, 5 μg kg^{-1}), in the presence of atropine, before (left) and after (right) administration of the tachykinin antagonist. Records from above: time in min; signal to mark the period of nerve stimulation, the drug injection and the onset of secretion; drops of saliva (the amount secreted, in μl, is also shown).

these circumstances large SP doses (1–2 μg kg^{-1} i.v.) gave rise to trace secretion only; about 2 h after injection of antagonist the secretory response to these large SP doses started to return. Also a dose of 0.5 mg kg^{-1} i.v. of the antagonist completely inhibited the effect of the standard dose of SP, while at a lower dose (0.1 mg kg^{-1} i.v.) the response to SP was halved.

NKA at a dose of 5 μg kg^{-1} i.v. caused the parotid and submandibular glands to secrete 11.3 ± 2.7 ($n = 4$) and 50.8 ± 9.2 μl saliva, respectively. The tachykinin antagonist (0.75 mg kg^{-1} i.v.) completely inhibited the response to the subsequent injection of NKA (Figure 1).

The secretory response to the parasympathomimetic drug methacholine (5 μg kg^{-1} i.v.) was not affected by the tachykinin antagonist (0.75 mg kg^{-1} i.v.). The parotid glands ($n = 20$) secreted 21.7 ± 1.9 μl saliva before and 23.4 ± 2.2 μl saliva 10–60 min (mean 30 min) after administration of the antagonist, while the corresponding figures for the submandibular glands ($n = 18$) were 10.5 ± 1.6 and 13.9 ± 1.9 μl saliva.

Parasympathetic nerve-evoked secretion

After administration of the tachykinin antagonist (0.75 mg kg^{-1} i.v.), the parotid ($n = 6$) and submandibular ($n = 6$) glands responded as promptly as before to parasympathetic nerve stimulation. Neither the threshold frequency for secretion, nor the frequency causing maximal response (parotid glands 40 Hz; submandibular glands 20 Hz) was changed in the presence of the antagonist. However, the magnitude of the salivary response to nerve stimulation was reduced. This was not the case if the nerve stimulation was repeated without the addition of the tachykinin antagonist (not shown). In the parotid glands (Figures 2 and 3), the antagonist evoked a statistically significant reduction at 10–60 Hz; at 40 Hz the response was 81.6 ± 7.0% of that before administration of the antagonist ($P < 0.05$). In the submandibular glands (Figures 2 and 3), a statistically significant reduction was observed at 2–40 Hz; at 20 Hz the response was 61.5 ± 8.0% of that before administration of the antagonist ($P < 0.02$). Other glands were stimulated at a single frequency only, before and after administration of the antagonist (0.75 mg kg^{-1} i.v.). The pattern was the same as when the whole range of frequencies was tested. In parotid glands the response to 40 Hz, 1 min, was 82.7 ± 6.6% ($n = 4$) of that in the absence of the antagonist ($P < 0.05$), while in submandibular glands the response to 20 Hz, 1 min, was 66.2 ± 8.0% ($n = 5$) of that in the absence of the antagonist ($P < 0.05$). Atropine (2 mg kg^{-1} i.v.) completely abolished the response remaining after administration of the tachykinin antagonist, except for two submandibular glands where a trace secretion occurred (less than 1% of the response in the absence of tachykinin antagonist and atropine); these observations were made during a stimulation period of 5 min at 40 Hz (parotid glands) or 20 Hz (submandibular glands) (Figure 3).

Atropine-resistant parasympathetic nerve-evoked seretion

In the absence of atropine (and tachykinin antagonist), the parotid ($n = 3$) and submandibular ($n = 3$) glands secreted 108.1 ± 22.3 (40 Hz) and 262.2 ± 13.1 (20 Hz) μl saliva during a period of 1 min, respectively. When the stimulation was repeated in the presence of atropine (2 mg kg^{-1} i.v.), the parotid and submandibular glands secreted 8.9 ± 2.4 and 149 ± 4 μl saliva, but now, during a 2 min period, respectively (Figure 1). This is in agreement with previous findings (Ekström *et al.*, 1988a), as was the observation of a marked increase in latency from start of stimulation to the appearance of saliva (parotid glands from 1.1 ± 0.1 to 37.3 ± 2.3 s; submandibular glands from 1.7 ± 0.3 to 23.4 ± 4.3 s). After administration of the tachykinin antagonist, the response to nerve stimulation during a 5 min period was either completely abolished or, in two submandibular glands, reduced to a very low level (less than 1% of the response obtained in the

