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Parasympathetic Non-adrenergic, Non-cholinergic Mechanisms in Salivary Glands and Their Role in Reflexly Elicited Glandular Responses

Anna Asztély

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II. Ekström, J., Asztély, A. & Tobin, G. (1996). NANC-induced parotid acinar degranulation in response to stimulation of the parasympathetic innervation in the anaesthetized rat. *Experimental Physiology* **81**, 935-942.

III. Asztély, A., Tobin, G. & Ekström, J. (1996). The role of circulating catecholamines in the depletion of parotid acinar granules in conscious rats in the cold. *Experimental Physiology* **81**, 107-117.

IV. Asztély, A., Tobin, G. & Ekström, J. (1994). Parasympathetic non-adrenergic, non-cholinergic reflex secretion of parotid acinar granules in rats pretreated with atropine and adrenoceptor antagonists. *Regulatory Peptides* **51**, 255-262.

V. Asztély, A., Tobin, G. & Ekström, J. (1994). Masticatory-salivary reflexes mobilize non-adrenergic, non-cholinergic secretory mechanisms in parotid glands of conscious rats. *Acta Physiologica Scandinavica* **151**, 373-376.

VI. Asztély, A., Ekman, R. Tobin, G. & Ekström, J. (1996). Depletion of vasoactive intestinal peptide and substance P in parotid glands of atropinized rats during reflex secretion. *Experimental Physiology* **81**, 297-303.

VII. Asztély, A., Havel, G. & Ekström, J. (1998). Vascular protein leakage in the rat parotid gland elicited by reflex stimulation, parasympathetic nerve stimulation and administration of neuropeptides. *Regulatory Peptides* **77**, 113-120.

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PARASYMPATHETIC NON-ADRENERGIC, NON-CHOLINERGIC MECHANISMS IN SALIVARY GLANDS AND THEIR ROLE IN REFLEXLY ELICITED GLANDULAR RESPONSES

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Since the days of Heidenhain (1872) the view has been, that in salivary glands the parasympathetic nerve-evoked vasodilatation is resistant to atropine, whereas the parasympathetic nerve-evoked secretion is easily abolished by the muscarinic receptor blocker. In recent years, however, it has become evident that in some species such as the rat and the ferret a flow of saliva, albeit at a reduced rate, occurs in response to parasympathetic stimulation despite pretreatment with atropine and α- and β-adrenoceptor antagonists, while in other species such as the cat salivary proteins are just released without any overtly secreted fluid. The present series of experiments focus, in particular, on the effect of the parasympathetic non-adrenergic, non-cholinergic (NANC) mechanisms in the exocytotic response of the parotid gland in the cat and the rat and further, on the role of these mechanisms in reflex secretion. Although, the sympathetic nerve is usually made responsible for the release of acinar secretory granules, prolonged parasympathetic stimulation caused a marked parotid acinar degranulation as assessed by morphometry. In the cat, the NANC mechanisms were responsible for the major part of the parasympathetic exocytotic response, while, in the rat, these mechanisms were responsible for (almost) the whole response. The further analysis showed infusion of the neuropeptide vasoactive intestinal peptide (VIP) to induce degranulation in the cat parotid gland without evoking any flow of saliva. The parasympathetic secretory NANC mechanisms would no doubt gain in significance if a role could be found for them under natural reflex conditions. The acinar degranulation in the parotid gland that occurs in the conscious rat in response to feeding has been attributed to sympathetic nerve activity and, in the chronically sympathectomized glands of rats under cold stress, to activation of the sympatho-adrenal system releasing catecholamines. However, in contrast to the prevailing view the present experiments showed the parasympathetic nerve and its NANC mechanisms to be reflexly mobilized in response to intake of hard chow. The chronically sympathectomized glands of rats under cold stress still lost acinar granules following adrenal medullectomy and pretreatment with adrenoceptor antagonists and atropine but not after additional parasympathectomy. The secretory NANC mechanisms were also at work in intact glands, where the catecholamine influence was acutely eliminated by adrenoceptor antagonists. Here, they were potentially responsible for the whole parasympathetic exocytotic response and for the major part of the response in the absence of any blockers. The NANC induced response was not elicited when the consistency of the food was changed to a liquified form, so the phenomenon depended on mastication rather than on taste. The reflexly elicited impulse traffic aiming at activating the salivary glands is most likely enhanced following atropinization, and in response to chewing the parotid gland was depleted on its content of VIP and substance P, both being secretagogues in this gland. Following feeding, the parotid gland was surrounded by an oedema. Evans blue was used to indicate changes in vascular permeability, and the result focused on neuropeptides of parasympathetic origin, notably neurokinin A, as cause of the oedema formation.

Key words: exocytosis, plasma protein leakage, parasympathetic stimulation, reflex secretion, mastication, non-adrenergic, non-cholinergic mechanisms, neuropeptides

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ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals.

I. Depletion of secretory granules from the feline parotid gland: action of NANC transmitters per se. (1994). *Acta Physiologica Scandinavica* **150**, 83-88. (Together with J. Ekström, H.F. Helander and G. Tobin).

II. NANC-induced parotid acinar degranulation in response to stimulation of the parasympathetic innervation in the anaesthetized rat. (1996). *Experimental Physiology* **81**, 935-942. (Together with J. Ekström and G. Tobin).

III. The role of circulating catecholamines in the depletion of parotid acinar granules in conscious rats in the cold. (1996). *Experimental Physiology* **81**, 107-117. (Together with J. Ekström and G. Tobin).

IV. Parasympathetic non-adrenergic, non-cholinergic reflex secretion of parotid acinar granules in rats pretreated with atropine and adrenoceptor antagonists. (1994). *Regulatory Peptides* **51**, 255-262. (Together with J. Ekström and G. Tobin).

V. Masticatory-salivary reflexes mobilize non-adrenergic, non-cholinergic secretory mechanisms in parotid glands of conscious rats. (1994). *Acta Physiologica Scandinavica* **151**, 373-376. (Together with J. Ekström and G. Tobin).

VI. Depletion of vasoactive intestinal peptide and substance P in parotid glands of atropinized rats during reflex secretion. (1996). *Experimental Physiology* **81**, 297-303. (Together with R. Ekman, J. Ekström and G. Tobin).

VII. Vascular protein leakage in the rat parotid gland elicited by reflex stimulation, parasympathetic nerve stimulation and administration of neuropeptides. (1998). *Regulatory Peptides* **77**, 113-120. (Together with J. Ekström and G. Havel).

CONTENTS

RESULTS AND DISCUSSION

INTRODUCTION

Salivary secretion is elicited by impulses in the autonomic nervous system as a response to various afferent stimuli. At rest, dryness of the oral mucosa and low-grade mechanical stimulation of the oral soft tissue maintain the nervous reflex drive on the secretory cells, and the saliva produced moistens, lubricates and protects the mucosa. Intermittently, in response to eating, the salivary glands are reflexly thrown into a state of high activity by stimulation of various types of receptors such as mechanoreceptors, gustatory receptors, olfactory receptors and nociceptors, and a large volume of saliva is secreted during a short period of time. An intense salivation is also associated with thermoregulation in some species including the rat, which by, under heat stress, spread their saliva on the fur for evaporating cooling (Emmelin, 1967; Hainsworth, 1967). In contrast to other exocrine glands of the alimentary tract, hormones, presently known, do not evoke secretion in salivary glands (with the possible exception of circulating catecholamines). However, there is a long-term hormonal influence on the morphology and function of importance for the glandular responses (Johnson, 1988; Anderson, 1998).

Stimulation of the parasympathetic innervation evokes a copious flow of saliva with a low concentration of protein. Sympathetic stimulation, on the other hand, evokes only a small flow of saliva, if any at all. The sympathetic saliva that is secreted contains a high protein concentration. In response to combined parasympathetic and sympathetic stimulation, both the secretion of fluid and the release of protein are usually enhanced. Under reflex conditions, the sympathetic secretory impulses are thought to act in a background of parasympathetic secretory impulses (Emmelin, 1979).

The various types of salivary glands serve various purposes. Parotid glands are particularly associated with mastication. Claude Bernard (1856) called attention to the facts that this type of gland is relatively larger in horses than in

dogs, the former animal chewing the food more extensively than the latter. He also described experiments in which the consistency of the food given to a horse varied, and found chewing in response to hay to induce secretion of larger volumes of parotid saliva than to fresh bread. In line with these studies the parotid gland of the rabbit secretes larger volumes of saliva in response to chewing pellets compared with chewing carrots (Gjörstrup, 1980). Long-term changes in the consistency of the food influence the parotid gland size. In the rat, a change from the standard pelleted diet to a liquid diet induces a rapid fall in gland weight (Hall & Schneyer, 1964; Ekström, 1973), while a change to a bulk-rich diet increases the weight (Wells & Peronace, 1967; Ekström, 1974). Since salivary reflexes are easily influenced by general anaesthesia, studies on the masticatory-salivary reflex on conscious mammals including humans are of particular interest. In an early report by Stoney (1873), mastication was shown to be an effective stimulus for parotid secretion in a patient with a parotid fistula, and in dental practice the "Lashley cup", placed over the orifice of the parotid duct, is a common device to collect parotid saliva. Studies in humans show the masticatory-salivary reflex to be elicited by activation of periodontal ligament mechanoreceptors and gingival mucosal tissue mechanoreceptors in response to the chewing force (Anderson & Hector, 1987; Jensen Kjeilen, Brodin, Aars & Berg, 1987; Linden, 1990; Scott, Hassanwalia & Linden, 1998).

