Secretory and Anti-inflammatory Actions of Some Gastro-intestinal Hormones in Salivary Glands

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Abstract

Secretory and anti-inflammatory actions of some gastro-intestinal hormones in salivary glands

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Textbooks generally state that the secretory activity of salivary glands is solely regulated by nerves. This view is challenged in the present *Thesis,* using the parotid gland of the anaesthetized rat as experimental *in vivo* model. By changing focus from secretion of fluid to secretion of protein and of acinar amylase a secretory role was found not only for gastrin and cholecystokinin but also for the non-traditional gastro-intestinal hormone melatonin. Neither intravenous infusion of the hormone-analogues pentagastrin and desulfated cholecystokinin-8 nor of melatonin evoked any overt fluid secretion from the duct-cannulated gland. However, a subsequent intravenous wash-out injection of the secretagogue methacholine revealed protein/amylase secretion in response to the infusion of the hormone/hormone-analogues. The hormone/hormone-analogues exerted their effect directly on the gland cells: it persisted in the presence of α- and β-adrenoceptor antagonists, after disconnecting the gland from its peripheral ganglia and after evisceration. The gland protein synthesis increased to pentagastrin. Cholecystokinin(CCK) -A and CCK-B receptor types as well as the melatonin receptors, MT1 and MT2, were expressed in the gland. By the use of hormone-receptor antagonists, protein secretion was shown to involve mainly the CCK-A receptors and the MT2 receptors, whereas both types of cholecystokinin receptors were involved in the protein synthesis. The secretion of protein/amylase and the synthesis of protein to the hormone/ hormone-analogues were partially dependent on nitric oxide generated by the activity of neuronal type NO-synthase, probably of parenchymal origin. Cholecystokinin may occur as a transmitter in the peripheral nervous system and, hypothetical, cholecystokinin might belong to the group of parasympathetic non-adrenergic, non-cholinergic transmitters that upon stimulation of the parasympathetic auriculo-temporal nerve evokes secretion of fluid and, in particular, secretion of protein/amylase. However, the stimulation of the parasympathetic innervation, in the presence of cholecystokinin receptor antagonists, gave no support for a transmitter role for cholecystokinin in the gland. Lipopolysaccharide injected intraductally towards the parotid gland induced inflammation, as shown by neutrophil infiltration and increased myeloperoxidase activity in the gland, and mobilization of β-defensins, being a part of the oral defense mechanism. Melatonin and cholecystokinin (sulphated CCK-8) administered intraperitoneally exerted anti-inflammatory actions in the inflamed gland as judged by reduced levels of the elevated activity of myeloperoxidase. The effect of melatonin was non-receptor mediated, while that of sulphated CCK-8 was partially dependent on CCK-A receptors. The inflammatory response involved NO generated from inducible NO-synthase and neuronal type of NO-synthase as shown by selective NO-synthase inhibitors, the neuronal type being most likely of parenchymal origin, since it was activated also in the chronically denervated gland. In conclusion, the secretory activity of salivary glands seems not only to be regulated by a cephalic phase of nervous activity but also by a gastric phase and an intestinal phase of endocrine activity. Circulating melatonin and cholecystokinin/gastrin may not only influence the secretory activity but may also protect the salivary glands from inflammation. The present findings have implications for salivary gland dysfunction/dry mouth and its treatment.

Keywords: Salivary glands, protein secretion, synthesis, inflammation, β-defensins, myeloperoxidase, cholecystokinin, gastrin, melatonin, nitric oxide, nerve stimulation.

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

Paper I: **Çevik Aras H**, **Ekström J.** Cholecystokinin- and gastrin-induced protein and amylase secretion from the parotid gland of the anaesthetized rat. *Reg Pept* 2006; 134: 89-96.

Paper II: **Çevik Aras H**, **Ekström J**. Pentagastrin-induced nitric oxide-dependent protein secretion from the parotid gland of the anaesthetized rat. *Exp Physiol* 2006; 9: 977-982.

Paper III: **Çevik Aras H**, **Ekström J.** Pentagastrin-induced protein synthesis in the parotid gland of the anaesthetized rat, and its dependence on CCK-A and -B receptors and nitric oxide generation. *Exp Physiol* 2006; 91: 673-679.

Paper IV: **Ekström J**, **Çevik Aras H**. Parasympathetic non-adrenergic, non-cholinergic transmission in rat parotid glands: Effects of choleystokinin-A and -B receptor antagonists on the secretory response. *Reg Pept* 2008; 146: 278-284.

Paper V: **Çevik Aras H**, **Ekström J**. Melatonin-evoked in vivo secretion of protein and amylase from the parotid gland of the anaesthetised rat. *J Pineal Res* 2008; 45(4): 413-421.

Paper VI: **Darnell M, Çevik Aras H, Ekström J.** Lipopolysaccharide induced-in vivo increases in ß-defensins of the rat parotid gland. *Arch Oral Biol* 2006; 51: 769-774.

Paper VII: **Çevik Aras H, Ekström J**. Anti-inflammatory effects of cholecystokinin and melatonin in the lipopolysaccharide-exposed rat parotid gland as indicated by myeloperoxidase activity. *In manuscript.*

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INTRODUCTION

Saliva exerts digestive and protective functions and further, it is necessary for the articulate speech. It is essential for chewing, bolus formation and swallowing. Saliva contains enzymes that degrade the food, notable amylase and lipase, and these enzymes continue their activity in the stomach at varying length of time. It is a solvent for tastants in food and carries them to the taste buds. A mucin-rich film of saliva covers and protects the oral structures and promotes the re-mineralization of the teeth. Bicarbonate and histidine-rich peptides exert saliva's buffering capacity. Saliva protects the oral tissues from noxious agents by its antibacterial, antiviral and antifungal activities through lysozymes, peroxidase, lactoferrin, immunoglobulin A, histatins, defensins and cathelicidin. A number of growth-promoting factors are of importance for wound healing. Furthermore, the constant flow of saliva cleanses the oral cavity from food debris and noxious agents. The protective functions of saliva are not restricted to just the oral cavity. Salivary antimicrobial mechanisms protect the upper respiratory tract from infections, acid in the oesophageus is buffered and mechanical and chemical damages to the mucosa in the oesophageus and stomach may be repaired by growth stimulating factors (Kaplan & Baum, 1993; Tenovou, 1998; Van Nieuw Amerongen et al., 2004). Salivation as a part of the thermoregulation in dogs (Gregersen, 1931) and rodents (Hainsworth et al., 1967) is a further example, that saliva may serve various functions.

In addition to the three paired major salivary glands, hundreds of minor glands contribute to the mixed saliva. The contribution of the various glands varies in volume as well as in composition. The mucin-rich fluid is mainly produced by the minor glands and the sublingual gland, while the large volumes of the watery fluid are produced by the parotid and submandibular glands (Sreebny & Broich, 1988).

In the salivary glands, the activity of calcium dependent ion channels account for fluid and electrolyte secretion. Briefly, upon stimulation, cytoplasmatic Ca^{2+} rises as a consequence of a release from internal stores and influx from the interstitium. Increased levels of intracellular Ca^{2+} activates basolateral K^+ - and apical Cl^- -channels to open and allow the two electrolytes to move down their concentration gradients to the extracellular compartment. The accumulation of Cl^- in the acinar lumen will drag Na^+ by a paracellular route to the lumen. The luminal increase of NaCl creates an osmotic gradient that may cause a rapid movement of large volumes of water into the lumen provided the gland blood flow is adequately adapted to the increased demands (Poulsen, 1998).

