Br. J. Pharmacol. (1990), **101**, 853–858

Effects of repeated infusions of substance P and vasoactive intestinal peptide on the weights of salivary glands subjected to atrophying influences in rats

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1 The long-term influence of substance P (SP) and vasoactive intestinal peptide (VIP) on rat salivary gland weight was investigated after parasympathetic denervation or on feeding soft food.

2 The parotid gland lost about one-third of its weight within 4–5 days following parasympathetic postganglionic denervation or change in dietary regimen, from pellets to liquid diet, thought to reduce nerve reflex activity.

3 Daily i.v. infusions with SP or VIP diminished or largely prevented the fall in parotid gland weight, whereas infusions with pentagastrin, bethanechol and saline had no effect. The infusions were preceded by administration of α- and β-adrenoceptor antagonists; these antagonists were also given to the control animals.

4 The effect of SP and VIP on the parotid gland weight appeared to be related to cell size rather than to cell number, as judged by measurements of RNA and DNA.

5 Observations on the two other major salivary glands underlined the fact that different gland types in the same animal behave differently. Parasympathetic preganglionic denervation (decentralization) lowered the weights of the sublingual and submandibular glands, whereas liquid diet only reduced the weight of the sublingual gland. SP and VIP did not affect the weights of the submandibular glands, but VIP prevented the slight fall in sublingual gland weight induced by liquid diet.

6 The present results suggest a trophic role in rats for SP and VIP on parotid glands and for VIP on sublingual glands. Such an influence may be exerted naturally as a result of their release from nerves containing these peptides around acini.

Introduction

In rats, the parotid gland loses 30–40% of its weight after parasympathetic denervation or diminished nerve reflex activation induced by dietary change, from pellets to liquid diet (Schneyer & Hall, 1966). Surprisingly, the weight of this gland does not decrease in response to prolonged treatment with antimuscarinic agents; in fact, it increases somewhat and this is not dependent on an intact sympathetic innervation (Ekström, 1974). In this species, stimulation of the parasympathetic innervation evokes a flow of parotid saliva in the presence of atropine and adrenoceptor antagonists (Ekström *et al.*, 1983a). Nerves occur in the rat parotid gland that contain substance P (SP, Sharkey & Templeton, 1984; Ayer-Le Lievre & Seiger, 1985), of the tachykinin family, and vasoactive intestinal peptide (VIP, Wharton *et al.*, 1979; Uddman *et al.*, 1980) and are found in fibres surrounding the acini. SP- and VIP-containing nerve fibres reach the parotid gland via the parasympathetic auriculo-temporal nerve, and SP and VIP are thought to be transmitters involved in the 'atropine-resistant', parasympathetic nerve-evoked secretion of parotid saliva (see Ekström, 1987; Ekström *et al.*, 1989a). In the present study we have considered the possibility that SP and VIP exert long-term influences on salivary gland size. Therefore, they were administered over a period of time in order to assess their efficacy in preventing the expected fall in parotid gland weight following parasympathetic denervation or reduced nerve reflex activity. For comparisons, some observations were made on the sublingual and submandibular glands as well as on the pancreatic gland.

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Methods

Altogether 145 adult, female Sprague-Dawley rats were used. Preliminary surgery was performed in animals anaesthetized by inhalation of diethyl ether. In order to denervate the parotid gland parasympathetically, the auriculo-temporal nerve was cut (Ekström, 1974; Alm & Ekström, 1976); in some experiments the chorda-tympani nerve (carrying preganglionic parasympathetic nerve fibres, Houssay *et al.*, 1962) was also cut where it crosses over the auriculo-temporal nerve, to cause parasympathetic decentralization of the sublingual and submandibular glands. These denervation and decentralization procedures were performed unilaterally. An intravenous polyethylene catheter for chronic use was inserted into the femoral vein and the tip was placed in the inferior caval vein. The other end of the catheter was inserted subcutaneously to an interscapular position and fitted with a modified Venflon wing (Viggo AB, Helsingborg, Sweden), which was sutured to the muscle fascia and allowed to protrude through the skin. This end of the catheter was sealed with a rubber membrane, through which repeated needle insertions could be made to gain access to the circulation. At the end of each experiment the venous position of the catheter and its function were checked. Food and water were given *ad libitum*. Liquid diet was prepared daily by mixing two parts of water with one part of a powder made from a commercial pelleted diet (Astra-Ewos, Södertälje, Sweden). Rats in the denervation/decentralization study and those serving as controls in the liquid diet study were maintained on this pelleted diet. The drug treatment started 12 h after denervation/decentralization (SP, 2 μg kg^{-1} min^{-1}; VIP, 5 μg kg^{-1} min^{-1}; and bethanechol, 20 μg kg^{-1} min^{-1}) or change to liquid diet (SP, 5 μg kg^{-1} min^{-1}; VIP, 5 μg kg^{-1} min^{-1}; and pentagastrin