The adjectives "cholinergic" and "adrenergic" were introduced by Dale (1933) to classify the nerve fibres on the basis of the chemical transmitter released from them, referring to acetylcholine and adrenaline (later shown to be *nor*-adrenaline by von Euler, 1946). He concluded that the postganglionic parasympathetic nerve fibres were, predominantly, and perhaps entirely, cholinergic and that the postganglionic sympathetic nerve fibres were predominantly, though not entirely adrenergic. During the past twenty years it has become increasingly evident that the autonomic nervous system uses a large number of transmitters, besides acetylcholine and noradrenaline, to influence

its effector organs (Burnstock, 1986). The "non-adrenergic, non-cholinergic" (NANC) effector responses elicited are not abolished by atropine, the classical muscarinic receptor antagonist, nor by antagonists to the α- and β-adrenergic receptors. A large number of peptides, and also nitric oxide (NO), have been found to serve as NANC transmitters in various effectors of the autonomic nervous system (Lundberg, 1996).

Retrospectively, Heidenhain´s finding in 1872 of an increased blood flow in the submandibular gland of the atropinized dog in response to electrical stimulation of the chorda-lingual nerve seems to be the very first report of a parasympathetic NANC phenomenon. Already before the turn of the century a couple of other parasympathetic NANC responses had, in fact, been reported, such as the contraction of the urinary bladder (Langley & Andersson, 1895) and the relaxation of the stomach (Langley, 1898). The atropine-resistant increase in blood flow, originally observed by Heidenhain, has been confirmed in a number of species. Over the years many factors have been supposed to account for the vasodilatation in salivary glands (Edwards, 1998): oxygen consumption, metabolic factors, bradykinin, hyperosmolarity, potassium ions, histamine, prostaglandins and muscarinic receptors inaccessible for atropine (the "proximity theory"). Mainly due to the works by Edwards (1980) and Lundberg (1980) and their co-workers the neuropeptide vasoactive intestinal peptide (VIP) has emerged as a likely parasympathetic NANC transmitter responsible for the vasodilatory response in the presence of atropine, and recently shown to depend on the formation of NO for its vasodilatory action.

As would be expected from the early experience of the effect of atropine on the vagal effect on the heart (Bezold & Bloebaum, 1867), Heidenhain found atropine to completely abolish the flow of saliva from the dog submandibular gland elicited by chorda-lingual nerve stimulation, a finding soon confirmed in the cat submandibular gland by Langley (1878). Since these days, the general view has been that parasympathetic nerve-evoked secretion in salivary glands is

easily abolished by atropine (Burgen & Emmelin, 1961). About hundred years later and using the rat as experimental animal Thulin (1976a,b) reported, in passing, a small flow of submandibular and parotid saliva that persisted upon electrical stimulation of the parasympathetic innervation despite pretreatment with atropine and adrenoceptor antagonists.

Since the early Eighties, the laboratory from which this *Thesis* originates has been engaged in studies on the parasympathetic NANC secretion in salivary glands of a number of species and on the role of neuropeptides in this phenomenon and further, on the long-term, trophic NANC regulation of salivary glands as revealed in polyamine metabolism, protein synthesis and gland size (Ekström, 1987, 1989; Nilsson, 1991; Månsson, 1991; Tobin, 1991). On their own the parasympathetic NANC mechanisms cause secretion of saliva as in the rat (Ekström, Månsson, Tobin, Garrett & Thulin, 1983b; Månsson & Ekström, 1991) and the ferret (Ekström, Månsson, Olgart & Tobin, 1988c) or just release of salivary proteins as in the cat (Ekström & Tobin, 1990). In the rat parotid gland the fluid response amounts, initially, to as much as 50 % of that in the absence of atropine. Characteristic features of the parasympathetic NANC evoked secretion of saliva are the relatively high threshold frequency (2-10 Hz) of stimulation required to elicit the phenomenon, the relatively long latency in onset of secretion and the fading response over time. These mechanisms can, however, be shown to be at work at much lower frequencies than those causing an overt fluid response. For instance, a release of protein occurs already at a frequency of 0.2 Hz in the ferret submandibular gland (Ekström & Tobin, 1989) and further at this low frequency and in the absence of atropine, the NANC mechanisms in the rat parotid gland interact positively with acetylcholine thereby enhancing the fluid response (Månsson & Ekström, 1991). In the rat sublingual gland, there is a NANC induced increase in the activity of ornithine decarboxylase, the key enzyme in the synthesis of polyamines, in response to prolonged stimulation of the parasympathetic innervation at 2 Hz applied in bursts (delivered for 1 s every 10th s) (Nilsson,

Rosengren & Ekström, 1991)

Several transmitters are likely to contribute to the nerve evoked NANC response, including VIP, pituitary adenylate cyclase activating peptide (PACAP), the tachykinins substance P and neurokinin A, calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY). In line with the variable responses to nerve stimulation, the response to administration of the neuropeptides varies. For instance, substance P, and to a less extent neurokinin A, evoke a copious flow of saliva in the rat and the ferret but cause no flow in the cat (Ekström & Wahlestedt, 1982; Ekström, Månsson & Tobin, 1987; Ekström *et al.* 1988c, see also Bertaccini & De Caro, 1965). VIP and PACAP evoke a small flow of saliva in the rat, which from the parotid gland is very protein-rich, but only protein release in the cat and the ferret (Ekström, Månsson & Tobin, 1983a; Ekström & Tobin, 1989, 1990; Mirfendereski, Tobin, Håkanson & Ekström, 1997). CGRP releases salivary proteins in the rat, while in the ferret CGRP evokes secretion of saliva poor in protein (Ekström, Ekman, Håkanson, Sjögren & Sundler, 1988a; Edwards, Ekström, Tobin & Mirfendereski, unpublished observations). NPY induces an *in vitro* release of salivary proteins from the glands of the rat, while *in vivo* this peptide evokes no flow of saliva (Sharkey, Mathison, Sharif & Davison, 1989; Ekström, Ekman, Luts, Sundler & Tobin, 1996).

Positive interactions with regard to fluid secretion and protein output occur between various peptides and between peptides and muscarinic and adrenergic agonists: in the rat parotid gland between substance P and VIP or CGRP, between a choline ester and VIP or CGRP, and between VIP and the α-adrenergic agonist phenylephrine (Ekström & Olgart, 1986; Ekström *et al.* 1988a; Bobycock & Chernick, 1989; Larsson & Olgart, 1989), and in the parotid and submandibular glands of the ferret between substance P and VIP (Ekström & Tobin, 1989), and in these glands of the cat between a choline ester and VIP (Lundberg, Änggård & Fahrenkrug, 1982; Ekström & Tobin,

1990). The synergistic effects achieved seem to be associated with the combined mobilization of the two intracellular pathways (Baum, 1988) "Ca^{2+}/ Inositoltriphosphate" (used by substance P and muscarinic and α-adrenergic receptor agonists) and "cAMP" (used by VIP and CGRP as well as by β-adrenergic receptor agonists).

In the rat, denervation experiments showed, the overwhelming proportion of the VIP, substance P and NPY containing nerve fibres to reach the parotid gland via the route of the parasympathetic auriculo-temporal nerve (Ekström, Brodin, Ekman, Håkanson & Sundler, 1984; Ekström *et al.* 1996). The NPY containing nerve fibres that innervate the blood vessels reach the gland via the sympathetic nerve supply (Schultz, Soinila, Tolonen, Häppölä, Uusitalo & Salo, 1994). Only a minor proportion of the CGRP containing nerve fibres travel via the auriculo-temporal nerve (Ekström *et al.* 1988a). The facial nerve, cervical dorsal root nerves and yet unknown pathways contribute to the major gland content of CGRP. The CGRP containing nerve fibres of these sources harbour substance P as well, and occur predominantly around ducts and blood vessels. These CGRP/SP containing fibres are thought to be of sensory origin. Upon electrical stimulation of the auriculo-temporal the rat parotid gland is gradually depleted on its gland content of the peptides, being an indirect measure of peptide release (Ekström, Brodin, Ekman, Håkanson, Månsson & Tobin, 1985; Tobin, Ekström, Ekman & Håkanson, 1994). More directly, VIP (cat, rabbit and ferret), NPY (cat) substance P (ferret) and CGRP (ferret) have been shown to appear in the venous drainage of the submandibular gland in response to stimulation of the chorda-lingual nerve in a number of animals (Bloom & Edwards, 1980; Lundberg, Änggård, Fahrenkrug, Hökfelt & Mutt, 1980; Fazekas, Gazelius, Edwall, Theodorsson-Norheim, Blomquist & Lundberg, 1987; Tobin, Ekström, Bloom & Edwards, 1991; Modin, Weitzberg & Lundberg, 1994). In the rat parotid gland, the NO synthesizing enzyme, NO synthase (NOS), is confined to the parasympathetic nerves as shown by denervation experiments (Alm, Ekström, Larsson, Tobin &

Andersson, 1997). Nerve fibres containing this enzyme are found close to the acinar cells and blood vessels. In the cat and ferret submandibular glands, parasympathetic nerve activity evokes flow of saliva and output of protein by mechanisms that involve the generation of NO upon which effect VIP is largely dependent (Buckle, Parker, Bloom & Edwards, 1995; Tobin, Edwards, Bloom & Ekström, 1997).

The parotid glands are supplied with only one type of acinar cells and further, these cells contain clearly delineated secretory granules storing pre-formed secretory proteins. In an investigation by Langley as early as 1879 on fresh live parotid tissue, he found the acinar cells of fasted animals to be packed with granules. In response to stimulation of the sympathetic nerve in the neck or injection of pilocarpine or a preceding feeding period, the acinar cells became markedly degranulated. The focus was on the rabbit parotid gland but the finding was the same in parotid glands of cats, dogs and rats. Although the parasympathomimetic drug pilocarpine in high doses may activate, at the ganglionic level, the sympathetic secretory pathway (Schneyer & Hall, 1966), the early observation made by Langley of acinar degranulation in response to administration of pilocarpine combined with his further notation of a similar effect, in the rabbit parotid gland, in response to stimulation of the "cerebral" nerve suggested, in fact, an influence of both branches of the autonomic nervous system on the exocytotic process. In recent years, much due to the extensive work on the rat parotid gland both *in vitro* and *in vivo* including reflex secretion, the sympathetic contribution to the acinar degranulation, mediated via β-adrenergic receptors and involving cAMP, has come to be emphasized, leaving no role for the parasympathetic nerve in the exocytosis (Garrett, 1988).