Proteomic analysis of saliva has so far demonstrated several hundreds of proteins/peptides (Hu et al., 2005). Multiple pathways exist for the secretion of proteins, the main distinction being the regulated granular secretion and the constitutive non-granular (vesicle) secretion (Castle & Castle, 1998). Along the regulated pathway, proteins are condensed, after leaving the Golgi apparatus, in maturing storage granules and transported to the apical part of the cell and released from large dense core granules by exocytosis in response to stimulation. The time from synthesis to exocytosis is several hours. The intracellular messenger cyclic AMP is usually associated with the exocytosis. The constitutive pathway is thought to represent the fundamental mechanism for secretion occurring in all cells. Along this pathway, the proteins, after leaving the Golgi apparatus, by-pass the granular storage stage and move towards both the apical and basolateral membranes from where they are continuously released. A vesicular release in response to stimulation is superimposed upon this continuous basal constitutive secretion. In salivary glands, biochemical findings show synthesized proteins to be released as fast as they are synthesized (Proctor, 1998).

During the passage of saliva through the duct system, the composition of saliva is modified, while the volume remains unchanged. The uptake of sodium ions and the secretion of potassium ions are well-known phenomena (Poulsen, 1998).

The unstimulated constitutive secretion of kallikrein into the ductule lumen is a further example (Garrett et al., 1996).

During the night and at rest, a small spontaneous secretion from the human minor glands dominates, while in response to a meal the major glands are stimulated to secrete by intense nervous activity. A number of reflexes, preferentially originating from the mouth, are elicited by taste, chewing, dryness and pain but also by smell and by the distension of the esophagus and the stomach and by acid in the oesophageus (Hector & Linden, 1999). Moreover, nausea and vomiting are well-known phenomena inducing salivation by both peripheral and central mechanisms. Fever and stress are examples of inhibitory influences on the outflow of impulses from higher brain centers of salivatory nuclei. The efferent part of the reflex arch consists of the autonomic parasympathetic and sympathetic nerves (Garrett, 1988).

Whereas the secretory elements of all glands are thought to be supplied with a parasympathetic innervation, the sympathetic secretory innervation may vary; the minor human glands as well as the rat sublingual gland are examples of glands lacking a sympathetic nerve supply of their acinar cells. Parasympathetic nervous activity may produce large volumes of saliva, whereas sympathetic nervous activity produces small volumes of saliva. Both nerves cause secretion of large amount of proteins. Since the volume of sympathetic saliva is small, sympathetic saliva is usually characterized as protein rich, while parasympathetic saliva, due to the large volume, is characterized as protein poor. Under physiological conditions, sympathetic activity is considered to occur in a background of on-going parasympathetic activity. Combined parasympathetic and sympathetic activity interact synergistically to enhance both the secretion of fluid and the secretion of protein (Emmelin, 1979; Garrett, 1988). In contrast to the positive interactions with respect to the secretion of saliva, the two divisions of the autonomic nervous system have opposite effects with respect to the regulation of the blood flow through the glands: parasympathetic activity increases the flow and sympathetic activity reduces the flow. However, the sympathetic vasoconstrictor nerve fibers are separate from the sympathetic

secretomotor nerve fibers and are not activated during reflex secretion. In case of a dramatic fall in blood pressure, e.g. as a consequence of bleeding, the vasoconstrictor fibers are activated (Emmelin, 1967; Edwards, 1998).

The classical neurotransmitters released from the peripheral postganglionic parasympathetic and sympathetic nerve endings are acetylcholine and noradrenaline, respectively, and the acinar cells are supplied with muscarinic receptors of various subtypes as well as of α_1- and β_1-adrenergic receptors. Apart from being exposed to acetylcholine and noradrenaline and, in addition to circulating catecholamines, a number of so called non-adrenergic, non-cholinergic co-transmitters stimulate the acinar cells. Some of these transmitters cause a large volume of secretion such as the tachykinins, while others cause just a small flow or no overt fluid secretion at all but add proteins to the saliva; parasympathetic vasoactive intestinal peptide, calcitonin gene related peptide and neuropeptide Y are examples of the latter. Interestingly, the various neuropeptides may interact synergistically with each other but also with the classical transmitters (Ekström, 1999). Though the parasympathetic nerves of the salivary glands contain the nitric oxide (NO) forming enzyme NO-synthase, the transmitter role of nitric oxide of parasympathetic origin is unclear. No doubt NO is involved in protein secretion, protein synthesis and mitotic activity but this is due to the activity of NO-synthase of non-nervous, parenchymal, origin rather than of nervous origin (Sayardoust & Ekström, 2003, 2006; Ekström et al., 2004).

Nerves do not only exert short-term influences on the glands, such as on the secretion of fluid and proteins, the contraction of the myoepithelial cells and the blood flow but also exert long-term influences on the receptor sensitivity to agonists, the synthesis of proteins and the gland size (Emmelin, 1967).

Hormones do also take part in the long-term regulation of salivary glands. The increased frequency of dry mouth in post-menopausal women is usually associated with the decrease in oestrogen production (Eliasson et al., 2003; Meurman et al., 2009). In rodents, hypophysectomy, thyroidectomy,

gonadectomy, adrenalectomy and experimental diabetes cause glandular atrophy, change in saliva composition and volume of saliva secreted (Shafer & Muhler, 1953; Ohlin, 1965; Takeuchi, 1977; Johnson, 1988; Anderson, 1994; Mahay, 2004).

In contrast, hormones are, on the whole, not thought to play any role in the short-term regulation of salivary glandular activities. Since the food is usually in the mouth for a very brief period a nervous regulation with its rapid onset and high precision in action may, from a functional point of view, seem more appropriate than an endocrine regulation to control the glands acutely. On the whole, few studies have been devoted to acute hormonal effects on salivary glands. Aldosterone has a great effect on the electrolyte composition on the parotid saliva of the sheep (Blair-West et al., 1967). Upon a latent period (90-120 min), of about two hours, it increases the sodium uptake in the duct system. However, its effect on human saliva is small. There are studies reporting a sparse fluid secretion from the rat submandibular gland upon administration of secretin and cholecystokinin (Iwabuchi & Masuhara, 1994; Takai et al., 1998) and two short notes reporting increases in amylase concentration in resting flow of saliva from the human parotid gland upon infusions of gastrin, secretin and cholecystokinin (Mulcahy et al., 1972; Matfertheiner P et al., 1980). Moreover, pentagastrin infusion increases the saliva content of epidermal growth factor in humans (Konturek et al., 1989). On the other hand, gastrin and secretin were without effect on volume or protein output of parotid saliva in the dog (Clendinnen et al., 1970), while gastrin inhibited on-going secretion of saliva in the sheep (Grovum & Leek, 1988), and secretin was without effect on mucous secretion in humans (Descos F et al., 1973). No systematic investigations on the secretory effect of gastro-instestinal hormones on salivary glands seem to be on record. The present study, using the rat as the experimental animal, focuses on the hormones gastrin, cholecystokinin and melatonin, which are all released in response to a meal (Konturek & Konturek, 2000; Heuther, 1994; Bubenik et al., 2000; Rice et al., 1995; Ingenito et al., 1986). Though melatonin is usually

associated with the corpus pineale and the circadian rythm, high concentrations are to be found in the intestines (Bubenik, 1980; Heuther, 1994).
Gastrin was the second hormone to be described (Edkins 1905). It is released from endocrine cells in the antrum and duodenum. It is initially synthesized as progastrin and finally processed to amidated gastrin. Gastrin may occur in a number of molecular forms, in humans mainly as G-17 and G-34 (Shulkes & Baldwin, 1997; Dockray et al., 2005). Gastrin stimulates acid secretion via two routes, by a direct action on the parietal cells and indirectly by causing the release of histamine from the enterochromaffin-like cells. It is also trophic to the gastric mucosa. Moreover, it stimulates the pancreatic gland to release protein. Gastrin is released by sight and smell of appetizing food as well as by food in the mouth through vagal activity and by food in the stomach through long reflexes (involving the central nervous system), short reflexes and digestive products (Kwiecien & Konturek, 2003). Pentagastrin possesses the same biological activity as native gastrin and is usually used for clinical and experimental purposes (Johnston & Jepson, 1967).