$10\,\mu g\,kg^{-1}\,min^{-1}$). These drugs (or sterile saline) were infused twice daily for 1 h over a period of five days. During the infusions the animals were awake and restrained to Bollman cages, to which they had been accustomed. The animals were given the α-adrenoceptor blocking agent dihydroergotamine ($0.5\,mg\,kg^{-1}$ i.p.) and the β-adrenoceptor blocking agent propranolol ($0.5\,mg\,kg^{-1}$ i.p.) 10 min before the i.v. infusion began. The control rats in the denervation/decentralization study and those given a pelleted diet in the liquid diet study were kept in Bollman cages and subjected to the adrenoceptor antagonists following the same protocol as mentioned above. The doses of adrenoceptor antagonists used were sufficiently large to prevent a possible sympathetic influence on the gland cells in connection with the infusions.

Twelve hours after the last infusion the animals were killed by diethyl ether and both parotid glands (and in some experiments the submandibular and sublingual glands as well as the pancreatic gland) were removed, weighed, and either heated to 110°C for 48 h (dry weights, denervation/decentralization study) or frozen and stored at −70°C for determination of gland protein, RNA and DNA (liquid diet study). In the latter study the mean value of left and right salivary glands of each animal was used for statistical calculations. RNA and DNA were extracted according to the procedure of Schmidt & Thannhauser (1945), modified by Schneider (1946) and then, measured as described by Mejbaum (1939) and Burton (1956), respectively. The standards used were RNA from baker's yeast (Sigma, Chemical Co, Mo, St Louis, U.S.A.) and DNA from calf thymus (Sigma). Protein was measured by the method of Lowry *et al.* (1951), the standard being bovine serum albumin. In preliminary experiments on non-denervated animals (kept on pellets) mean arterial blood pressure was monitored continuously (pressure transducer) via a catheter placed into a femoral (anaesthetized animals) or tail artery (awake animals). To measure flow of parotid saliva in anaesthetized rats (chloralose, $100\,mg\,kg^{-1}$ i.v.), the parotid duct was cannulated and drops of saliva falling from the cannula were photoelectrically recorded and collected over 10 min periods in tubes and weighed. Drugs were infused either via a femoral vein or a tail vein (awake animals); the animals were pretreated with α- and β-adrenoceptor blockers as mentioned above.

Drugs

Adrenaline tartrate (ACO, Solna, Sweden), carbamyl β-methylcholine chloride (Bethanechol, Sigma), dihydroergotamine methansulphonate (Sandoz, Basel, Switzerland), pentagastrin (ICI Pharmaceuticals, Macclesfield, U.K.), propranolol hydrochloride (ICI), SP (Sigma), and VIP (kindly supplied by Professor V. Mutt, Karolinska Institutet, Stockholm, Sweden).

Group matching and statistics

In the groups to be compared, the rats were age- and weight-matched. At the end of the experimental period no differences between the body-weights of the rats in each group were found (see legends to figures). Student's *t* test for unpaired data was used, and the Bonferroni method was applied to make multiple comparisons (Wallenstein *et al.*, 1980). *P* values <0.05 were considered to be significant. Values given are means ± s.e.mean.

Results

Mean arterial blood pressure and salivary secretion

With respect to blood pressure, the pattern was the same in anaesthetized and non-anaesthetized animals. After an initial drop in pressure at the start of the infusion the bethanechol- and SP-treated animals regained their pre-infusion levels of blood pressure, while in the VIP-treated rats the recovery was not complete; the pressure was about 100 mmHg during the major part of the infusion period (Figure 1, awake animals). Total amount of parotid saliva secreted over 60 min in response to SP ($2\,\mu g\,kg^{-1}\,min^{-1}$) was $700 \pm 84\,\mu l$ ($n = 3$ glands and rats) and to bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$) $616 \pm 91\,\mu l$ ($n = 3$). During infusion of SP the salivary flow rate declined, while it was steady during the bethanechol infusion. There was a small flow of viscous saliva in response to VIP ($5\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$). However, the cannula was repeatedly plugged which made measurements of the volume secreted difficult.