Despite the prevailing concept, it was recently shown possible, by choosing a high frequency of stimulation of the parasympathetic innervation, to induce acinar degranulation of the parotid gland of the anaesthetized rat (Ekström,

Garrett, Månsson & Tobin, 1988). Furthermore, the acinar cells still lost their secretory granules when the stimulation was performed in the presence of atropine and adrenergic receptor antagonists, showing the involvement of NANC mechanisms in the exocytotic process. When turning to the cat parotid gland, parasympathetic stimulation, but not sympathetic stimulation, was found to induce granular depletion of the acini (Emmelin & Garrett, 1989).

It is evident that the NANC effects in the transmission of parasympathetic secretory impulses would gain in physiological significance if a role could be demonstrated for them under reflex conditions. When the experimental work of this *Thesis* began some evidence was already at hand suggesting a reflex contribution of the NANC mechanisms. In a study by Reid & Titchen (1988) on the anaesthetized and atropinized sheep, the continuous flow of saliva from the sympathectomized parotid gland was shown to accelerate and the salivary protein concentration to increase in response to distension of the distal thoracic oesophagus. Furthermore, the parotid gland of the conscious rat, sympathetically denervated 1-2 weeks in advance, and pretreated with atropine and α- and β-blockers had been shown to lose acinar granules and amylase activity in response to feeding (Ekström, Helander & Tobin, 1993). With the parotid gland of the rat as the main model organ, the present *Thesis* focuses particularly on the contribution of the parasympathetic NANC mechanisms to the reflexly elicited secretory response. The loss of acinar secretory granules and glandular amylase activity was presently used as indices of glandular activity. The number of acinar secretory granules was morphometrically assessed, while the amylase activity was estimated enzymatically.

The release of proteins from exocrine glands seems to follow two main pathways (Kelly, 1985; Proctor, 1998). One is the well-established so called regulated pathway, where, after leaving the Golgi apparatus, proteins are condensed in the maturing storage granule compartment, transported to the apical part of the cell and released from large dense core granules to the

lumen by exocytosis upon receipt of a signal. The formation of new granules is a process thought to take several hours (Amsterdam, Ohad & Schramm, 1969; Castle, Jamieson & Palade, 1972). The other pathway is the constitutive (vesicular) one, where proteins by-pass the granular storage stage. Instead newly formed molecules travel directly from the Golgi apparatus to the apical and basolateral plasma membranes of the cell by a vesicular mechanism allowing a release of proteins as fast as they are synthesized. Superimposed upon a continuous basal vesicular release is the vesicular release in response to external stimuli. Little morphological evidence of constitutive secretory vesicles has been presented in salivary cells, one reason no doubt being the fact that the vesicles lack a dense core (Zastrow & Castle, 1987). There is, however, biochemical evidence for a non-granular protein secretion in the glands (Asking & Gjörstrup, 1987; Garrett, Zhang, Proctor, Anderson & Shori, 1996).

The acinar cells of the rat parotid gland occupy about 90% of the gland. Amylase is the major exportable enzyme, being 35% of total secretory protein (Robinowitch, Keller, Johnson, Iversen & Kauffman, 1977). 55% of the gland amylase activity is located in the granules (Scramm & Danon, 1961). Release of stored secretory proteins as well as resynthesis of proteins have been studied extensively in the rat parotid gland. This gland shows a diurnal cycle related to the nocturnal feeding habits of the animals. During the day, the gland stores of granular secretory products increase, while the protein synthesis decreases. In response to intake of hard chow, the secretory products are expelled and the protein synthesis increases during the night. Maintenance of rats on a liquid diet over a period of time abolishes the diurnal cycle and reduces protein synthesis and protein gland content as well as cell size and the number of acinar secretory granules (Sreebny, Johnson & Robinowitch, 1971; Johnson, 1988). Electrical nerve stimulation shows both divisons of the autonomic nervous system to promote protein synthesis in the rat parotid gland (Asking & Gjörstrup, 1987).

In the first section of this *Thesis* the effect of electrical stimulation of the parasympathetic nerve was investigated in the parotid gland of the cat and the rat. In the cat (1A), it was wondered whether the parasympathetically nerve-evoked acinar degranulation previously reported (Emmelin & Garrett, 1989) engaged NANC mechanisms. This was found to be the case, and a possible involvement of VIP was examined. Further, the acinar degranulation previously described in response to high frequency stimulation in non-atropinized rats as well as in atropinized rats (Ekström *et al.* 1988b) was morphometrically assessed (1B). Stimulation was also performed in the presence of an inhibitor of protein synthesis to find out whether this would affect the number of persisting acinar granules (1B). For comparison, the effect of sympathetic stimulation was investigated also.

In the second section, the feeding response in the cold (2-4°C) was studied. An extensive acinar degranulation in chronically sympathectomized parotid glands of rats, exposed to cold and fed a pelleted diet, has been attributed to circulating catecholamines (Garrett, Harrop & Thulin, 1985). Howerver, in that study the parasympathetic innervation of the gland was intact, and the effects of adrenal medullectomy or adrenergic receptor antagonists were not examined. By including these procedures, and atropine in addition, it was possible to demonstrate similar (or even greater) effects as in those glands subjected only to the sympathetic denervation. In the further analysis, a possible role for circulating catecholamines was considered following combined sympathetic and parasympathetic denervation (2B).

In the third section, the parasympathetic NANC contribution to the reduction in acinar granules and amylase activity in non-sensitized parotid glands of the rat in response to feeding was investigated (3A). Furthermore, it was wondered whether the glandular response depended on masticatory-salivary reflexes (3B). In further series of experiments, it was investigated whether

reflex mobilization of NANC mechanisms in the rat parotid gland was reflected in the neuropeptide content of the gland (3C).

Some neuropeptides such as the tachykinins and CGRP are known to promote microvascular protein leakage and tissue swelling, and are important mediators in sensory neurogenic inflammatory responses in the airways, eye, skin and the urinary bladder (Lundberg & Saria, 1987; Lembeck, 1988; Maggi, 1993; Håkanson & Wang, 1996). The parotid gland of the rat, activated under reflex conditions or by electrical stimulation of the parasympathetic innervation was often found surrounded by an oedema. Since the oedema developed in the absence as well as in the presence of atropine and α- and ß- adrenoceptor antagonists a role for NANC mechanisms in the tissue swelling was considered in the last section of the *Thesis* (4).

In summary, the present *Thesis* pays attention to the following questions:

* To which extent are the NANC mechanisms involved in the parasympathetic exocytotic response of the rat parotid gland and further, are these mechanisms involved in the parasympathetic exocytotic response of the cat parotid gland?
* Do the parasympathetic NANC mechanisms contribute to the exocytotic response to feeding in the chronically sympathectomized parotid gland under cold stress and further, is there any role for circulating catecholamines in the exocytotic response under cold stress?
* Are the NANC mechanisms involved in the exocytotic response to feeding in non-sensitized glands and further, is the neuropeptide gland content affected in response to reflex activation?
* Are the NANC mechanisms mobilized by masticatory reflexes and further, are these mechanisms involved in the development of a parotid periglandular oedema?

MATERIAL AND METHODS

Adult cats (I) and rats of a Sprague-Dawley strain (II-VII) were used as experimental animals. The cats were anaesthetized with pentobarbitone (40 mg/kg i.p., further anaesthetic was injected i.v. as required). To perform preliminary surgery on the rats, either inhalation of diethyl ether or administration of methohexital (40/mg kg, i.v.) or combined administration of pentobarbitone (20 mg/kg, i.p.) and ketamine (50 mg/kg, i.m.) was used to cause anaesthesia. To cause anaesthesia in acute experiments, the rats were anaesthetized with pentobarbitone (40-45 mg/kg, i.p., further anaesthetic was injected i.v. as required). Before the acute experiment food, but not water, was withheld for periods of 24 h with respect to cats and for 24-33 h with respect to rats (but for some experiments in paper VII, where the rats had free access to food). Eventually, the animals were killed with an overdose of pentobarbitone. The studies were approved by the Ethics Committee for Animal Experiments, Göteborg, Sweden.

Preliminary surgery

The denervation procedures were performed 10-12 days before the acute experiment. Sympathetic (postganglionic) denervation was achieved by bilateral avulsion of the superior cervical ganglion (III, VI). The auriculo-temporal nerve was cut where it emerges from the base of the skull aiming at parasympathetic (postganglionic) denervation (III, IV). To cause adrenal medullectomy (III), the adrenals were reached retroperitoneally, an incison was made in the capsule and the medulla was squeezed out and removed. The medullectomy was carried out on both sides; during the first post-operative week the rats were given a 0.5% saline solution to drink. To provide a conduit for intravenous administration of Evans blue in rats subjected to feeding experiments (VII), the jugular vein was exposed on one side, a fine polyethylene catheter was inserted and the other end tunnelled subcutaneously

to an interscapular position and protruded through the skin.

Capsaicin pretreatment

To cause degeneration of sensory nerve fibres, capsaicin of a total dose of 250 mg/kg was given over two days, 50 and 100 mg/kg on the 1st day and 100 mg/kg on the 2nd day (Holzer-Petsche and Lembeck, 1984) two weeks before the acute experiment (VI, VII). The toxin (25 mg/ml), in a vehicle of 20% ethanol and 10% Tween 80 in isotonic saline, was injected s.c. Before the injection, aminophyllin (5 mg/kg, i.p.), terbutaline (0.1 mg/kg, i.p.) and ketamine (50 mg/kg, i.m.) were administered to achieve analgesia and to avoid bronchoconstriction. Controls were injected with these drugs and the vehicle.

Blocking agents

To achieve blockade of the α-adrenergic receptors and the ß-adrenergic receptors phentolamine mesylate and propranolol were used, respectively. Muscarinic receptor blockade was achieved by the use of atropine sulphate. These blockers were administered i.v. or i.p. 10-15 min prior to the test. Control animals were instead given saline in the same way. The doses chosen of the receptor antagonists cause an effective blockade (Ekström *et al.* 1993; IV).