Cholecystokinin is located in endocrine cells of the duodenum, and was first shown to contract the gallbladder (Ivy & Oldberg, 1928) and later found to cause the release of enzymes from the pancreas (Harper and Raper, 1943). Also cholecystokinin may occur in various molecular forms, in humans cholecystokinin-33 and -22 dominate (Rehfeld, 2004). Apart from the effects already mentioned, cholecystokinin also stimulates bicarbonate secretion from the gastric mucosa and the exocrine pancreatic gland as well as pepsinogen secretion, and it inhibits gastric emptying and further, provides signals for satiety. Cholecystokinin is thought to be released by intraluminal products of digested fat and protein (Wank, 1995).

Cholecystokinin shares the same carboxyl heptapeptide as gastrin at the end of the biologically active part of the molecule. Consequently, they may act at the same receptors and evoke the same response. The cholecystokinin/gastrin family interacts with at least two receptors, cholecystokinin A and B receptors,

which belong to the G-protein-coupled receptor family (Wank, 1995). A third type of high-affinity receptor for cholecystokinin in the stomach, the gastrin receptor (Soll et al., 1984), turned out to be identical with the cholecystokinin B receptor. The two types of receptors are highly homologous, having 48% amino acid identity. Besides these traditional receptors, homo- and heterodimerization of cholecystokinin A and cholecystokinin B receptors have been demonstrated (Cheng et al., 2003).

Melatonin was discovered in the pineal gland by Lerner (1958). It is synthesized in a variety of tissues. In the intestinal mucosa, melatonin is confined to the enterochromaffin cells, also storing the related serotonin. The amount of melatonin in the intestines is about 400 times that in the pineal gland (Bubenik, 1980; Huether, 1994). Melatonin stimulates duodenal bicarbonate secretion and pancreatic amylase secretion (Sjöblom & Flemström, 2003; Jaworek et al., 2004). Many of the melatonin's regulatory roles are mediated through high affinity and G protein-coupled receptors, melatonin 1 and melatonin 2 receptor subtypes. A close relationship between food intake and melatonin release from the intestines has been reported (Bubenik, 2002; 2008).

Salivary gland inflammation may cause hyposalivation, Sjögren´s syndrome being the classic example (Grišius & Fox, 1998). However, a reduced flow of saliva may also allow microorganisms to invade the salivary gland duct system and the secretory cells, resulting in chronic gland inflammations (Van-Winkelhoff & Boutaga, 2005). As a consequence of the inflammation, the oral health is jeopardized. In recent years, β-defensins, a group of small antimicrobial peptides of the innate immune system have come into focus as a part of the defense system. They have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungi and virus. β-Defensins are expressed along the epithelial surfaces of the digestive tract, including the oral mucosa, gingival tissue, tongue and salivary glands, and they appear in the saliva (Schutte & McCray, 2002; Dunche et al., 2001).

Interestingly, cholecystokinin and melatonin, in particular, are reported to display anti-inflammatory actions, e.g. inhibiting neutrophil infiltration and neutralizing reactive oxygen and nitrogen compounds, and may be thought of as potential therapeutic options (Carrasco et al., 1997; Ling et al., 2001; Reiter, 2001; Barlas et al., 2004; Jahovic et al., 2004; Topal et al., 2005). Nitric oxide seems to play a key role during inflammation, and may both promote and inhibit the process.

Nitric oxide is synthesized from L-arginine and molecular oxygen by the enzyme nitric oxide synthase (NOS). Nitric oxide has the ability to diffuse through cell membranes. Three isoforms of NOS have been identified, the neuronal type (nNOS), the endothelial type (eNOS), both constitutively expressed, and the inducible type (iNOS). Neuronal NOS is expressed both in nervous tissue and in epithelial cells. Neuronal NOS as well as endothelial NOS are activated in response to a generated calcium signal, for instance, by nerve activity or the exposure of the blood vessels to shear stress. Nitric oxide formed in response to the two constitutive NO-synthases activates soluble guanylate cyclase to form cyclic GMP (Moncada et al., 1991). The inducible NOS is activated by cytokines and bacterial lipopolysacharide, and nitric oxide may originate from neutrophils and macrophages. Nitric oxide released upon the activity of inducible NO-synthase is not thought to work through the cGMP-pathway. It is cytotoxic (partly because it interacts with oxygen derived radicals) not only for invading microorganisms but also for the host immune cells. In Sjögren´s syndrome, inducible NO-synthase is present in acinar and ductal cells (Konttinen et al, 1997).

In the first section of this *Thesis,* a trace secretion from the submandibular gland, but not from the parotid gland, occurred in response to the cholecystokinin analogue desulfated CCK-8 and to the gastrin homologue pentagastrin (1).

In previous studies from this laboratory on parasympathetic non-adrenergic, non-cholinergic transmitter mechanisms in salivary glands, neuropeptides were found to cause secretion of proteins and to evoke exocytosis of acinar secretory

granules without any accompanying *overt* secretion of fluid (Ekström, 1999). The "occult" neuropeptide-evoked protein secretion was revealed by a subsequent wash-out flow of saliva in response to volume-producing secretagogues administered to the blood stream. By applying this *in-vivo* approach, a basal secretion of protein/amylase was initially demonstrated and possible influences on this secretion were tested in the next section (2).

This basal secretion served as reference to the response of the submandibular (section 3) and parotid glands (section 4) upon administration of the hormone/hormone-analogues. The outcome made the parotid gland to become the primary study object. In this gland, amylase is the major exportable protein, being 35% of total secretory protein (Robinovitch et al., 1977). It reflects acinar secretion, while total protein reflects both acinar and ductal secretion. The following experiments were designed to define whether the hormone/hormone-analogues evoked secretion by a direct effect on the gland cells. In this connection, the types of receptors involved were examined as well as their glandular expression.

In the next section (5), the effect on the glandular synthesis of protein following exposure to pentagastrin was investigated. Receptors involved in the observed increase were examined by the use of antagonists and further, the consequence of the receptor blockade on the basal protein synthesis was defined.

Next (6), the involvement of NO generation in the protein/amylase secretion (pentagastrin and melatonin) as well as in the protein synthesis (pentagastrin) was considered. Moreover, particular attention was paid to the contribution of the neuronal type of NO-synthase to the generation of NO (Ekström & Sayardoust, 2003).

Cholecystokinin may serve as a transmitter in the autonomic nervous system (Rhefeld, 1980), and was suggested to act as parasympathetic non-adrenergic, non-cholinergic transmitter evoking secretion of saliva from the submandibular

gland of the rat (Takai et al., 1998). In section 7, the possibility that not only cholecystokinin of gastro-intestinal origin but also cholecystokinin of nervous origin contribute to the regulation of the secretory activity of the parotid gland was explored.

In the last section (8) of this *Thesis*, an experimental model was worked out for studies on inflammation and its resolution, using the rat parotid gland exposed to the retrograde ductal injection of bacterial lipopolysaccharide. Myeloperoxidase activity reflected the neutrophil infiltration of the gland tissue, and the antimicrobial β-defensins were found to increase. In a number of experiments, the effect of melatonin and cholecystokinin on the increase in myeloperoxidase activity following the local injection of lipopolysaccharide was followed and further, the involvement of inducible and neuronal NO-synthases was explored.