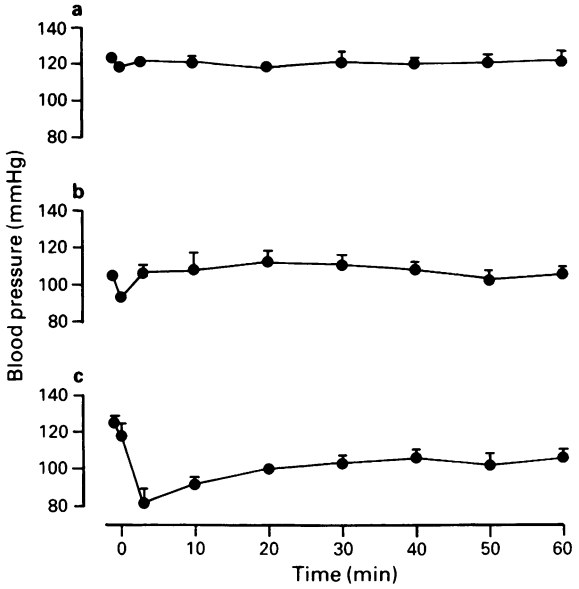

Figure 1 Mean arterial blood pressure monitored in (9) awake rats infused i.v. with either (a) bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$), (b) substance P ($2\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$) or (c) vasoactive intestinal peptide ($5\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$) over a period of 60 min. The animals were pretreated with α- and β-adrenoceptor blocking agents.

Control experiments with adrenoceptor antagonists

Control experiments in 10 rats after adrenoceptor blockade showed adrenaline ($10\,\mu g\,kg^{-1}$ i.v.) to be ineffective in evoking secretion of saliva from the parotid and submandibular glands and the response to stimulation of the sympathetic innervation (20 Hz in bursts of 1 s every 10 s, see Anderson *et al.*, 1988) to be reduced by 80%. Three hours after the injection of the antagonists a certain recovery in the secretory response had occurred. However, the responses were far from pre-injection levels. It should also be noted that much larger doses of adrenoceptor antagonists than presently used are ineffective in lowering salivary gland weights in rats (Brenner & Stanton, 1970).

Parasympathetic denervation or decentralization

Parotid glands Figure 2a shows the gradual fall in the weight of the parotid gland after denervation. The expected fall in weight caused by denervation was diminished or largely prevented by infusions of SP and VIP. The weights of denervated glands of rats treated with these peptides were significantly heavier than those of denervated glands of untreated controls (+27%, $P < 0.05$, and +32%, $P < 0.01$, respectively, Figure 3a,b). After VIP treatment the weights of denervated glands were not significantly different from those of the intact contralateral glands from the control animals. After bethanechol, the weights of denervated glands were higher than those from

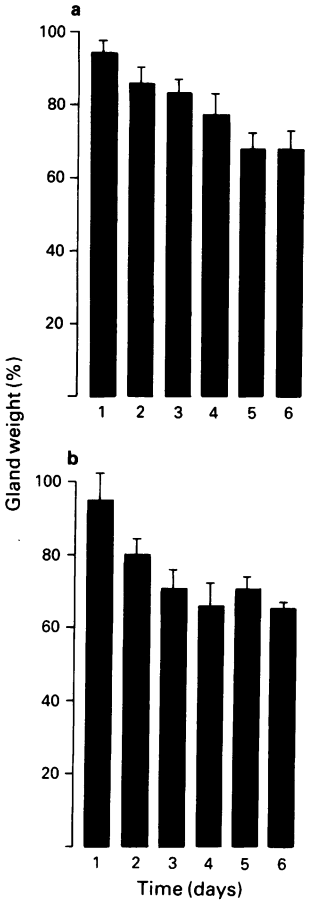

Figure 2 Effects on parotid gland (wet) weights over time following (a) parasympathetic denervation and (b) change in dietary regimen, from pellets to liquid diet. (a) At each time of observation following the unilateral denervation three rats were used. The weight of the denervated gland was expressed as a percentage of the weight of the contralateral gland (the contralateral glands did not show any compensatory hypertrophy with time; weight of contralateral glands 166 ± 5 mg, $n = 18$). (b) At each time of observation following the change to liquid diet three rats were used. The gland weights (mean of left and right glands) were expressed as percentages of mean gland weight of the same control group of six rats given pelleted diet (143 ± 5 mg). Values are means and bars show s.e.mean.