Feeding and cold exposure

The rats were kept in a room (21° C) with lights on from 5 a.m. to 7 p.m. Rats were kept together and they were given a standard pelleted diet as well as water *ad libitum*. During the fasting period each rat was placed separately in a cage with a floor of wire-netting. In the feeding experiments, the pelleted food was reintroduced for periods varying between 60 min and 90 min (9-11 p.m., light off). In case of cold stress (III), the animals were placed in a cold room (2-5 °C, light off) 15 min before feeding (9-11 p.m., light off); in some experiments non-fed control rats remained in room temperature but the positions of their cages were shifted.

Change in the consistency of the food

A liquid diet was prepared daily by mixing 1.5 part of water with one part of a powdered form of the standard pelleted diet (V). The animals kept, 2-4, in cages supplied with bottoms of wire-nettings for 7 days, were offered the liquid diet and water *ad libitum*. Then, they were fasted as above, and finally offered the liquid diet once again or the standard pelleted diet (9-11 p.m., light off).

Estimation of food intake

Post-mortem, the stomach was removed, opened and the gastric content was weighed to obtain the dry weights (110°C for 4-5 days).

Observations on salivary secretion

In both cats (I) and rats (II, IV) the parotid duct was exposed by a skin incision in the cheek close to the mouth. The duct was cannulated by a fine glass cannula or polyethylene tube. Saliva was collected in pre-weighed ice-chilled polyethylene tubes.

Electrical stimulation of the autonomic nerves

The parasympathetic auriculo-temporal nerve was exposed and cut medial to the mandible, where it emerges from the base of the skull. The peripheral end of the nerve was stimulated continuously (8 V, 5 ms) by a bipolar electrode and a Grass S48 stimulator, in the cat for 90 min at 10 Hz (I) and in the rat for various periods, 10, 20, 40 or 80 min, at 40 Hz (I, II, IV, VII). The cervical sympathetic nerve trunk was exposed in the neck, cut and the ascending nerve was placed on a bipolar electrode and stimulated over a period of 60 min at 50 Hz for 1 s every 10th s (IV).

Infusions of bethanechol and VIP

To provide a conduit for intra-arterial infusions in the cat, the end of a fine

hypodermic needle, on a narrow-bore polyethylene tubing, was introduced into the carotid artery in the neck. VIP or bethanechol was infused continuously for 90 min in the heparinized animal by means of a pump. VIP was infused at a dose rate of 0.5 μg/kg/min, while bethanechol (20-80 μg/kg/min) was infused at a dose rate aiming at producing the same salivary flow rate as the nerve stimulation (I). Tissue pieces of the contralateral gland, to serve as control tissue, was removed before the start of the infusions.

Administration of Evans blue

Evans blue (VII) of room temperature, was slowly infused i.v. (either in the jugular vein, the femoral vein or the tail vein depending on the type of experiment) over a period of 1 min in a dose of 20 mg/kg made up in saline to give a final volume of 1 ml. The Evans blue was taken from a filtered (Munktell paper no. 1F) stock solution containing 20 mg of the dye per ml saline. At the end of the experiment, the thorax was opened on the anaesthetized animal. A glass cannula was inserted into the ascending aorta via the left ventricle of the heart, and the animal was perfused with 45 ml cold saline at a pressure of about 8 kPa. The salivary glands, the urinary bladder and the stomach were removed, the stomach to weigh its gastric content and the urinary bladder to be used as reference organ with respect to the accumulation of Evans blue.

Morphometric assessments

Tissue specimens from the lower lobe of the parotid gland were put in the fixative (3% formaldehyde, 4% glutaraldehyde and 0.05% picric acid in 0.16 M potassium phosphate buffer, pH 7.2-7.4) and cut into small pieces; the fixation continued for 2 h at room temperature. Following postfixation in 1% buffered OsO_4 and dehydration in rising concentrations of ethanol, the tissues were embedded in Polybed (Polysciences, Warrington, USA). Using an ultramicrotome, 0.7 μm thick sections were cut and subsequently stained with Toluidine Blue and pyronin in a borax buffer. A light microscope was

equipped with 10 x 10 square mesh graticule in the eyepiece where each square corresponds to 100 μm^2 and a 100 oil-immersion lens (NA = 1.25). Furthermore, in order to obtain the highest possible resolution, an oil-immersion system was used for the condenser (NA = 1.4). The morphometric analyses (Carlsöö, Danielsson & Helander, 1974; Weibel 1979) were carried out on randomly selected and coded blocks. Five sections from two blocks for each gland were assessed. Only technically perfect preparations were examined. Areas of acinar cells were looked for, thus introducing a possible underestimation of ductule volume density. An area of acinar cells was selected, then the glass slide was slightly shifted left-ward to offer a new acinar area for inspection, and it was this second region that was used for morphometry. For each section the number of granules in ten squares was counted. Using the point-counting method, the volume densities were first estimated for stroma and for the epithelium. Within the epithelium, the volume densities of the cell nuclei and of the ducts were then calculated. The numerical density of the secretory granules was expressed as numbers per 100 μm^2 of acinar epithelial cytoplasm. To estimate whether the degree of granular depletion of acinar epithelial cytoplasm occurred uniformly throughout the gland the ratio standard error of the mean (x 100) over mean number of granules (per 100 μm^2 of acinar epithelial cytoplasm) of the five sections examined of each gland was calculated and used as "granule variance index" in paper IV. Systematic errors due to swelling, shrinkage or section thickness were not corrected for (Holmes effect, see Helander 1978).

The time-consuming morphometric assessment was performed by two of us (A.A. and J.E.). To find out the interindividual difference, exactly the same areas were examined and a number of comparisons was made on the calculated numerical density of acinar granules (per 100 μm^2 epithelial cytoplasm) of the current preparations according to a method of Eränkö (1955). The difference, expressed as a percentage of the mean value was about 6%, which is a value well within acceptable limits.

Assay of protein and amylase

The salivary protein content was analysed by the method of Lowry, Rosebrough, Farr and Randall (1951) (I). The glandular amylase activity was measured by an enzymatic colorimetric test (Boehringer- Mannheim) using α-4-nitrophenylmaltoheptaoside as substrate (Hägele, Schaich, Rauscher, Lehmann, Bürk & Wehlefeld, 1982) (III, IV, V).

Radioimmunoassay of gland content of NPY, substance P and VIP

Immunoreactive NPY (VI) was quantitated using a rabbit antiserum in a final dilution of 1:40 000 (Dr. P. C. Emson, Cambridge, UK). It crossreacts with peptide PYY to 30% but does not cross-react with bovine pancreatic polypeptide, VIP, or peptide histidine isoleucine (Jansen, Uddman, Ekman, Olesen, Ottoson & Edvinsson, 1992). Antiserum directed against the C-terminal part of substance P (VI) was used in a final dilution of 1:350 000 with ^{125}I-Tyr^{8}-substance P as tracer. The antiserum does not cross-react with other known tachykinins (Brodin, Lindefors, Dalsgaard, Theodorsson-Norheim & Rosell, 1986). To assay VIP (IV) an antiserum that recognizes the N-terminal 15-amino acid sequence of VIP and which does not cross-react with peptidine histidine isoleucine amide or any known regulatory peptide was used (Fahrenkrug & Shaffalitzky de Muckadell, 1977; Ekström *et al.* 1984). The final dilution of the antiserum was 1:16 000 and ^{125}I-VIP served as tracer.

[^{3}H]Leucine incorporation

To measure incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble material of parotid glands, 40 μCi of the amino acid dissolved in 1 ml of saline was injected i.v. 15 min after the end of an 80 min long period of stimulation of the auriculo-temporal nerve. The animal was killed 15 min later, and then the parotid glands on both sides were rapidly removed and prepared for analysis (II). The protein synthesis inhibitor cycloheximide (20 mg/kg i.p.) was injected before the start of the stimulation period (Goldblatt, Archer &

Eastwood, 1975; Asking & Proctor, 1989).

Measurement of Evans blue

Evans blue was extracted from the organs by incubation at 50° C for 20 h in formamide (VII). The dye was quantified by determining the optical density of the formamide extract at 620 nm (Pharmacia LKB Ultrospec III), and the absorbance was compared with a standard curve for 0.25-10 ug/ml Evans blue in formamide as described by Saria and Lundberg (1983).

Drugs

Aminophylline (Parke-Davis), atropine sulphate (Sigma), calcitonin gene-related peptide (Peninsula), capsaicin, cycloheximide, Evans blue (Fluka), formamide (Merck), ketamine (Parke-Davis), methohexital (Eli Lilly), neurokinin A (Peninsula), phentolamine mesylate (Ciba-Geigy), pilocarpine (Sigma), pituitary adenylate cyclase activating peptide (Peninsula), propranolol hydrochloride (ICI), substance P (Peninsula), terbutaline (Astra Draco) vasoactive intestinal peptide (kindly supplied by professor V. Mutt, Karolinska Institute, Stockholm, Sweden),

Comparisons and analysis of data

When possible, the contralateral gland in the same animal served as control. This was the case when the effects of electrical stimulation of the parasympathetic and sympathetic innervations were investigated. Before stimulation of the sympathetic innervation began (in the rat), the parotid gland on the opposite side was removed. This was done as a precaution, since the contralateral gland in this species has been suggested to recieve a few sympathetic nerve fibres from the ipsilateral side (Alm, Asking, Emmelin & Gjörstrup, 1984).

When it was not possible to base the comparisons between the glands in one and the same animals, comparisons were made between experimental and

control animals of the same age and body-weight, and the rats (and glands) to be used as controls were subjected to the same treatment as the experimental rats (and glands) but for the very test under study. It is of importance to point out that in order to avoid influences of the circadian secretory cycle of the parotid gland with respect to both structure (Albegger, Müller & Albegger, 1975) and biochemical composition (Sreebny *et al.* 1971), both experimental and control rats were fasted and further, killed at the same time. It should also be mentioned that the lower lobe of the parotid gland on one side was used for the morphometric analysis, while the whole contralateral gland was used to measure the amylase activity, thus making it impossible to make comparisons as to these two parameters within the same animal.