In summary, the objective of this *Thesis* is to define the roles of the traditional gastro-intestinal hormones gastrin and cholecystokinin as well as the role of the non-traditional gastro-intestinal hormone melatonin in the regulation of glandular activities associated with secretion and with special attention to:

1) The secretion of proteins and the synthesis of proteins.
2) A direct or indirect action of the hormones on the glands, the types of receptors involved and the nitric-oxide dependency.
4) Cholecystokinin as a possible parasympathetic non-adrenergic, non-cholinergic transmitter; and further, to define an anti-inflammatory role in the lipopolysaccharide-induced gland inflammation with respect to:
5) Melatonin and cholecystokinin, mediating receptors and the involvement of neuronal and inducible types of nitric oxide-synthase in the inflammatory response.

MATERIAL AND METHODS

Adult female rats of a Sprague Dawley strain were used. They were maintained on a pelleted standard diet and tap water *ad libitum*. Prior to the acute experiments, the rats of Papers II–V were fasted over-night (but for a study group in V). The experiments were approved by the local animal welfare committee, Gothenburg, Sweden.

For the chronic experiments, the rats were anaesthetized with a combination of pentobarbitone (20 mg/kg, I.P.) and ketamine (50 mg/kg, I.M.); buprenorphine (0.015 mg/kg, S.C.) was given as an analgesic.

For the terminal experiments, the rats were anaesthetized with pentobarbitone (55 mg/kg, I.P., additional doses were given intravenously as required). The body temperature was kept at about 38º C by using a heating blanket connected to a rectal probe. A tracheal cannula was fitted. The rats were provided with a femoral venous catheter on both sides; one serving as a conduit for hormone/hormone-analogue infusion and the other for injection of methacholine and various blockers. In some types of the experiments, receptor antagonists were administered intraperitoneally (VII).

At the end of the experiments, the animals were killed, still under pentobarbitone-anaesthesia, by exsanguinations after cutting the abdominal aorta.

Preliminary surgery

Combined parasympathetic postganglionic denervation and sympathetic postganglionic denervation was achieved by avulsion of the auriculo-temporal nerve (Khosravani et al., 2006) and avulsion of the superior cervical ganglion (Alm & Ekström, 1977). The wound was sutured and the animals were allowed to recover. The acute experiments were performed 1-2 weeks postoperatively (allowing time for nerve degeneration).

Duct-cannulations: collection of saliva or retrograde ductal injections

The parotid duct was exposed on the masseter muscle, close to the mouth, by a skin incision. The submandibular duct was exposed under the myelohyoideus muscle. To collect saliva a fine polyethylene tube (filled with distilled water) was inserted, under the dissection microscope, into the duct. Saliva was either collected on pre-weighed filter papers or in ice-chilled pre-weighed Eppendorf™ tubes and then they were re-weighed. The saliva was frozen (-20° C) and stored (-70° C). The specific density of the saliva was taken to be 1.0 g/ml.

For retrograde ductal injection (Alm & Ekström, 1976), a syringe filled with LPS (a concentration of 0.2 mg/ml in sterile saline) or just saline as control, attached to a fine polyethylene tube was inserted into the duct and 0.04ml of the solution was injected towards the gland during 5 seconds. The tube was then cut open for 5 seconds to allow overflow if any, and was closed 2-3 min later by heating; then, the wound was sutured. The gland on the contralateral side was untreated.

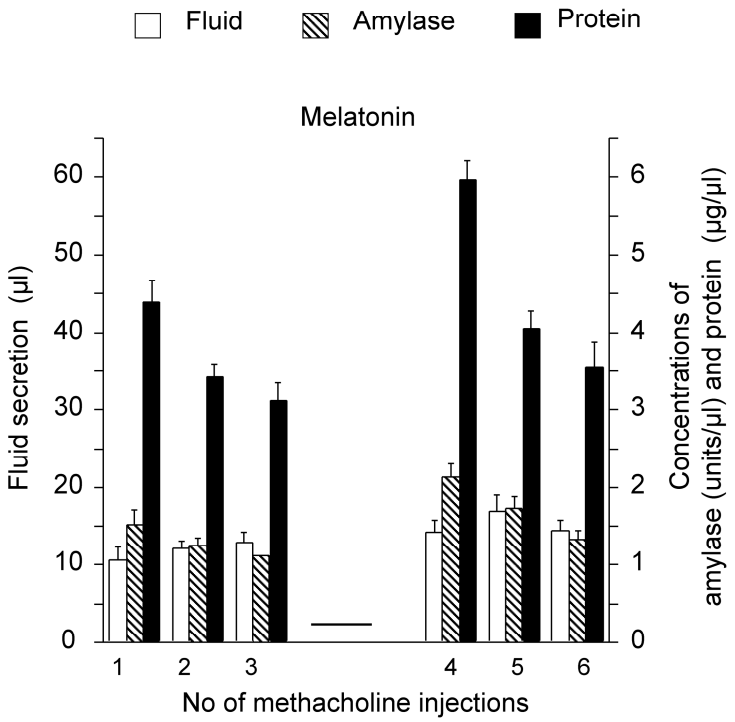
Terminal surgery: denervation and/or evisceration

Combined parasympathetic and sympathetic denervation of the parotid gland was the same surgical procedure as for the chronic procedure (see above, I, V). Evisceration was performed by ligating the coeliac artery (which interrupts the arterial blood supply to the liver), the superior mesenteric artery and the hepatic vein. The esophageus and the rectum were divided, and the stomach, spleen, pancreas and the small and large intestines were removed (I, V; in V denervation and evisceration were performed in the same rat).

Fluid secretion: submandibular gland

Pentagastrin and desCCK-8 were infused intravenously over a period of 1 hour. There was no secretion from the parotid gland but a trace secretion appeared from the submandibular gland (I). Saliva was collected over 5- min periods by pre-weighed filter paper. The experiments were performed in the presence of α- and β-adrenoceptor antagonists as well as atropine.

Metacholine-evoked wash-out secretion: the standard protocol



The Figure, showing the mean responses of seven rats, illustrates the experimental protocol. Three intravenous bolus injections of methacholine (5 µg/kg) given with 10 min intervals, were followed by a 5 min pause (during which, when appropriate, blockers were given), then a 10 min long period of intravenous infusion of hormone/hormone-analogues or saline (horizontal line; in the present case melatonin 25 mg/kg) followed. Ten minutes after the end of the infusion, methacholine was once again injected every 10 minute over 30 minutes. For statistical evaluation, the response to the fourth methacholine injection was compared to that of the third, set to 100%. The methacholine response was prompt and with a short duration (< 1 min). The saliva produced in response to an injection was collected in ice-chilled pre-weighed tubes. The experiments were performed in the presence of α- and β-adrenoceptor antagonists.

Stimulation of the parasympathetic auriculo-temporal nerve

The auriculo-temporal nerve was stimulated at supramaximal voltage (7 V, 2 ms) using a Grass S48 stimulator with an isolation unit (Grass SIU 5A), at a frequency of either 10 Hz or 40 Hz (IV). Saliva was collected in pre-weighed ice-chilled tubes during each stimulation period. The experiments were performed in the presence of α- and β-adrenoceptor antagonists and, when appropriate, atropine as well. Six stimulation periods were applied to nerve, each of a duration of 3 min in the atropinized rats and of 2 min in the *non*-atropinized rats. The interval between two stimulation periods was 5 minutes. Immediately after the *end of the second* stimulation period, the CCK-A receptor antagonist lorglumide was injected intravenously. In the same animal, the CCK-B receptor antagonist itriglumide was injected intravenously immediately after the *end of the fourth* stimulation period. As to controls, the same stimulation protocol was followed but saline replaced the hormone-receptor antagonists. For the statistical evaluation, the response to the third stimulation period (in the presence of the CCK-A receptor antagonist) was compared to that of the second stimulation period (set to 100%) and the response to the fifth stimulation period (in addition, in the presence of the CCK-B receptor antagonist) to that of the fourth stimulation period (set to 100%).