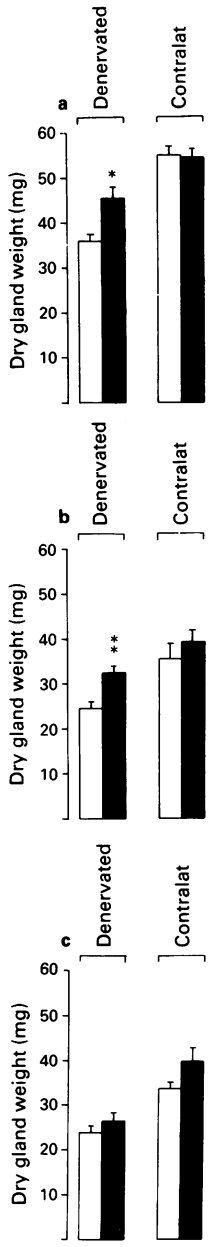

Figure 3 Effects on the weights of denervated and contralateral parotid glands of i.v. infusions (one hour twice daily for 5 days) of (a) substance P (SP, $2\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 274 ± 8 g, $n = 9$, and untreated rats 276 ± 8 g, $n = 9$), (b) vasoactive intestinal peptide (VIP, $5\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 215 ± 7 g, $n = 6$, and untreated rats 222 ± 8 g, $n = 6$) and (c) bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 219 ± 8 g, $n = 6$, and untreated rats 217 ± 4 g, $n = 6$). Before each infusion period the animals were given α- and β-adrenoceptor blocking agents; the untreated, control rats were subjected to the same protocol. The weights of the glands of the drug-treated animals (solid columns) were compared with those of corresponding glands of untreated, control animals (open columns). Values are means and bars show s.e.mean. $^*P < 0.05$, $^{**}P < 0.01$.

controls (+11%, Figure 3c), but the difference did not achieve statistical significance. Also the weights of contralateral glands of bethanechol- and VIP-treated rats tended to be higher than those of corresponding glands of controls (+11% and +18%, respectively), but again the differences were not statistically significant.

Sublingual and submandibular glands The dry weights of decentralized sublingual and submandibular glands of control rats after 5 days of saline infusion were 35% less (5.1 ± 0.2 mg versus 7.9 ± 0.2 mg, $n = 11$, $P < 0.001$) and 23% less (40.4 ± 1.9 mg versus 52.2 ± 2.0 mg, $n = 13$, $P < 0.001$), than those of intact contralateral glands. Neither in the SP-treated (nine observations for each type of gland) nor in the VIP-treated animals (four observations) were the decentralized glands heavier than the corresponding decentralized glands of untreated animals.

Liquid diet

Parotid glands The fall in gland weight following the change from pellets to liquid diet is shown in Figure 2b. The fall in weight was not diminished by saline (−42%, $P < 0.001$) (Figure 4a) but was less after SP and VIP. The SP and VIP-treated glands were significantly heavier than those of saline-treated animals (SP +32%, VIP +45% $P < 0.001$). In fact after VIP treatment the gland weight did not differ significantly from that of the animals on the pelleted diet. In this study the dose of substance P used was larger than that in the denervation/decentralization study. However, this increase in dose did not prevent a reduction in gland weight. The gland weight of pentagastrin-treated rats was not different from that of saline-treated ones. Bethanechol was not tested in this part of the study. Total gland protein in saline-treated rats on a liquid diet was reduced by as much as 52% ($P < 0.001$), whereas the reduction after SP- and VIP-treatment was less