Statistical significances of differences were calculated by Student´s t-test for paired or unpaired values or by one way of analysis of variance followed by Fisher´s protected least significant difference. Probabilities of less than 5 % were considered significant. Values are means ± S.E.M.

RESULTS AND DISCUSSION

Mean numerical density of the acinar secretory granules (per 100 μm^2 epithelial cytoplasm) of parotid glands of fasted cats and rats was about 25 and 30. These values are in the range reported for the mouse parotid gland (42, Carlsöö *et al.* 1974). The acute test situations caused very little changes, if any, to the gland volume densities of acinar epithelium and stroma. Furthermore, the nuclear volume density was not affected, suggesting but small, if any, changes in cell size in response to the acutely applied stimulus. For instance, the mean nuclear density of parotid glands of the rats subjected to continuous stimulation of the parasympathetic innervation for 80 min, and secreting a large volume of saliva, was the same as in the contralateral unstimulated glands (being 3 % of gland volume). Chronic inactivation of the parotid gland, induced by for instance a liquid regimen, caused glandular atrophy and here, the mean nuclear density increased (to about 8 % of gland volume) indicating decreased cell size in agreement with previous reports (Wilborn & Schneyer, 1970).

Formation of vacuoles in exocrine cells is often thought to reflect excessive stimulation and has been associated with water secretion. However, vacuoles have also been demonstrated in rat parotid acinar cells under reflex conditions (Garrett, 1987). In the present investigation, vacuoles were only observed sporadically in the glands, but for the cat parotid gland subjected to prolonged infusion of the parasympathomimetic drug bethanechol (I). Glands stimulated by reflexes or by electrical nerve stimulation were usually surrounded by an oedema of varying size, often without any morphometric support for the presence of an intraglandular oedema.

(1A) Effects of electrical stimulation of the parasympathetic auriculo-temporal nerve on the cat parotid gland

Stimulation of the parasympathetic innervation (10 Hz continuously for 90 min), in the presence of α- and β- adrenergic antagonists, caused the parotid gland to secrete a large volume of saliva (I). Morphometric assessment (Fig. 1) showed the numerical density of the acinar secretory granules in the stimulated gland to be reduced by 62 % (when compared with the unstimulated contralateral gland). When the stimulation was performed in the presence of atropine as well, there was, as expected, no secretion of saliva from the gland at all, but the numerical density of granules was reduced by as much as 42 %. Intracarotid infusion of VIP evoked no flow of saliva from this gland in the cat. However, VIP induced a decrease by 26 % in the numerical density of granules. The parasympathomimetic drug bethanechol was infused into the carotid artery at a dose rate aiming at producing the same salivary flow rate as the nerve stimulation. Although the bethanechol infusion and the nerve stimulation resulted in the secretion of similar amounts of saliva and outputs of protein, the reduction in the numerical density of granules in response to bethanechol was much less than that to the nerve stimulation, being 27 %.

It may be concluded that acetylcholine is not the sole transmitter responsible for the acinar degranulation previously found in the cat parotid gland in response to parasympathetic stimulation (Emmelin & Garrett, 1989). VIP, or a structurally related peptide, is a likely transmitter involved in the NANC exocytotic response. In the cat parotid gland, VIP is present in nerve fibres close to the acinar cells (Lundberg, Martling & Hökfelt, 1988) and upon administration it releases proteins (Ekström & Tobin, 1990). The results suggest that the NANC transmitters are potentially responsible for as much as two-thirds of the loss in parotid acinar granules that occurs upon stimulation of the parasympathetic innervation in the non-atropinized cat. Since bethanechol caused about the same total output of protein but less than half the loss in

granular number compared with the responses to the nerve stimulation (in the absence of atropine), the parasympathomimetic drug seems to have mobilized both the constitutive route and the regulated route for protein secretion.

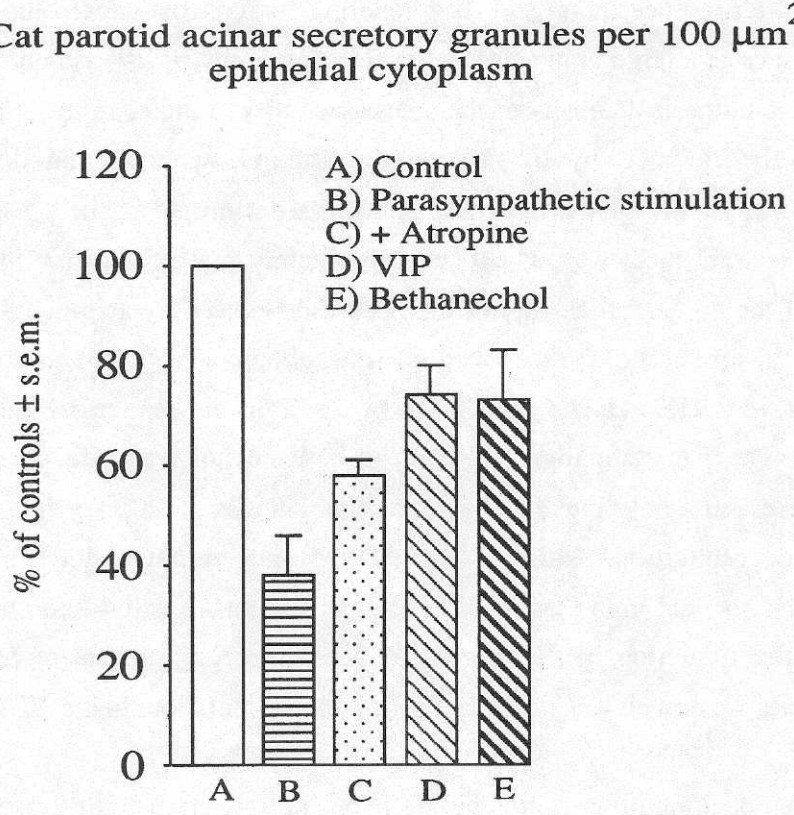

Figure 1.

(1B) Effects of electrical stimulation of the parasympathetic auriculo-temporal nerve or the cervical sympathetic trunk on the rat parotid gland

Continuous stimulation of the auriculo-temporal nerve, in the presence of α- and β-adrenergic receptor antagonists, at a frequency of 40 Hz, being the maximal frequency for the fluid response, caused the parotid glands to secrete copiously at a fairly constant rate over the stimulation periods (40 min and 80

min) (II). In agreement with previous observations (Ekström *et al.* 1988b; Månsson & Ekström, 1991), the flow rate in the atropinized rats declined rapidly. In the atropinized rats, the numerical density of parotid acinar granules was reduced by 30 % (40 min) and 27 % (80 min) as compared to the unstimulated glands (Fig. 2). In the non-atropinized rats, the corresponding reductions were 30 % and 39 %. The figures obtained at 40 min suggest that up to this time, the NANC transmitters are potentially responsible for the whole exocytotic response. The acinar granular depletion at 80 min was larger in the non-atropinized rats than in the atropinized rats, so acetylcholine seems also to contribute to the exocytotic response.

The results obtained might give the impression, that the releasable pool of secretory granules is limited to about one-third of the granular content. This was, however, not found to be the case. Stimulation of the ascending cervical sympathetic nerve trunk in an intermittent mode (50 Hz for 1 s every 10 th s) to avoid vasoconstriction (Anderson, Garrett & Proctor, 1988) for 60 min, in the presence of atropine, caused the gland to secrete only a small amount of saliva. However, the gland lost as much as 65 % of its number of granules (Fig. 2). The glandular responses were completely abolished by the pretreatment with α- and β- adrenergic antagonists (IV).

Prolonged parasympathetic stimulation at a frequency of 10 Hz or less has been found to induce neither acinar granular depletion nor loss in amylase activity in the rat parotid gland (Garrett & Thulin, 1975; Asking & Gjörstrup, 1987; Asking & Proctor, 1989). Consequently, the large output of amylase in the parasympathetic saliva under these experimental conditions is thought to be the result of a constitutive vesicular release. The result of the present study implies that the release of amylase previously found during the latter half of the 80 min period of stimulation in the non-atropinized animals (Ekström *et al.* 1988b) is likely to reflect a constitutive release.

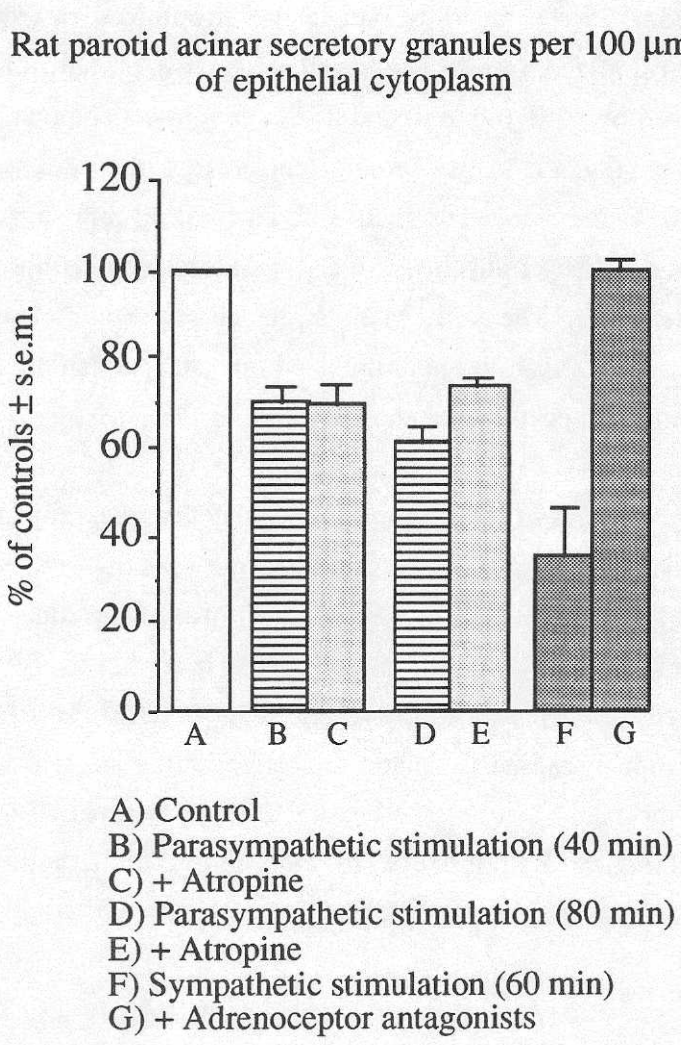

Figure 2.