Assay of protein and amylase

The salivary protein content was analysed by the method of Lowry *et al.* (1951) and expressed in terms of concentration (µg/µl saliva), using bovine serum albumin as standard (I, II, IV, V). The salivary amylase activity was measured by an enzymatic colorimetric test (Roche Diagnostics), using α-4-nitrophenylmaltoheptaoside (4NP-G_7) as substrate (Hägele et al., 1982). One unit (U) of catalytic activity of α-amylase is defined as the hydrolysis of 1 µmol of 4NP-G_7 per min per µl, being equivalent to the definition of the international unit. The salivary amylase was expresessed in terms of concentration (U/µl) or as total otput (U).

Protein synthesis: [^{3}H] leucine incorporation

To measure the incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble material of the parotid gland, 500 µCi/kg of leucine in 0.5 ml saline, was injected intravenously 30 min after the end of an hour long intravenous infusion period of pentagastrin (20 µg/kg/h). The experiments were performed in the presence of α- and β-adrenoceptor antagonists as well as atropine. CCK-receptor antagonists and nitric oxide synthase inhibitors were administered before the infusion period. The animal was killed 15 min after the [^{3}H]leucine administration by exsanguination, and the parotid glands of both sides were removed, washed in saline, pressed between gauze pads, placed on filter paper to remove adherent tissue (if any) and to absorb additional fluid, weighed, frozen (-20° C) and stored (-70° C) until processed within a week (III). Following the processing of the gland tissue, the final precipitate was analysed in a scintillation counter. The amount of radiolabelled leucine was expressed as disintegrations per min per mg of gland tissue. In each study group, two rats were paired, based on age and body weight, before the experiment began, one to serve as experimental animal and the other as control animal. The pair of animals was treated concomitantly and in the same way apart from the difference in type of infusion (and pretreatment of drugs) and furthermore, the glands of each pair were processed and analysed at the same time.

Immunoblotting: CCK- and melatonin-receptors

Western blot technique was used to determine the expression of CCK- and melatonin- receptor proteins in the salivary glands (and for comparison the pancreatic gland and antrum of the stomach). The tissues were homogenized and centrifuged. The supernatants were analysed for protein content with an assay from Bio-Rad Laboratories AB, using serum albumin as standard. Applying equal amounts of protein, the proteins in the homogenates were electroblotted and later application of first primary and later secondary antibodies performed. A high-performance chemoluminescence film was developed and the membranes were scanned (Paper I, V).

Tissue myeloperoxidase activity: neutrophil infiltration

Tissue myeloperoxidase activity was determined according to Hillegas et al. (1990) and Kuebler et al. (1996) (VI, VII). Though, the assay is not thought to be influenced by other peroxidases (Kuebler et al., 1996), a selective inhibitor (Dapsone, diaminodiphenylsulfone) of peroxidases (and not of myeloperoxidase), was used to ascertain the specificity of the assay (Thomas et al., 1994). One unit of enzyme activity was defined as the amount of myeloperoxidase present that caused a change in absorbance of 1.0 per min at 37˚C. Myeloperoxidase activity was expressed as total activity (units per whole gland per minute).

Histology: neutrophil infiltration

To confirm glandular cell infiltration, as a response to lipopolysaccharide, in the parotid and submandibular glands, tissue specimens were stained for hematoxylin and eosin (VI).

ELISA assay: β-defensins

Based on a sandwich ELISA protocol (Faurschou *et al.,* 2002) an indirect ELISA for detection and quantification of rat β-defensins 1, 2 and 3 was designed (VI). The amounts of β-defensins were expressed in terms of µg per 100 mg gland tissue.

Chemicals

Lipopolysaccharide (Escherichia coli LPS 0111:B4; purified by gel-filtration chromatography), Pentagastrin, cholecystokinin octapeptide desulfated (desCCK-8) or sulfated (sCCK-8), melatonin, CCK-A receptor antagonist CR-1409 (lorglumide), CCK-B receptor antagonist CR 2945 (itriglumide), melatonin receptor antagonist luzindole, metacholine hydrochloride, non-selective nitric oxide-synthase inhibitor L-NAME, inducible nitric oxide-synthase inhibitor L-NIL, atropine sulphate and propranolol hydrochloride and Dapsone (diaminodiphenylsulfone) were from Sigma Chemicals, phentolamine mesylate was from Novartis Pharma AG, [^{3}H]leucine was from Amersham Biosciences,

neuronal nitric oxide-synthase inhibitor N-PLA from Cayman Chemical Company and pentobarbitone sodium was from Apoteksbolaget, Stockholm, Sweden.

Statistical analyses

Statistical significances of differences were calculated by Student's *t*-test for paired or unpaired values or by one-way analysis of variance (ANOVA) 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

Basal values: methacholine- and nerve evoked saliva

The mean volume of saliva secreted from the submandibular gland was 16 µl in response to an intravenous bolus dose of methacholine (5 µg/kg), while the mean salivary protein concentration was 0.5 µg/µl. The volume of parotid saliva secreted was 9.5-11.7 µl to methacholine 5 µg/kg; the mean salivary concentration of protein was within the range of 2.0-5.1 µg/µl, while that of amylase activity was 0.6-1.6 U/µl.

In response to parasympathetic stimulation at 10 Hz, the parotid gland secreted a mean of 52 µl/min saliva in the absence of atropine, and in its presence 8 µl/min, the mean amylase concentration being 1.0 U/µl and 10 U/µl, respectively. At 40 Hz, in the presence of atropine, the mean flow rate was 23 µl/min and the amylase concentration was 6 U/µl.

Since in the studies using the wash-out design, the post-infusion volume of saliva to methacholine was the same at that to the pre-infusion volume of saliva, protein and amylase were expressed as concentrations (Paper I, II, IV and V). On the other hand, volume changes occurred when using the experimental protocol for nerve stimulation. Therefore, figures for the total output of amylase were also used.

(1) Fluid secretion

There was no resting flow of saliva from the submandibular and parotid glands in the anaesthetized animals. In the submandibular gland, but not in the parotid gland, pentagastrin and desCCK-8 evoked a trace secretion in the presence of muscarinic- and α- and β-adrenergic- receptor blockade (I). No secretion of saliva was observed in response to melatonin from the two types of glands (V, and unpublished observation with respect to the submandibular gland).

(2) Basal (constitutive) secretion

A continuous secretion of protein/amylase occurred from the both glands (I, II, V). This was revealed by an increase of the interval of the repeated wash-out injections of methacholine (from 10 min to 25 min). The concentration values being elevated by 10-30% in the parotid saliva and by 70% in the submandibular saliva (only protein), while the volume of saliva secreted to methacholine remained unchanged. (I, II, V)

The elevation occurred in the presence of α- and β-adrenergic receptor blockade, and was unaffected by the various hormone-receptor antagonists and NO-synthase inhibitors tested. The increase is presently attributed to on-going constitutive secretion. In this connection, it should be mentioned that the phenomenon occurred also after acute denervation of the glands and evisceration (I, V).

(3) Protein secretion of the submandibular gland

Pentagastrin and desCCK-8 evoked higher figures for the protein concentration in the wash-out saliva than control values (125% and 114%, respectively, *versus* 70%) but a significant difference was not attained under the present experimental set-up (I). The volume of the wash-out saliva to methacholine remained unchanged. Subsequent observations on the secretion of protein were focused on the parotid gland.

(4a) Protein and amylase secretion of the parotid gland

The protein concentrations of the wash-out saliva to methacholine (as compared to pre-infusion values) were significantly higher than in corresponding controls, in response to pentagastrin by 141% (158% and 145% *versus* 11%, I, II), for desCCK-8 by 74% (85% *versus* 11%, I) and for melatonin by 29-70% (53-94%, depending on dose, *versus* 24%, V). Similar effects were observed with respect to the amylase concentration, being for pentagastrin by 105% (127% *versus* 22%, II), desCCK-8 by 45% (68% *versus* 23%, I) and melatonin by 27-64% (53-90%, depending on dose, *versus* 26%, V). Apart for melatonin, the observations were performed on non-fasted rats. However, food intake did not affect the magnitude of the response to melatonin (V).