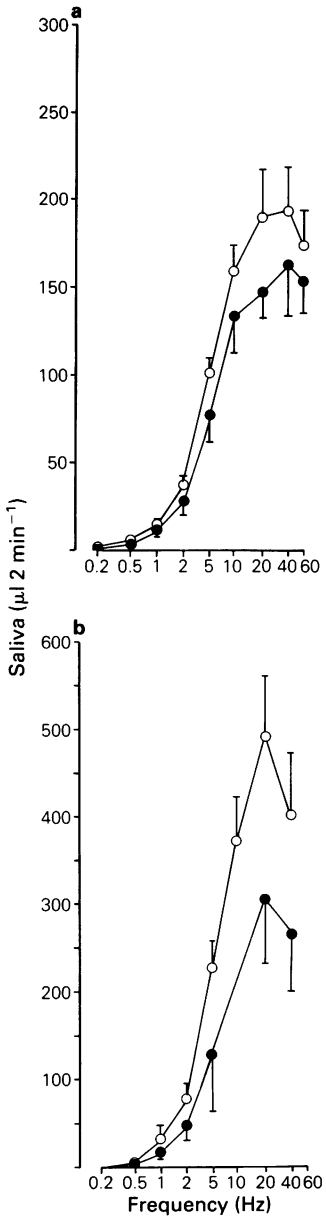

Figure 2 The effect of the tachykinin antagonist (D-Arg[1], D-Cl_2Phe[5], Asn[6], D-Trp[7,9], Nle[11])-substance P, 0.75 mg kg^{-1} i.v., on the amount of saliva secreted from the ferret (a) parotid ($n = 6$) and (b) submandibular ($n = 6$) glands in response to stimulation of the parasympathetic nerves at various frequencies. Ordinate scale: amount of saliva, in μl, per 2 min. Abscissa scale; frequency of stimulation in Hz. (○) Before and (●) after administration of the tachykinin antagonist. Values are means and vertical lines indicate s.e. mean; in some cases s.e. mean was smaller than the size of the symbols.

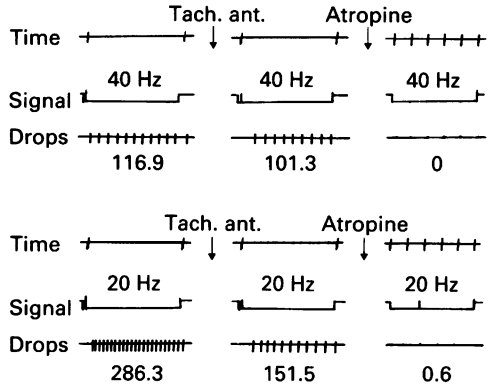

Figure 3 Secretion of parotid saliva in one ferret (upper row) and secretion of submandibular saliva in another ferret (lower row) in response to stimulation of the parasympathetic nerves at 40 (parotid gland) or 20 Hz (submandibular gland) before (1 min, left) and after the tachykinin antagonist (D-Arg[1], D-Cl_2Phe[5], Asn[6], D-Trp[7,9], Nle[11])-substance P, 0.75 mg kg^{-1} i.v. (1 min, middle) or the tachykinin antagonist and atropine, 2 mg kg^{-1} i.v., (5 min, right). Records from above: time in min; signal to mark the period of nerve stimulation and the onset of secretion; drops of saliva (the amount secreted, in μl, is also shown).

absence of atropine and tachykinin antagonist) (Figure 1).

Protein and amylase in the saliva

The protein concentration of parotid and submandibular saliva secreted in response to SP (0.2 μg kg^{-1} i.v.) was 948 ± 169 ($n = 8$) and 673 ± 154 ($n = 7$) μg ml^{-1}, respectively. The protein concentration of saliva secreted in response to nerve stimulation in the presence of the tachykinin antagonist (0.75 mg kg^{-1} i.v.) was the same as that in the absence of the antagonist. In parotid saliva ($n = 4$), stimulated at 40 Hz, it was 1185 ± 139 μg ml^{-1} before and 1162 ± 189 μg ml^{-1} after the administration of the antagonist, while in submandibular saliva ($n = 6$), stimulated at 20 Hz, the corresponding figures were 1176 ± 148 and 1268 ± 155 μg ml^{-1}. The protein concentration of the submandibular saliva ($n = 7$) secreted following stimulation at 20 Hz after administration of atropine (in the absence of the tachykinin antagonist) was significantly less than that before atropine (579 ± 63 vs 1216 ± 69 μg ml^{-1}, $P < 0.01$).

The amylase activity in parotid and submandibular saliva did not differ from blank values (< 0.05 u ml^{-1}).

Observations on the rat

In five submandibular glands, the tachykinin antagonist (1–2.5 mg kg^{-1} i.v.) completely or almost completely abolished the response to SP at a dose of 0.2 μg kg^{-1} i.v. (before/after: 25.3 $\pm$ 3.0/2.2 $\pm$ 0.8 μl saliva), while both the sympathetic nerve-evoked response (5 Hz, 1 min) (before/after: 10.3 $\pm$ 1.5/9.9 $\pm$ 1.2 μl saliva) and the methacholine-evoked response at 5 μg kg^{-1} i.v. (before/after: 14.8 $\pm$ 3.2/13.4 $\pm$ 3.0 μl saliva) were unaffected.