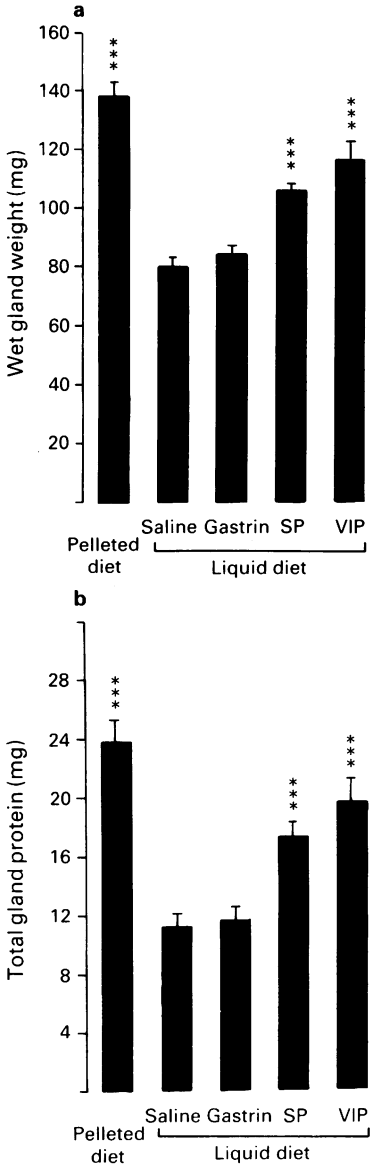

Figure 4 Effects on (a) weight and (b) total protein of parotid glands of rats maintained on liquid diet of i.v. infusions (one hour twice daily for 5 days) of saline (bodyweight 219 ± 5 g, $n = 6$), pentagastrin (Gastrin, 10 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 220 ± 7 g, $n = 6$), substance P (SP, 5 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 215 ± 3 g, $n = 6$) and vasoactive intestinal peptide (VIP, 5 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 218 ± 10 g, $n = 6$). For comparison, the glands of a group of rats kept on pelleted diet are included (bodyweight 225 ± 2 g, $n = 9$). Before each infusion period the animals were given α- and β-adrenoceptor blocking agents; the animals given pellets underwent the same treatment. Values are means and bars show s.e.mean. Differences between the glands of saline-treated rats and those of each of the other four groups of rats were analysed. $P < 0.001$.

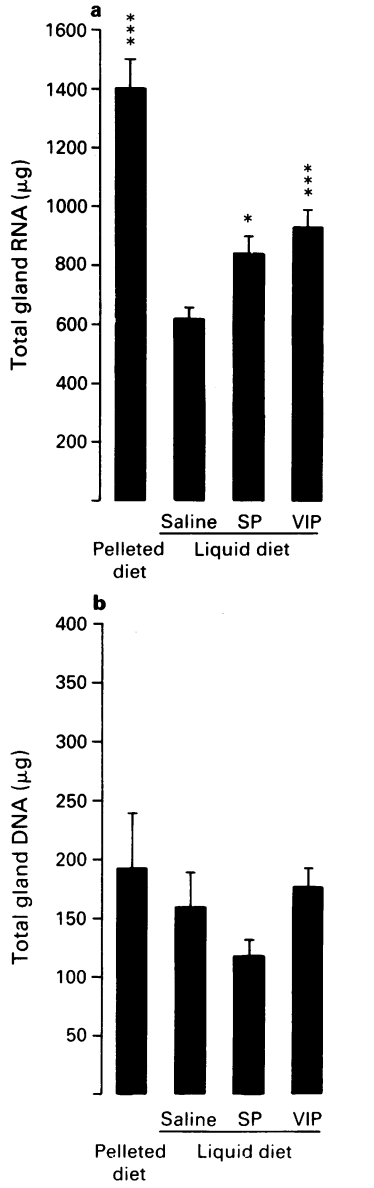

Figure 5 Effects on total amounts of (a) RNA and (b) DNA in parotid glands of rats maintained on liquid diet of i.v. infusions of saline, substance P (SP) and vasoactive intestinal peptide (VIP). For further details, see legend of Figure 4. * $P < 0.05$, *** $P < 0.001$.