The volume of the wash-out saliva in response to methacholine remained unchanged. Therefore, any change in the concentrations of protein and amylase activity reflects a change in the total output of protein and amylase. The changes in acinar amylase output went in parallel with the changes in total protein output.

The elevated protein and amylase outputs were not a reflection of synergistic interactions between the hormones and methacholine, as shown by a change in the standard protocol (I, V). Instead of injecting the first post-administration wash-out dose of methacholine 10 min after the end of the period of hormone infusion, an injection of methacholine was given already at 5 min and then, followed by “the ordinary” injection of methacholine 5 min later (i.e. 10 min after the end of infusion period). If a potentiating effect was at work at 10 min, enhanced responses would be expected despite the preceding methacholine injection just 5 min after the infusion period. With respect to both pentagastrin and melatonin, neither amylase nor proteins were significantly elevated compared to the pre-infusion period. Importantly, the amylase and protein responses to the injection 5 min after the end of the infusion were markedly increased, while the volume of saliva remained unchanged.

The hormones most probably evoked protein/amylase secretion by a direct action on the secretory cells of the glands (I, II, V). The responses were still evoked after excluding potential influences exerted by the central nervous system or autonomic ganglia and circulating catecholamines as well as the stomach, the intestines, the spleen and the liver.

(4b) Cholecystokinin and melatonin receptors involved in the protein/amylase secretion

The CCK-A receptor antagonist lorglumide prevented completely the protein/amylase response to pentagastrin and desCCK-8. In the presence of the CCK-B receptor antagonist itriglumide, the glands still responded to both desCCK-8 and pentagastrin. However, the pattern was not uniform, since the response to desCCK-8 was unaffected by itriglumide, while the response to pentagastrin was significantly less than in the absence of itriglumide (I, Fig. 2). The melatonin receptor blocker luzindole, prevented the expected protein/amylase increase to melatonin. Luzindole displays a higher affinity for MT2 receptors than MT1 receptors. Presently, it is not possible to decide whether just MT2 receptors or also MT1 receptor subtypes were involved in mediating the secretory response.

The pancreatic amylase secretion evoked by melatonin is thought to depend on a melatonin-induced release of cholecystokinin, which in turn activates a vago-vagal reflex eliciting the pancreatic gland to secrete amylase (Leja-Szpak et al., 2004). As shown by the use of the CCK-A receptor antagonist lorglumide the melatonin-induced protein/amylase response of the parotid gland did not require cholecystokinin as intermediary agent.

(4c) Expression of cholecystokinin and melatonin receptors

Appropriate receptor antibodies recognized both CCK-A and CCK-B receptor proteins in the parotid and submandibular glands (I). The expression of these receptors did also appear in chronically denervated parotids. Likewise, both MT1 and MT2 receptor proteins were identified in the parotid gland (V). The specific

binding of the receptor proteins was tested in the presence of blocking peptides or in the absence of primary antibodies, and in neither case did the expected protein bands appear.

(5) Protein synthesis

A 17% increase in the protein synthesis of the parotid gland, as judged by the incorporation of [^{3}H] leucine into trichloroacetic acid-insoluble materials, followed upon infusion of pentagastrin over one hour (under muscarinic and α- and β-adrenoceptor blockade, III). In the presence of the CCK-A receptor antagonist lorglumide or the CCK-B receptor antagonist itriglumide, the expected increase to pentagastrin was not only absent, but also further reduced, below the basal value (by 16-12%) of controls (not exposed to the CCK-receptor antagonists). The further reduction (below control value) suggested that the CCK-receptors were exposed not only to exogenous pentagastrin but also to endogenous stimulation. Support for this idea was gained when the control glands (under basal conditions) were subjected to either of the two CCK-receptor antagonists and displaying a decrease in protein synthesis of about the same magnitude (by 22-17%). Evidently, the effect of the two CCK-receptor antagonists was non-additive, since upon combined administration the magnitude of the reduction was not further increased. The non-additive effect may suggest that both receptor types, share a common pathway intracellularly.

In the rat parotid gland, most of the protein synthesis is directed towards producing secretory products (Sreebny, 1971). The synthesis of protein and amylase is thought to be the consequence of a direct agonist action temporally dissociated and independent of the agonist-evoked secretory activity (Grand & Gross, 1969). This view is supported by the fact that both types of CCK-receptors were involved in the protein synthesis, while the protein/amylase secretion seemed mainly to depend on the CCK-A receptor (2d).

Since, the CCK-receptor antagonists affected the *protein synthesis* under “basal condition”, but did not affect the *protein/amylase secretion* during “basal

condition", it may be concluded that the intracellular mechanisms associated with cholecystokinin-receptor mediated *protein synthesis* is more sensitive to low graded stimuli than the intracellular mechanisms associated with cholecystokinin-receptor mediated *protein secretion*.

The CCK-receptor mediated stimulation of an early and rapid protein synthesis in the parotid gland presently observed is probably associated with the synthesis of secretory proteins rather than with hypertrophy or hyperplasia. While prolonged treatment with pentagastrin or cholecystokinin is without effect on the weight of the rat parotid gland, the pancreatic gland gains in weight in response to the treatment (Maystone & Barrowman, 1971; Månsson et al., 1990; Axelson et al., 1996).

On-going studies of ours (H. Çevik Aras, T. Godoy and J. Ekström) show also an infusion of melatonin to induce protein synthesis (35%) in the rat parotid gland, partially dependent on neuronal type NO-synthase and affected by luzindole.

(6) NO-dependent and NO-*non*dependent protein secretion and protein synthesis

Roughly, two-thirds of the increase in *protein and amylase secretion* (II) in response to pentagastrin or melatonin could be related to NO-generation. The selective neuronal type of NO-synthase inhibitor *N*-PLA (Zhang et al., 1997) showed about the same efficacy as the non-selective NO-synthase inhibitor L-NAME.

The expected increase (23%) in *glandular protein synthesis* to pentagastrin was prevented by the two NO-synthase inhibitors (III), thus illustrating its NO-dependency. NO-synthase inhibition did not affect the "basal" protein synthesis, in agreement with previous work (Sayardoust & Ekström, 2004). However, the "basal"protein synthesis was reduced (17-23%) by the cholecystokinin-receptor antagonists (III). Thus, already under basal conditons, the cholecystokinin-receptors of the parotid gland were subjected to stimulation, probably by

circulating hormones, and this did not require the generation of NO. The results suggest that *strong* rather than *weak* stimuli of the CCK-receptors are necessary to activate the neuronal type NO-synthase. The varying degree of NO-dependency presently found, is in agreement with earlier observations in the parotid gland (Sayardoust & Ekström, 2003).

(7) Effects of cholecystokinin receptor antagonists on the parasympathetic transmission

No support was gained for the view that cholecystokinin is one of the transmitters involved in mediating the parasympathetic non-adrenergic, non-cholinergic secretion of fluid and of protein/amylase in the parotid gland of the rat (IV). The fluid and amylase secretion rate (under α- and β-adrenoceptor blockade) at a stimulation frequency of 10 Hz or of 40 Hz of the auriculo-temporal nerve was neither affected by the CCK-A receptor blocker lorglumide nor by the CCK-B receptor blocker itriglumide, and this was the case for both non-atropinized and atropinized rats. Thus, under physiological conditions cholecystokinin and gastrin, acting on the cholecystokinin receptors of the glands, are likely to be of endocrine origin rather than of nervous origin.