Discussion

The results of the present study suggest that there is a parasympathetic nervous release of tachykinins in ferret salivary glands that contributes to the secretory response. The new, potent tachykinin antagonist (D-Arg^{1}, D-Cl$_2$Phe^{5}, Asn^{6}, D-Trp^{7,9}, Nle^{11})-SP (Håkanson *et al.*, 1988) was found to inhibit completely the secretory effect of exogenous SP and NKA, and to reduce parasympathetic nerve-evoked secretion by 15–20% in parotid and 35–40% in submandibular glands. Further, the tachykinin antagonist abolished the 'atropine-resistant' parasympathetic nerve-evoked response in both glands. Rat salivary glands secrete upon administration of SP (Lembeck & Starke, 1968; Ekström & Wahlestedt, 1982) and upon stimulation of the parasympathetic nerve in the presence of atropine (Thulin, 1976; Ekström *et al.*, 1983; 1987b). In this species the tachykinin antagonist was also found to block the secretory effect of SP.

The action of the tachykinin antagonist seemed to be specific. The response to the parasympathomimetic, methacholine, was unaffected by the antagonist, which excludes an 'atropine-like' effect or a general depressive action on the secretory cells. In the case of the submandibular gland, preganglionic nerve fibres of the chorda-lingual nerve were being stimulated (Ekström *et al.*, 1988a) and reduced responses following administration of the antagonist might be due to impairment of ganglionic transmission. The sympathetic secretory innervation of ferret salivary glands seems to be poor (Ekström *et al.*, unpublished observation). The rat submandibular gland has a rich sympathetic secretory innervation (Ohlin, 1965) and was, therefore, considered a suitable preparation for the stimulation of the preganglionic sympathetic nerve fibres. However, in the presence of the antagonist, in a dose larger than that used in the ferret, sympathetic stimulation caused salivary secretion of the same magnitude as before the administration of the antagonist. This argues against a ganglionic blocking effect and a general neurosuppressive effect of the antagonist.

'Atropine-resistant', parasympathetic nerve-evoked salivation has been demonstrated in the three major salivary glands of the rat (Ekström, 1987; Ekström *et al.*, 1987b) and in the parotid and submandibular glands of the ferret (this study; Ekström *et al.*, 1988a). Recently, it was also shown in the sheep parotid and submandibular glands (Reid & Titchen, 1988). A relatively high frequency of stimulation is required before this 'atropine-resistant' secretion becomes apparent; in the ferret, the threshold frequency is 10–20 Hz in parotid glands and 2–10 Hz in submandibular glands. These frequencies coincide with those where the secretory response was found to be significantly reduced by the tachykinin antagonist. Interestingly, the 'tachykinin antagonist-sensitive' fraction of the response evoked by high frequency nerve stimulation was greater than the 'atropine-resistant' fraction (this study; Ekström *et al.*, 1988a), particularly so in the parotid gland (15–20% vs 5%); the difference might be explained by co-operation of co-released transmitters. Sympathetic nerve fibres are not thought to reach salivary glands via the parasympathetic nerve (Garrett, 1982). Nevertheless, in the present study the ferrets were pretreated with adrenoceptor blockers to exclude adrenergic effects, if any, on the secretory cells upon parasympathetic nerve stimulation.

The protein concentration of both parotid and submandibular saliva resulting from parasympathetic nerve stimulation was unaltered by the tachykinin antagonist. The protein concentration of 'atropine-resistant' nerve-evoked saliva from the submandibular gland was reduced. The protein concentration of the SP-evoked submandibular saliva fell into the range of that of the 'atropine-resistant' nerve evoked saliva.

Both parotid and submandibular saliva were found to lack amylase. This was predicted by Poddar & Jacob (1977), because the ferret salivary glands have no true serous cells.

Since (a) the 'tachykinin antagonist-resistant' parasympathetic nerve-evoked response of the ferret was abolished by atropine and, conversely, (b) the 'atropine-resistant' parasympathetic nerve-evoked response was abolished by the tachykinin antagonist and (c) the protein concentration of SP-evoked secretion was about the same as that of the 'atropine-resistant' parasympathetic nerve-evoked secretion, it is tempting to suggest that tachykinins are involved in the non-adrenergic, non-cholinergic nerve-evoked salivation. Other neuropeptides, such as vasoactive intestinal peptide (VIP; Wharton *et al.*, 1979; Uddman *et al.*, 1980; Wathuta, 1986) and calcitonin gene-related peptide (Ekman *et al.*, 1986; Ekström *et al.*, 1988b), have been demonstrated in salivary glands. They may play a role together with the tachykinins. VIP, like SP, occurs in nerve fibres

around acini of the ferret salivary glands (Ekström *et al.*, unpublished observation), but does not give rise to salivary secretion. However, VIP enhances the secretory response to exogenous SP and NKA (Ekström *et al.*, 1988a). Of interest in this connection is the observation that the SP-evoked secretory response in the ferret is enhanced by parasympathetic nerve stimulation in the presence of atropine, at subthreshold secretory frequencies (Ekström *et al.*, 1988a). Furthermore, recent observations (Ekström & Tobin, unpublished) show that VIP causes release of protein from ferret salivary glands.

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