Incorporation of tritiated leucine into trichloroacetic acid-insoluble material of parotid glands in the non-atropinized rats subjected to nerve stimulation revealed a 6-fold increase in the protein synthesis after the 80 min long period of stimulation (II). Pretreatment with cycloheximide abolished the increase in protein synthesis without affecting the fluid response in the non-atropinized as well as in the atropinized rats and further, and most importantly in this

connexion, without affecting the degree of acinar degranulation. The fact that the cycloheximide-pretreated animals showed an acinar degranulation not larger than that in the corresponding non-cycloheximide-treated animals, indicates that 80 min is too short a time period to allow newly formed granules to become visible at the light-microscope level in non-treated animals. This is consistent with previous observations showing the first signs of reaccumulation of acinar granules following an almost complete acinar degranulation, induced by isoprenaline, to occur after 6 hours (Amsterdam *et al.* 1969). Furthermore, pulse labelling experiments show about the same time course for newly formed secretory proteins to be released from granules (Castle *et al.* 1972).

(2A) Effects of feeding under cold stress on the chronically sympathectomized rat parotid gland

In sympathectomized glands of rats fed a pelleted diet and exposed to cold, the numerical density of acinar granules was reduced by 42 % and the total activity of amylase by 54 % as compared to sympathectomized glands of non-fed rats in the cold (III). Cold stress is known to activate the sympatho-adrenal system (Hartman & Hartman, 1923; Hartman, McCordock & Loder, 1923; Cannon, Querido, Britton & Bright, 1927). Therefore, it seemed reasonable to previous investigators (Garrett *et al.* 1985) to attribute the acinar granular depletion in the cold to circulating catecholamines liberated from the adrenal medulla. Despite the fact that the food consumption of the animals sympathectomized and subjected to bilateral adrenal medullectomy plus treatment with atropine without or with the adrenergic receptor blockers was, in the cold, only one-third of that of the animals subjected to sympathectomy only, the responses of the animals subjected to the extended treatment were enhanced (III). Following adrenal medullectomy and pretreatment with atropine the reduction in the number of granules and total amylase activity amounted to 68 % and 74 %, respectively, and in addition, in the presence of α- and β-adrenergic receptor antagonists to 60 % and 65 %. A further

analysis showed that when parasympathetic denervation was included in the surgical procedure, the feeding response was abolished (III). Thus the feeding response in the cold of sympathectomized parotid glands seems to reflect parasympathetic NANC activity rather than the action of circulating catecholamines.

(2B) Effects of circulating catecholamines on the chronically sympathectomized and parasympathectomized rat parotid gland

To study a possible contribution of the adrenals to the parotid acinar degranulation the parasympathetic as well as the sympathetic nerve influence were eliminated (III). Furthermore, to avoid any influence on the acinar cells from those few cholinergic nerve fibres that might escape the denervation procedure (Ekström, 1974; Alm & Ekström, 1976) the animals were atropinized. By just exposing the animals to cold, a slight reduction in the numerical density of granules occurred, by 17%. Intake of pelleted food in the cold reduced the numerical density of granules by 55% compared to non-fed animals in room temperature. Evidently, the adrenal medullae were of importance for the response. In the denervated gland of the medullectomized rats, there was no degranulation following exposure to just cold and further in response to food, the magnitude of the acinar degranulation, being 29%, was less than that in the non-medullectomized rats despite the fact that the food intake was the same in the two groups of rats. The persisting degranulation was obviously exerted via adrenergic receptors, since the combined pretreatment with α- and β-adrenergic antagonists abolished the degranulation.

Following bilateral removal of the superior cervical ganglion, as in the present study, adrenergic nerve fibres occur only occasionally in the parotid glands (Ekström *et al.* 1988b). Adrenergic nerve terminals elsewhere (Khalil, Livett & Marley, 1986) and perhaps , in addition, extra-adrenal chromaffin tisssue (Ricordi, Shah, Lacy, Clutter & Cryer, 1988) are likely sources for the

catecholamines mediating the persisting response after adrenal medullectomy. Interestingly, cold exposure of equithesin-anaesthetized rats over a 60 min period caused a continuous rise in the plasma levels of noradrenaline and adrenaline, and while the rise in the plasma level of adrenaline was prevented by adrenalectomy, that of noradrenaline was not (Khalil *et al.* 1986).

Sympathectomy or parasympathectomy causes the secretory cells of the parotid gland of the rat to become increasingly sensitive to secretory agonists over a period of time (Ekström, 1980), and the highest degree of sensitization is to be expected after combined sympathectomy and parasympathectomy. In the above related series of experiments of the present study the contralateral gland was sympathectomized. By comparing the effect of the various procedures on the glands of both sides, it appears that a high degree of sensitivity of the secretory cells is probably necessary for acinar degranulation to occur in response to circulating catecholamines. Thus, in one and the same atropinized animal, the sympathectomized plus parasympathectomized gland responded to cold exposure, while the contralateral sympathectomized gland did not and further, medullectomy reduced the response to feeding of the gland subjected to the combined denervations but not of the gland subjected to sympathectomy only.

(3A) Effects of feeding on the rat parotid gland supplied with an intact innervation

Most likely, chronic sympathectomy creates favourable conditions for demonstrating NANC mediated responses in salivary glands. In response to sympathetic denervation the parotid gland of the rat develops a supersensitivity to the neuropeptides substance P (Ekström & Wahlestedt, 1982) and VIP (Ekström *et al.* 1983a) injected intravenously. The neuropeptide content of the parasympathetic salivary innervation may also increase following sympathetic denervation (Ekström *et al.* 1984; Ekström *et al.* 1988a). Thus, the observed effects in chronically sympathectomized parotid glands in connexion with food

intake (III; Ekström *et al.* 1993) may be regarded as a consequence of the sympathectomy. It is, in fact, said that no acinar degranulation in the parotid gland occurs in response to food intake in rats subjected to sympathetic decentralization (i.e. cutting the preganglionic nerve) 24 hours before feeding (Harrop & Garrett, 1974). Nevertheless, by using another experimental approach avoiding surgery and using morphological assessment (IV), a different result was obtained than that of Harrop & Garrett (1974). The loss in granular number and total amylase activity in the absence of any receptor blocker in response to food intake amounted to 52 % and 38 %, respectively (Fig. 3). The acute elimination of the catecholaminergic influence by administration of α- and β-adrenergic antagonists resulted in a corresponding fall by 31 % and 32 %. In the rats treated with both atropine and adrenoceptor antagonists a fall of the same magnitude, by 32 %, occurred as to both the numerical density of granules and total amylase activity. The effects observed after blockade of the sympathetic influence on the adrenergic receptors, depended most likely on an intact parasympathetic innervation, since these effects did not occur when the auriculo-temporal nerve was cut in advance. Thus it may be concluded, that the parasympathetic NANC mechanisms are potentially responsible for the whole parasympathetic response with respect to acinar degranulation and loss in amylase activity and for the major part of this response in the absence of any antagonists in rats showing a food intake twice as large as in the other groups of rats.

(3B) On the participation of masticatory-salivary reflexes in the NANC evoked secretory effects of the rat parotid gland

The role of chewing in the NANC evoked acinar degranulation and loss in amylase activity was investigated by changing the consistency of the standard diet, from hard dry pellets to liquefied chow (V). The animals had to be accustomed to the liquid diet before they were prepared to accept it readily

upon feeding. Therefore, all animals were kept on a liquid regimen for a week before the acute feeding experiment. However, maintenance of rats on liquid regimen over a period of time induces atrophy of the parotid gland (Hall & Schneyer 1964; Ekström, 1974) and further, the total amounts of substance P, VIP and CGRP in the gland decrease (Månsson, Ekman, Håkanson & Ekström, 1990). Therefore, it might be argued that the NANC mechanisms cannot be activated following liquid regimen due to lack of sufficient amounts of releasable transmitters. As a consequence of this possibility, the protocol included not only animals offered liquid chow in the final test but also animals offered hard chow. The disused glands activated in response to the intake of the hard chow showed a fall in the numerical density of acinar granules by 50 % and in total amylase activity by 70 % (Fig. 3). These changes were, in fact, more marked than those observed in rats maintained on the standard diet (IV), and might be combined with previous findings of enlarged fluid responses to secretagogues at submaximal doses, in disused parotid glands of rats on liquid diet and thought to reflect supersensitivity (Ekström & Templeton, 1977). Despite the fact that the group of rats offered liquid diet in the final experiment, consumed twice as much food as those offered the pelleted diet, the parotid glands of those rats consuming the liquid diet showed no fall in the numerical density of acinar granules or in the total amylase activity. The reflexly elicited NANC response to the pelleted diet did evidently not depend on taste, since any sapid components of the hard chow would be more available for gustatory stimulation in the liquefied form. It may be concluded that the NANC evoked acinar degranulation and loss in amylase activity are part of salivary reflexes set up in response to mastication.