A characteristic feature of the non-adrenergic, non-cholinergic secretion is that it fatigues in response to repeated sequences of stimulation and, in particular, when a high frequency of stimulation is applied to the nerve. The phenomenon is attributed to the depletion of the neuropeptide stores of the nerve terminals (Ekström, 1999). This depletion might be the explanation to the decreased fluid response from the submandibular glands, observed by Takai and coworkers (1998), upon repeated parasympathetic stimulation at a high frequency after administration of lorglumide.

(8a) Lipopolysaccharide (LPS)-induced gland inflammation and β-defensins

The presence of the three subtypes of the antimicrobial β-defensins was demonstrated in the rat parotid gland using an ELISA assay. In response to the intraductal injection of LPS, the levels of β-defensin 1 and 3 were elevated 3 to 6

hours post-injection (by 41-30% and 15-65%, respectively), while the level of β-defensin 2 was unaffected during the observation period. When examined at 6 hours, the LPS-treated parotid glands showed a marked glandular infiltration of neutrophils and further, the myeloperoxidase activity was increased 9 to 10-fold; at the early stage (3 hours), the increase was as much as 12-fold (VI, VII).

The three subtypes of β-defensins were not detected in the submandibular and sublingual glands. Though, the myeloperoxidase activity was 6-fold increased in response to LPS in the submandibular glands, neither of the β-defensins was detected. Previous in vitro findings do also show various patterns of response to LPS, which might suggest different roles for the three subtypes in the defense mechanisms of the glands (Feucht, 2003; Fahlgren, 2004).

(8b) Anti-inflammatory actions of cholecystokinin and melatonin

A direct relationship between tissue myeloperoxidase activity and the number of neutrophils exists (Bradley et al., 1982). Both cholecystokinin and melatonin markedly, and dose-dependently, reduced the myeloperoxidase response to the intraductal administration of LPS (VII). Whereas both sulfated and desulfated forms of CCK-8 have been shown to exert anti-inflammatory actions, most previous works have focused on the sulfated form (Carrasco, 1997; Ling et al., 2001; Cong et al., 2002; Wu et al., 2008), which was also the form presently studied. The expected 10-fold rise in the activity of myeloperoxidase (at 3 hours following the intraductal LPS administration) was just 3.5-fold in the presence of either cholecystokinin or melatonin administered intraperitoneally. The anti-inflammatory action exerted by cholecystokinin was partially due to the involvement of CCK-A receptors, but not to CCK-B receptors. As judged by the lack of effect of luzindole to attenuate the anti-inflammatory action of melatonin, melatonin did not exert its action by MT2 receptors. Though luzindole also has a certain affinity for MT1 receptors (Dubocovich et al., 1998), it might be too early to classify the anti-inflammatory action of melatonin (with respect to myeloperoxidase activity) as a non-receptor phenomenon.

(8c) Anti-inflammatory actions of non-specific and specific NO-synthase inhibitors

The LPS-induced increase in myeloperoxidase activity was abolished by the non-specific NO-synthase inhibitor L-NAME (VII). Each of the specific inhibitors for inducible NO-synthase, L-NIL, and for neuronal NO-synthase, *N*-PLA, halved the response. The neuronal type of NO-synthase mobilized by LPS was of non-nervous origin. This was confirmed in the glands subjected to combined chronic postganglionic parasympathetic and sympathetic denervation, the marked increase in myeloperoxidase activity in response to LPS was more than halved by *N*-PLA.

Whether sCCK-8 and melatonin exerted their anti-inflammatory action by inhibiting NO-generation in the inflamed parotid gland is unknown and it is presently under study. Melatonin has been shown to inhibit LPS-induced activity of inducible NO-synthase in liver and lungs in rats (Crespo et al., 1999).

GENERAL DISCUSSION

The nervous and hormonal control of the exocrine secretion in the digestive tract is usually divided into separate phases depending on the location of the stimulus: the *cephalic phase* initiated by sight and smell of the food or by food in the mouth, the *gastric phase* by distension, a high pH and peptides and the *intestinal phase* by a low pH, osmolarity, distension, amino acids and fatty acids. The gastric secretion is conveniently divided into those three distinct phases, though it should be realized that they overlap each other except at the start of the food intake. Whereas nervous activity dominates during the cephalic phase, endocrine activity dominates during the intestinal phase (Konturek & Konturek, 2000; Raybould et al., 2003).

The present *Thesis,* using the rat parotid gland as experimental *in vivo* model organ, gives a new perspective to the control of salivary secretion by adding, to the well-established cephalic phase, both a gastric and an intestinal phase as possible sites for the control of salivary gland secretion. Recent observations of ours do also show all three major salivary glands of the rat to be supplied with cholecystokinin A and B receptors and further, by melatonin 2 receptors (J. Ekström, T. Grunditz, H. Çevik Aras, A. Riva & H. Helander, to be published). With respect to the parotid gland, presently in focus, the type A of cholecystokinin receptors was preferentially located to the acini, while the type B was preferentially located to the ducts; the melatonin 2 receptor was more evenly divided between the two locations. Moreover, we found the major salivary glands in humans to be supplied with these types of receptors. Preliminary findings did also show acini of human parotid gland tissue exposed to pentagastrin to induce ultrastructural changes associated with secretory activity and further, to release protein (Loy et al., 2008).

The CCK-A receptor shows a high affinity for cholecystokinin and sulfated cholecystokinin analogues but a low affinity for gastrin and desulfated cholecystokinin analogues. The CCK-B receptors, on the other hand, show about the same affinity for gastrin and for cholecystokinin and its desulfated analogues. In the present study, no attempt was made on *basis of agonist potency* to decide which type, A or B, of cholecystokinin receptors was involved in the responses. The cholecystokinin receptors lorglumide and itriglumide are considered selective for CCK-A and CCK-B receptors, respectively (Makovec et al., 1987; 1999). However, the relative affinities for various antagonists are usually based on findings under *in vitro* conditions and might therefore not be directly applied to the functional *in vivo* situation with respect to potency and selectivity (Ding & Håkanson, 1996). Thus, conclusions should be drawn with caution. The *use of antagonists* in the present study, showed the CCK-A receptor to be involved in the secretion of protein/amylase, while the contribution of the CCK-B receptor to the secretory response was less unambiguous. Both the CCK-A and the CCK-B receptors were involved in the synthesis of protein. The CCK-A receptor is

usually associated with the motoric activity of the gastro-intestinal tract but is also involved in pancreatic secretion, while the CCK-B receptor is associated with acid secretion and gastric mucosal growth. Both receptor types seem to participate in the secretion of pepsinogen (Blandizzi et al., 1999). Though, luzindole is described as a melatonin 2 receptor preferring antagonist, it is too early to exclude a role for melatonin 1 receptor in the methacholine evoked secretion of protein. The melatonin 1 receptor type was expressed in the parotid gland but so far its glandular localization is unknown. Melatonin seems not to occur in peripheral nerves and though, this may be the case for cholecystokinin (Larsson & Rehfeld, 1979) no support for a transmitter role for cholecystokinin was found in the present preparation.