(Figure 4b). Thus, total gland protein was significantly higher in SP- and VIP-treated rats than in the saline-treated ones (SP +53%, VIP +73%, $P < 0.001$). The value for the VIP-treated animals was not significantly different from that for the animals on the pelleted diet. In pentagastrin-treated rats, total gland protein was the same as in the saline-treated animals. The reduction in the total amount of RNA was 56% ($P < 0.001$) in the glands of saline-treated animals (Figure 5a). The amount of RNA was significantly larger in SP- and VIP-treated rats than in saline-treated ones (SP +35%, $P < 0.05$, VIP +50%, $P < 0.001$). The total amount of DNA in the glands of the saline-treated rats was not significantly different from that in the glands of rats maintained on pellets (Figure 5b). Neither were there any statistically significant differences between gland DNA content of saline-treated animals and that of SP- and VIP-treated rats, although the DNA content after SP treatment was notably low. The nucleic acids were not analysed in the glands of the pentagastrin-treated rats.

Sublingual and submandibular glands Sublingual glands of rats kept on a liquid diet and infused with saline (5.9 ± 0.2 mg dry weight, $n = 6$) weighed significantly less (−17%) than glands of rats given pellets (7.1 ± 0.3 mg, $n = 9$, $P < 0.05$). However, the glands of VIP-treated rats (7.4 ± 0.4 mg, $n = 4$) were significantly heavier than those of saline-treated rats (+25%, $P < 0.01$). The sublingual gland weights of SP- (6.3 ± 0.6 mg, $n = 5$) and pentagastrin- (6.6 ± 0.4 mg, $n = 6$) treated rats were not significantly different from those of saline-treated animals.

The weight of the submandibular gland, which was not affected by the liquid diet, did not change in response to the various infusions.

Pancreas The pancreatic gland (dry) weights of rats given pellets (287 ± 11 mg, $n = 4$) and liquid diet combined with infusions of either saline (265 ± 20 mg, $n = 6$) or VIP (242 ± 19 mg, $n = 4$) were about the same, while those of rats

treated with pentagastrin (346 ± 26 mg, $n = 6$) were significantly greater ($P < 0.05$, compared with saline-treated rats).

Discussion

The fall in weight on the parotid gland as a result of parasympathetic denervation or liquid diet was diminished or largely prevented by treatment with SP or VIP. Obviously, the effect of the two neuropeptides was not due to an increase in glandular water content, as judged by dry weights and total amounts of gland protein and RNA. Treatment with the parasympathetic drug bethanechol and pentagastrin had no significant effect on the parotid gland weight. However, the bethanechol treatment tended to reduce the magnitude of the weight loss of the denervated gland and to cause the contralateral gland to gain in weight. That pentagastrin, in the dose used, had trophic potential was shown in the pancreas, since the pancreatic gland weight increased as previously found (Mayston & Barrowman, 1971).

The neuropeptides were administered in the presence of α- and β-adrenoceptor antagonists. This was to exclude the possibility that a peptide-induced release of noradrenaline from sympathetic nerve terminals, or of catecholamines from the adrenal medulla (Malhotra & Wakade, 1987), caused the glandular effects observed in response to SP and VIP. A β-adrenoceptor-mediated hypertrophy of salivary glands in rats is a well-known phenomenon (see e.g. Schneyer, 1962; Brenner & Stanton, 1970). In the denervated glands a release of acetylcholine or other substances from parasympathetic nerve terminals elicited by SP and VIP can also be excluded as a cause of the effect of these peptides on glandular weight. Furthermore, the finding that SP and VIP affected significantly the denervated gland, but not the contralateral gland in the same animal underlines the fact that a parasympathetic background activity was not a prerequisite for the phenomenon to occur. Thus, it seems unlikely that the effect of SP and VIP observed in the rats given the liquid diet was due to an indirect mechanism, depending on the presence of parasympathetic nerve fibres in the glands. Previously, intraperitoneal injections of the nonmammalian tachykinin physalaemin (over a period of 15 days) have been shown to cause an increase in parotid and submandibular gland weights of rats, whereas eledoisin, another nonmammalian tachykinin, did not cause any increase in gland weight (Bertaccini *et al.*, 1966; Cantalamessa *et al.*, 1975); in these studies pretreatment with adrenoceptor antagonists was not undertaken.