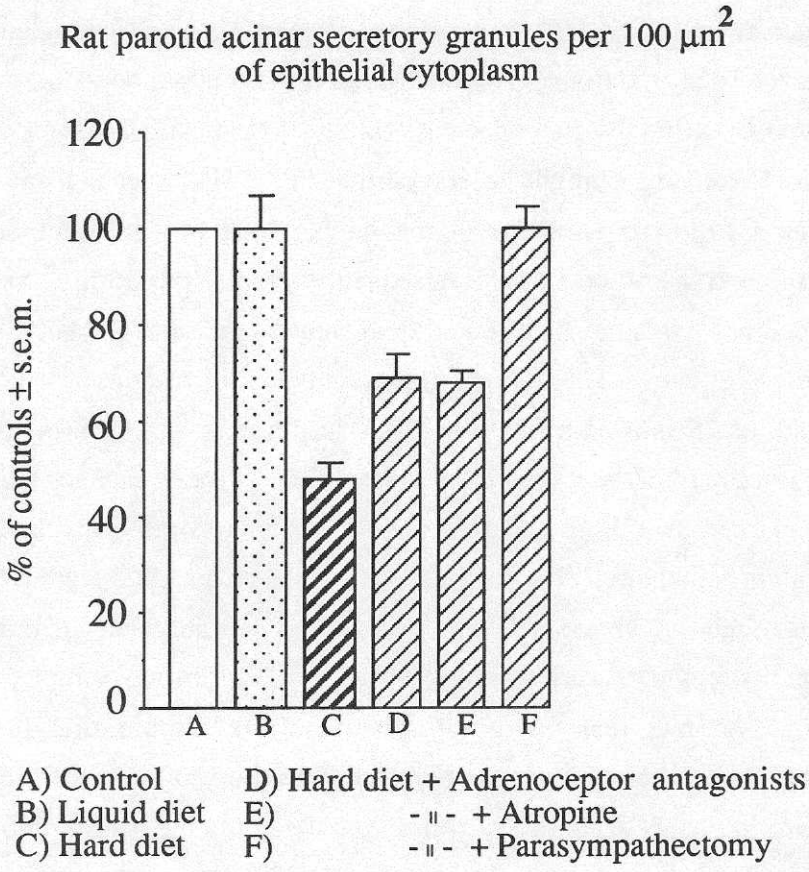

Figure 3.

(3C) Effects of reflex activation on the neuropeptide content of the rat parotid gland

The classical transmitters, acetylcholine and noradrenaline, are synthesized in the nerve terminals and the stores of these transmitters are not easily depleted. In contrast, the stores of neuropeptides in the nerve terminals are dependent on their replenishment by axonal transport of preformed, packaged peptides from the nerve cell body (Håkanson & Sundler, 1983). Concomitant with a gradual

decline in parotid secretion of fluid and outputs of protein and amylase during on-going electrical stimulation of the parasympathetic innervation at a high frequency (40 Hz) in the anaesthetized and atropinized rat, there is a progressive reduction in the gland content of VIP, substance P, CGRP and NPY (Tobin *et al.* 1994; Ekström *et al.* 1996) together with a decrease in the number of large densed-cored vesicles storing peptides in the parasympathetic nerve terminals innervating the acini (Ekström, Garrett, Månsson, Rowley & Tobin, 1989). Upon a 60 min period of nerve stimulation the gland contents of VIP and substance P are reduced by 75 % and the content of NPY by 50 % both in the absence and presence of atropine (and adrenergic receptor blocking agents), whereas the gland content of CGRP is less affected.

In rats pretreated with atropine and the adrenergic receptor blockers, the total amounts of VIP and substance P were reduced by 23 % and 42 % in response to feeding, respectively (VI). Similarly, in rats only treated with atropine the feeding response resulted in a 25 % decrease as to VIP and in a 40 % decrease as to substance P. The third peptide examined, NPY, showed no decrease. Despite the fact that the food intake was twice as large in the non-drug treated rats than in the drug treated rats, there were no decreases in the contents of the three peptides in the absence of blockers. Evidently, neither an intact sympathetic innervation nor a capsaicin sensitive (sensoric) innervation was necessary for the depletion to occur in the presence of atropine (with and without the adrenoceptor antagonists). However, under these circumstances the reduction in the VIP content was more profound than in the intact glands, being 33-47 %, and here, NPY showed a decrease of 15 %, while the reduction in the content of substance P was 33-34 %. The differences in magnitude of responses between sympathectomized glands and intact glands may reflect adaptation to the sympathectomy. Interestingly, the larger relative losses in contents of VIP and NPY following sympathectomy (in the presence of atropine and adrenergic antagonists) may contribute to the larger losses in the numerical density of granules and amylase activity occuring in these glands in

response to feeding as compared to those glands not sympathetically denervated (Ekström *et al.* 1993; IV). The decreases in peptide contents observed are thought to reflect an imbalance between peptide release from nerve terminals and their replenishment by axonal transport. In the absence of muscarinic receptor blockade the peptidergic mechanisms were evidently economized.

(4) Vascular protein leakage in the rat parotid gland

A role for NANC mechanisms in inducing microvascular leakage in parotid gland tissue was examined by measuring changes in vascular permeability for plasma proteins labelled by Evans blue. In this type of experiment care was taken to save the periglandular fluid oedema, so the removed glands were not, as in the other types of experiments, gently pressed between gauze pads to remove water, if any. After feeding , the total amount of extractable Evans blue from the glands (and any additional water) was increased by 116 % in the absence of any blockers, by 154 % in the presence of atropine, by 55 % in the presence of atropine and the adrenergic receptor blockers and by 126 % in those rats that had been pretreated with the sensory neurotoxin capsaicin two weeks in advance and then given the three autonomic blockers. In response to parasympathetic stimulation (40 Hz) for 10 min and 20 min, respectively, the total amounts of extractable Evans blue from the stimulated gland (and additional periglandular oedema) was 81 % and 124 % higher than in the contralateral unstimulated gland. In the presence of atropine and α- and β-adrenergic receptor antagonists, the stimulation periods also gave rise to an accumulation of Evans blue, the corresponding percentage increases being 52 % and 50 %. When the parenchyma and the periglandular oedema were analysed separately in rats subjected to parasympathetic stimulation for 20 min in the absence of any receptor antagonists, the extractable amount of Evans blue was increased by 56 % and 177 %, respectively. Treatment with capsaicin reduces the parotid gland content of substance P by 11 % and CGRP by 36 %, but not that of VIP, PACAP and NPY (Ekström *et al.* 1989; Mirfendereski *et*

al. 1997). However, the presence of sensory capsaicin-sensitive nerve fibres was evidently not a prerequisite for the accumulation of Evans blue.

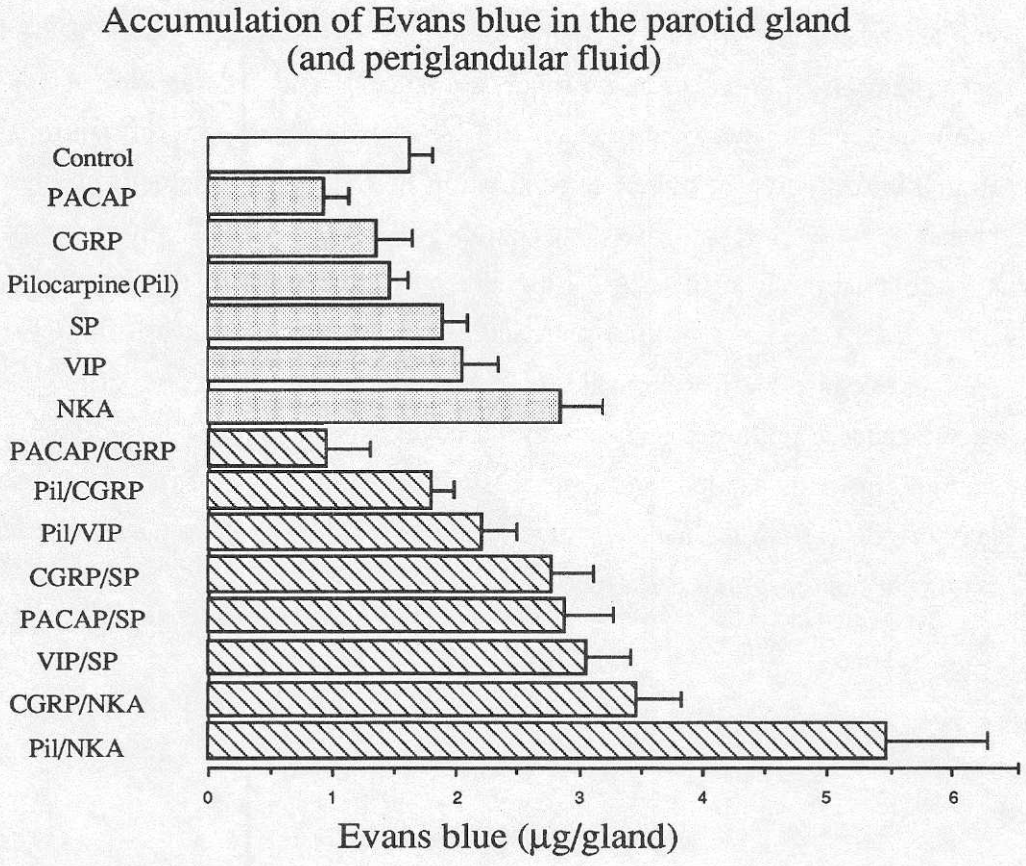

Figure 4.

In the further analysis, the effects of a number of peptides confined to the parasympathetic nerve were tested. Intravenous administration of the tachykinin neurokinin A resulted in a 75 % increase in the accumulation of Evans blue in the parotid gland, while the tachykinin substance P as well as VIP, PACAP and CGRP lacked effects with respect to Evans blue (Fig. 4). However, substance P combined with either VIP, PACAP or CGRP increased the accumulation of the dye. Further, the parasympathomimetic drug pilocarpine, which in itself caused no increase in the Evans blue accumulation

of the parotid gland, enhanced the neurokinin A induced response, showing in this case a 236 % increase. Pilocarpine combined with VIP or CGRP was, however, without effect. When turning to the sublingual+ submandibular glands, treated as an entity, neurokinin A as well as pilocarpine caused accumulation of the dye and further, subtance P and VIP as well as the combination substance P and CGRP showed synergistic interactions. Bradykinin, known to induce accumulation of Evans blue in many tissues, probably via the release of sensory neuropeptides, and presently illustrated by a 24-fold increase in the urinary bladder, was without effect in the glands. Interestingly, neither a profuse secretion nor a high glandular blood flow was enough to cause plasma extravasation as illustrated by the outcome of the use of substance P and VIP, respectively. In conclusion mediators of importance for protein extravasation, and oedema fluid formation in the salivary glands may originate from the parasympathetic innervation and involve the combined actions of neuropeptides and acetylcholine.