In the present study, the hormone/hormone-analogues mediated their secretion and synthesis of proteins partially by NO generation by the activity of neuronal type NO-synthase. Their direct action on the parotid gland makes it likely that the neuronal type of NO-synthase mobilized was of parenchymal origin rather than of nervous origin (Sayardoust & Ekström, 2003). In the rat parotid gland NO-synthase is present in the parasympathetic innervation but not in the sympathetic innervation (Alm et al., 1995, 1997). However, in parotid gland the amount of fluid, protein output, protein synthesis and mitotic activity upon stimulation of the parasympathetic innervation is independent of NO generation (Sayardoust & Ekström, 2006). Likewise, neither the volume of saliva nor the outputs of protein and amylase in response to methacholine (under control conditions) were presently affected by the non-selective NO-synthase inhibitor or by the neuronal NO-synthase inhibitor. In contrast, the protein output, protein synthesis and mitotic activity in the rat parotid gland in response to β-adrenoceptor activation by sympathetic nerve stimulation or by a β-adrenoceptor agonist are partially dependent on NO-generation due to the activity of neuronal type NO-synthase (Sayardoust & Ekström, 2003, 2004; Ekström et al., 2004). Compelling evidence for the idea of the involvement of neuronal type NO-synthase *of parenchymal origin* is gained in chronically denervated parotids, where β-adrenoceptor agonists (but not muscarinic agonists) cause the secretion of protein that is

partially blocked by a selective neuronal type NO-synthase inhibitor (Sayardoust & Ekström, 2003). Interestingly, NO generation may be dissociated from the secretory response. In parotid acinar cells from the rabbit, NO generation caused by methacholine is completely inhibited by a non-selective NO-synthase inhibitor but the methacholine-induced amylase secretion is not affected (Tsunoda et al., 2003). Moreover, NO is generated during gland inflammation and also here engaging neuronal type of NO-synthase in denervated glands, see below.

The intracellular signalling pathways mobilized by desCCK-8, pentagastrin and melatonin eliciting secretion and synthesis of proteins in the parotid gland are presently unknown. They evoke, however, a "profile" similar to that of β-adrenoceptor agonists and vasoactive intestinal peptide rather than to that of muscarinic agonists, α-adrenoceptor agonists and substance P. The former group, using cAMP intracellularly, produces a small flow of parotid saliva with a high protein concentration, whereas the latter group, using Ca^{2+} intracellularly produces a large flow of saliva with a low protein concentration (Baum & Wellner, 1999). In the present study, a large part of the protein secretion and protein synthesis depended on NO generation by the activity of neuronal type of NO-synthase, which is also a common feature for β-adrenoceptor agonists and vasoactive intestinal peptide (Sayardoust & Ekström, 2003; 2004). In contrast, agonists of muscarinic receptors or α-adrenoceptors do not mobilize NO for protein secretion or protein synthesis (Sayardoust & Ekström, 2003; Ekstöm et al., 2007). The NO non-dependent responses are likely to be associated with cAMP, while the NO/cGMP-signalling system may mobilize calcium and or prolong the action of cAMP due to the inhibition of the enzymatic degradation of cAMP by phosphodiesterases (Imai et al., 1995; Looms et al., 2000; Tritsiaris et al., 2000). In the pancreas, the CCK-A receptor is associated with both the Ca^{2+} and the cAMP pathways to release zymogen granules (Williams, 2001). The intracellular signal-transduction events for CCK-B receptors, mainly studied in nervous tissue, are poorly characterized (Noble & Roques, 1999). This is also the case for the melatonin receptors where the studies are mainly on nervous

tissue. In many cases, melatonin inhibits the mobilization of various messengers, including cAMP, cGMP and Ca^{2+} (Vanecek, 1998).

As would be expected inducible NO-synthase was activated in response to the intraductal injection of lipopolysaccharide in the parotid gland. Though, there seemed to be a role for neuronal type of NO-synthase of non-nervous origin in the inflammatory response, NO-generation by the activity of neuronal type of NO-synthase in response to sympathetic activity or to cholecystokinin/gastrin and melatonin is not likely to induce inflammation. Activation of inflammatory cells and cytokine release are pre-requisites for triggering the inflammatory response (Morris & Billiar, 1994; Cloutier & McDonald, 2003).

No doubt the relatively high doses of the hormone/hormone analogues tested in the anaesthetized animal did not mimic physiological conditions. Under natural conditions, gastrin, cholecystokinin and melatonin are likely to act in concert with the transmitters to regulate the glandular activites. In future experiments, it would be of interest to test the feeding responses of awake animals in the presence of the hormone receptor antagonists. Interestingly, in previous feeding experiments on the rat, a certain loss of glandular amylase activity and increase in protein synthesis occur in parasympathetically denervated parotid glands under muscarinic and α- and β-adrenoceptor blockade (Ekström et al., 1993; Ekström & Reinhold, 2001), implying a role for bloodborne substances such as the hormones (Liddle et al., 1984; Kitano et al., 2000).

Proteomics applied to the saliva (Helmerhost & Oppenheim, 2007; Messana et al., 2008) will most likely reveal the secretion of a number of substances in response to gastrin, cholecystokinin and melatonin and, how these hormones may influence the processing of the secretory products. Stimulation of the salivary glands during the gastric and intestinal phases may add protective and healing substances to the saliva of benefit for the oral structures and (when swallowed) for the esophageus, the stomach and the intestines. This is exemplified by observations of Konturek and co-workers, showing pentagastrin

to increase the salivary levels of epidermal growth factor and, as a result, promoting ulcer healing (Konturek PC, 1996). Moreover, salivary gland products are not only delivered to the lumina but also to the circulation, implying an endocrine role for salivary glands (Isenman, Liebow & Rothman, 1999). Thus, it may be worth to consider the possibility that the hormones, in the present study may influence endocrine secretion from the glands.

There is the possibility that changed blood levels of gastrin, cholecystokinin and melatonin, as consequence of diseases or drug treatments may influence the composition of saliva. Gastrinaemia occur under conditions such as *Helicobacter pylori* infections, gastrinoma (Zollinger-Ellison syndrome) and treatments with protone-pump inhibitors and histamine 2-receptor antagonists (removing the brake on gastrin release). In patients with Zollinger-Ellison syndrome, the output of epidermal growth factor is, in fact, elevated (Sarosiek et al., 2000). On the other hand, reduced blood levels of cholecystokinin have been reported in patients suffering from coeliac disease and diabetes (Berna & Jensen, 2007). Upon melatonin treatment of sleep disorders such as jet-lag and shift-work the blood level of melatonin increases (Bubenik, 2002).

In our inflammation model, sCCK-8 and melatonin were found to be effective anti-inflammatory agents and their effect was reduced (sCCK-8) or unaffected (melatonin) by the receptor antagonists tested as judged by the myeloperoxidase activity. Both melatonin (Rice et al., 1995) and gastrin (Ingenito et al., 1986) appear in the saliva, and the amounts increase during a meal. And while focus has recently been on melatonin as a part of the defense of the oral cavity (Cutando et al., 2007a; Czesnikiewicz-Guzik et al., 2007), a role for cholecystokinin/ gastrin may also be at hand. Saliva contains particularly high concentrations of melatonin, about 25% of that in plasma (Laakso et al., 1990). Melatonin is highly lipophilic and may diffuse into intracellular compartments and exercise effects on nuclear binding sites independent of their effects on cell-membrane receptors (Acuna-Catroviejo et al., 1994; Carlberg & Wiesseberg, 1995). In the oral cavity, melatonin exerts antioxidative, immuno-modulatory and

anti-cancerogenic effects and further, promotes bone formation and growth by stimulating collagen fibers (Cutando et al., 2007a; Czesnikiewicz-Guzik et al., 2007). Salivary melatonin may have important implications in the treatment of periodontal diseases, infections of the oral mucosa, wound healing following oral surgery, oral cancer and, as presently illustrated, gland inflammation (Gomez-Moreno et al., 2007; Cutando et al., 2007a, b).

Decreased volumes of saliva as well as changed saliva composition give rise to dry mouth. The quality of life of people suffering from dry mouth is dramatically impaired. The prevalence of dry mouth is 15-40%, and it is more common among women than men and it increases with age (Nederfors et al., 1997; Ship et al., 2002). A new regulatory view of the functions of salivary glands and hormone-exerted anti-inflammatory actions in the oral cavity may offer new possibities in the treatment of salivary dysfunctions.

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