The effect of SP and VIP on parotid gland weight was not solely a reflection of their capacity to increase the flow of saliva, since the dose of VIP used produced much less secretion of saliva (but a very protein-rich one, Ekström *et al.*, 1983b) than bethanechol, and the dose of SP used produced about the same amount of saliva as bethanechol. The same conclusion can be drawn by comparing the effects of the β-adrenoceptor agonist isoprenaline, which causes a very large increase in parotid gland weight but only a small, protein-rich, salivary flow, with those of pilocarpine, which causes none or only a slight increase in gland weight but a copious flow of saliva (Schneyer, 1969; Ekström, 1977). The enlargement of the rat parotid gland occurring in response to β-adrenoceptor stimulation is associated with increases in adenosine 3′:5′-cyclic monophosphate (cyclic AMP, Butcher & Putney, 1980). In this gland, VIP is thought to act via cyclic AMP as intracellular messenger (Dehaye *et al.*, 1985), but in contrast to β-adrenoceptor stimulation the VIP activation of the gland cells also involves calcium (Christophe *et al.*, 1989); parasympathomimetics and SP are also thought to act via the calcium pathway (Putney, 1986). Evidently, there must be complex interactions which maintain the normal gland size since, for example, agonists thought to use the same intracellular pathway differ in their effectiveness in causing trophic responses. Furthermore, different gland types within the same animal behave differently, as illustrated in the present study.

Following a liquid diet, Schneyer (1970) obtained a reduced amount of RNA, but unchanged amount of DNA, in the parotid gland, indicating that the weight loss was probably attributable to a decrease in the size of individual cells and not to a decrease in the number of cells. Similar findings were obtained in the present study. The fact that RNA, but not DNA, was found to be higher in the glands of SP- and VIP-treated animals than in the saline-treated animals, favours the idea that these peptides affected cell size rather than cell number. Notably, with respect to DNA, there was in fact a tendency for a decrease to occur in response to treatment with substance P.

There was no statistically significant increase in the weights of the contralateral parotid glands (supplied with an intact innervation) in response to the various drug infusions. Increased sensitivity to chemical stimuli, such as SP and VIP, of the secretory cells of the parotid glands subjected to parasympathetic denervation (Ekström, 1980; Ekström & Wahlestedt, 1982; Ekström *et al.*, 1983b) or liquid diet (Ekström & Templeton, 1977) may have created favourable conditions for revealing trophic effects.

The sublingual, but not the submandibular, glands showed a slight fall in weight in response to liquid diet, as previously observed (Wells & Peronace, 1967). In the present study, VIP, but not SP, prevented the reduction in sublingual gland weight. The weights of the atrophied, parasympathetically-decentralized sublingual and submandibular glands, which do also develop supersensitivity to SP and VIP (for references, see above), were not affected by the treatment with these neuropeptides. A comparison of the different behaviour of the three major salivary glands to the various agents, shows that the parotid glands are particularly susceptible to treatment with SP and VIP. This may be combined with the observation that prolonged treatment with antimuscarinic agents reduces the weights of the sublingual and submandibular glands of the rat (Ohlin & Perec, 1966; Ekström, 1974), but not the weight of the parotid gland which increases slightly (Ekström, 1974).

Polyamines are known to be coupled to protein synthesis and cell growth (Tabor & Tabor, 1984). Interestingly, in rat parotid and sublingual glands the polyamine formation increases in response to infusions (for 3 h in the anaesthetized rat) with VIP, in particular, but also to SP (Ekström *et al.*, 1989b). Further, in these glands polyamine formation is influenced by non-adrenergic non-cholinergic transmitters in response to stimulation of the parasympathetic innervation (Ekström *et al.*, 1989c). The present results seem to suggest a trophic role for SP and VIP: loss of SP and VIP bombardment on secretory cells after parasympathetic denervation and liquid diet may contribute to the parotid gland atrophy. After parasympathetic denervation SP and VIP disappear from this gland (Ekström *et al.*, 1984) and these peptides are also reduced in the gland by liquid diet (Månsson *et al.*, 1990). Interestingly, *in vitro* studies on mammalian cell cultures suggest that SP and VIP are growth-promoting factors (Dalsgaard *et al.*, 1989).

Supported by grants from the Swedish Medical Research Council (5927), Nordisk Insulin Foundation Committee, Dir. Albert Påhlssons foundation and the Medical and Odontological Faculties in Lund.

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(Received March 24, 1990
Revised June 24, 1990
Accepted July 25, 1990)