GENERAL DISCUSSION

During recent years the awareness of parasympathetic NANC mechanisms in salivary glands and their influence on secretion of saliva, gland metabolism, protein synthesis and gland size has been growing. Support for a physiological role for these mechanisms under such a natural condition as food intake may be gained from the findings presented in this *Thesis*.

In humans (Lashley, 1916; Kerr 1961) as well as in a number of various animals such as rabbits (Anderson, Hector & Linden, 1985), sheep (Patterson, Brightling & Titchen, 1982), horses and mules (Colin, 1854) food is chewed on one side at a time, and the flow rate of saliva is higher on the chewing side than on the contralateral side. This is in contrast to the experimental animal presently chosen to study reflex glandular responses on, since the rat chews on both sides at a time and secretes at equal flow rates from the submandibular and parotid glands of both sides (Weijs & Dantuma, 1975; Matsuo, Yamamoto, Ikehara & Nakamura, 1994).

The previously demonstrated parotid acinar degranulation in the rat in response to eating hard chow, which has been attributed to sympathetic nerve activity or circulating catecholamines under cold stress (Garrett, 1987), is likely to involve a parasympathetic NANC component. First, the NANC transmitters were potentially responsible for the whole or for the major part of the parotid acinar degranulation in response to stimulation of the parasympathetic innervation in the anaesthetized animal. Secondly, the sympathetically denervated parotid gland in medullectomized rats treated with adrenoceptor blockers and atropine still lost secretory granules in response to feeding under cold stress but not when parasympathectomy was included in the procedures. Thirdly, the acute elimination of the adrenoceptor-mediated drive on the non-sensitized parotid glands showed the parasympathetic NANC transmitters to be potentially responsible for the major part of the acinar

degranulation in response to feeding in the absence of any receptor blockers.

The parasympathetic neuropeptides VIP and substance P were released in such amounts that the gland contents of these peptides decreased in response to eating under muscarinic receptor blockade. Infusion of VIP causes parotid acinar degranulation in the cat without any accompanying fluid secretion, a phenomenon also occurring in the ferret parotid gland (Ekström & Ekström, 1998). The action of VIP on the acinar cells is also a likely cause of the NANC evoked exocytosis in the rat parotid gland. However, an action of substance P in the exocytotic response is possible. Agonists using the "Ca2+/ Inositoltriphosphate" intracellular pathway may induce exocytosis as illustrated by the effect of bethanechol in the cat parotid gland and of the effect of substance P infusion in the ferret parotid gland (Ekström & Ekström, 1998).

Prolonged continuous stimulation at a high frequency (40 Hz) of the whole parasympathetic nerve trunk or infusion of secretagogues over a period of time is no doubt unphysiological. Nevertheless such a protocol provided reproducible findings about distinctions in effector responses arising from the action of different agonists. Under normal reflex conditions a wide range of impulses is likely to occur intermittently, in a variable number of nerve fibres at any one time and allowing various agonists to act in synergy. Frequencies above or in the range of that presently applied to the parasympathetic innervation have, in fact, been reported in parasympathetic salivary nerves under reflex conditions in the sheep (Carr, 1977) and the rat (Matsuo, Morimoto and Kang, 1998). The present findings under reflex conditions do also support the idea that frequencies in the range presently used to electrically stimulate the parasympathetic innervation may occur under natural events.

The losses in number of parotid acinar granules and in glandular amylase activity were not always in proportion. Apart from the fact that there may be regional gland tissue differences (the morphometric assessment was only

performed on a selected part of the gland, while the whole contralateral gland was used for amylase assay) and the fact that the two parameters were not always determined in the same animal, a close positive correlation between these parameters may not necessarily have to exist. For instance, amylase occurs also outside the granules, in gland lumina and in cytoplasmic vesicles to be released via the constitutive route (Kelly, 1985). Furthermore, a re-synthesis of amylase may occur concomitantly with the secretion of amylase upon electrical stimulation of the parasympathetic innervation, as shown in non-atropinized parotid glands of the rat using a frequency of stimulation of 10 Hz (Asking & Gjörstrup, 1987). In Results and Discussion attention was drawn to a possible constitutive release of proteins and amylase upon stimulation of the parasympathetic innervation, and this might also have occurred in the disused glands of the rats kept on the liquid diet, when suddenly thrown into activity by chewing as a consequence of the intake of the pelleted diet.

The era of research on the NANC effects on salivary secretory cells started with the observation of a flow of saliva upon stimulation of the parasympathetic innervation that persisted in the presence of atropine and adrenergic receptor blockers. However, so far a NANC induced flow of saliva *per se* in response to stimulation of the parasympathetic innervation has only been found in rats and ferrets. In sheep, the NANC mechanisms accelerate the resting parotid flow of saliva upon parasympathetic nerve stimulation (Reid & Titchen, 1988). In contrast, release of salivary proteins may be a general feature. For instance, VIP with and without accompanying fluid secretion has been implicated in the release of salivary protein in all the species which have so far been studied including the rat, ferret, pig, sheep, cat, dog and calf (Ekström *et al.* 1983a; Reid & Heywood, 1988; Ekström & Tobin, 1989, 1990; Tobin & Ekström, 1992; Calvert, Heck & Edwards, 1998).

As judged by the foregoing, comparisons based on changes in the numerical

density of granules rather than on changes in glandular amylase activity seem to be the procedure of choice. However, the different degrees of acinar depletion can probably not be used for direct calculations of the relative contributions of the various transmitters and pathways involved in the electrically nerve-evoked exocytotic response as well as in the reflexly-elicited exocytotic response. For instance, interactions amongst agonists may occur. Pharmacological or surgical interruption of one pathway for glandular activation may increase the demands on the masticatory apparatus and, as a result, short-term compensatory mechanisms may occur in other pathways. Furthermore, atropinization may influence the neuropeptide release from the nerve terminals (Tobin *et al.* 1994). Surgical interference may be followed by long-term compensatory mechanisms in the remaining nerves, such as increases in transmitter levels (Ekström, 1978; Ekström *et al.* 1984; Ekström *et al.* 1988a), and in the secretory cells, such as the development of supersensitivity (Emmelin, 1965; Ekström, 1980). Under natural conditions, NANC mechanisms, adrenergic mechanisms, cholinergic mechanisms and, when liberated, supported by the action of circulating catecholamines are most likely working in concert to achieve the most purposeful reflex response, and in this case, the neuropeptide content of the nerve terminals are economized.

The parasympathetic NANC mediated parotid acinar degranulation and amylase release reflexly evoked in response to food intake were evidently dependent on mastication. It was recently shown that chewing also evoked a flow of saliva from the duct-cannulated parotid gland in the conscious rat in the presence of atropine and adrenoceptor antagonists, albeit at a reduced flow rate (Ekström, 1998). However, the potential contribution of the NANC mechanisms *per se* to the fluid response appeared small. As was shown in the present *Thesis*, the NANC mechanisms reflexly activated by chewing were potentially responsible for the whole parasympathetic exocytotic response and for the major part of the exocytotic response in the absence of any blocker. Although the present *Thesis* stresses the importance of mastication for

mobilization of the NANC mechanisms, the mobilization of these mechanisms is not excluded in response to other oral stimuli. In fact, taste or aversive reflexes causing salivary secretion seem to involve the action of the parasympathetic NANC mechanisms. Ascorbic acid applied onto the tongue of the conscious rat evoked a small flow of parotid saliva in the presence of blockade of the classical autonomic receptors, and this persisting NANC response was abolished by a tachykinin antagonist (Ekström, 1998).

NANC secretory mechanisms may be of importance for human salivary glands but presently, functional studies on human glands are few. In humans, the acinar cells of the parotid and submandibular glands (Uddman, Fahrenkrug, Malm, Alumets, Håkanson & Sundler, 1980; Hauser-Kronberger, Albegger, Saria & Hacker, 1992; Heym, Webber & Adler, 1994) as well as those of the minor glands (Hauser-Kronberger, Hacker, Kummer & Albegger, 1995) are richly innervated by VIP-containing nerve fibres. The supply of NPY-containing nerve fibres is less abundant, whereas the supply of substance P- and CGRP- containing fibres associated with the acini is rare or absent. The blood vessels of the glands are well supplied with VIP- and NPY-containing nerve fibres, but are also innervated by substance P- and CGRP- containing fibres; these peptides are in some fibres co-localized, suggesting, in this case, a sensory origin. Substance P and VIP were found to be without effect on the *in vitro* release of potassium, thought to indicate water secretion, from fragments of the human submandibular gland. However, VIP elevated the tissue content of cAMP, known to be associated with protein secretion (Larsson, Dunér-Engström, Lundberg, Fredholm & Änggård, 1986).

Furthermore, in the clinic, parotid swelling, with or without pain, is one of the more common conditions affecting the gland, and while thought to depend on the formation of oedema and streching of the glandular capsule, the fundamental cause is often unknown (Shapiro, 1973; Leipzig & Obert, 1979). A number of case reports concern the transient swelling of the parotid gland

associated with general anaesthesia and the use of belladonna alkaloids as premedication (Vorhier, 1978). The findings of protein extravasation and the formation of parotid periglandular oedema in the rat in connection with intake of hard chow is surprising but reflects evidently a natural event as judged from the present findings. Various neuropeptides of the parasympathetic innervation including tachykinins, CGRP and VIP seemed to play a role in these phenomena. Therefore, it might be of interest to combine these presently described neuropeptide actions with the salivary gland swelling in humans.

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