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ULTRASTRUCTURAL EVIDENCE OF A SECRETORY ROLE FOR MELATONIN IN THE HUMAN PAROTID GLAND

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In vivo animal studies show that pentagastrin, cholecystokinin and melatonin cause the secretion and synthesis of salivary proteins. Melatonin occurs in large amounts in the gut and is released into the blood on food intake. *In vitro* experiments suggest that pentagastrin exerts secretory activity in human salivary glands, as judged by ultrastructural changes, reflecting secretion, and an actual protein output. Currently, it is hypothesised that melatonin induces secretory exocytotic events in the human parotid gland. Human parotid tissues were exposed to a high single concentration of melatonin *in vitro*, processed for high resolution scanning electron microscopy and then assessed morphometrically with the emphasis on the membrane of the intercellular canaliculi, a site of protein secretion. Compared with controls and in terms of density, the melatonin-exposed parotid tissues displayed increases in protrusions (signalling anchored granules) and microbuds (signalling membrane recycling and/or vesicle secretion) and decreases in microvilli (signalling cytoskeletal re-arrangement related to exocytosis), phenomena abolished or very largely reduced by the melatonin receptor blocker, luzindole. In conclusion, acinar serous cells of parotid tissue displayed *in vitro* exocytotic activity to melatonin, signalling protein secretion. Whether, under physiological conditions, melatonin influences the secretion of human parotid glands remains to be explored, however.

Key words: *human parotid gland, melatonin, luzindole, melatonin receptors, gastrointestinal hormones, high resolution scanning electron microscopy, transmission electron microscopy, secretion*

INTRODUCTION

The regulation of the secretion of fluid and proteins from salivary glands is usually thought to be solely the result of nervous activity (1, 2). In recent years, however, some hormones/hormone analogues have been found to cause the exocytosis of acinar granules and to increase the protein content of the saliva without affecting the flow. In the rat, pentagastrin, which has the same biological activity as native gastrin (3), cholecystokinin and melatonin cause the prompt release of acinar amylase from the parotid gland upon intravenous administration (4-6). In pieces of human parotid gland tissue exposed to pentagastrin, exocytotic "dynamic" events have been demonstrated at both light- and electron-microscopic levels, indicating secretory activity, and, in addition, the protein concentration of the tissue incubate has been found to increase (7). Whereas gastrin and cholecystokinin are well-known gastrointestinal hormones, melatonin is usually associated with the pineal gland. Nevertheless, by far the largest source of melatonin in the body is the mucosa of the gastrointestinal tract, where it is synthesised by the enterochromaffin cells (8, 9). Animal experiments show that the melatonin concentration in the peripheral blood, and in the gastrointestinal tract, increases in response to food intake (9).

It is possible that, in human salivary glands, circulating melatonin, released in connection with a meal, acts in concert with autonomic neurotransmitters and some gastrointestinal hormones to influence the salivary response. Considering that usually only a few, small pieces of surgical specimens from a human salivary gland are available for studies and that the number of contributing patients is restricted, we applied our *in vitro* technique to reveal morphological changes reflecting secretion (10-13) and focused on the effect of a high single concentration of melatonin to establish whether, if at all, the secretory cells would respond to melatonin. Our working hypothesis of a secretory effect by melatonin was tested on the acinar serous cells of the parotid gland tissue. Interest focused in particular on the membrane of the intercellular canaliculi, as a large part of protein secretion takes place at this site (11). Here, ultrastructural phenomena reflecting anchored secretory granules (visible as protrusions by scanning electron microscopy), recycling membranes and/or vesicles (visible as microbuds), and cytoskeletal re-arrangements allowing secretory granules to reach the luminal membrane (visible as the collapse of microvilli) are all used as indicators of secretory activity (7, 14). As the results showed that melatonin does in fact induce exocytotic events, we further investigated whether the effect of melatonin was affected by the presence of the melatonin receptor

antagonist, luzindole, previously found to be effective in inhibiting the melatonin-evoked protein secretion in rats (6).

MATERIALS AND METHODS

Fragments of human parotid glands were obtained during orofacial surgery from 11 patients (6 males and 5 females) aged 40 – 73 years. The tissues under study came from non-irradiated glands and appeared normal under light microscopy. The present study conforms with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from each patient and permission was granted by the local ethics committee (ASL 8, Cagliari, Italy). The aspect of any gender or age differences with respect to gland morphology was not taken into account.

Tissue preparation

Samples were immediately cut into 1 mm^3 pieces and put into 10 ml of oxygenated inorganic medium (NaCl 79 mM, KCl 4.9 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1 mM, TRIS 16 mM, D-glucose 5 mM, $NaHCO_3$ 44 mM; 13) kept at 37°C. The bath concentration of melatonin, 1 mM, was chosen from the literature (15, 16) and found to give distinct and reproducible observations. After 15 minutes of exposure to the hormone, the fragments of the glands were removed. To test the effect of luzindole (100 µM), the inhibitor was added to the medium five minutes before melatonin. Specimens incubated in the same medium, and under the same time protocol, but in the absence of the hormone (and luzindole) served as controls.

After stimulation, treated and control specimens were fixed in a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2), for two hours at room temperature, embedded in epoxy resin and treated for transmission electron microscopy (TEM; 17). Semi-thin sections were stained for light microscopy (LM) with toluidine blue and examined in a Leica DMR HC. Ultra-thin sections were observed with a Jeol 100S TEM operated at 80 kV.

For high resolution scanning electron microscopy (HRSEM), some fragments were fixed in a mixture of 0.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 15 minutes at room temperature. After rinsing in a phosphate buffered saline (PBS), specimens were cut into 150 µm slices and subjected to our variant (18, 19) of the OsO_4 maceration method. To view the cytoplasmic side of the membrane, we used a rotating agitator during rinsing to remove all cellular organelles. In order to highlight HRSEM images, slices of tissue were sputtered with platinum (2 nm thickness). Observations were made using a Hitachi S4000 FEG HRSEM operated at 15 – 20 kV.

Drugs

All drugs were purchased from Sigma (St. Louis, MO, USA).

Statistical analysis

To evaluate secretion by morphological changes, in HRSEM images of the cytoplasmic side of intercellular canaliculi, the mean number of microvilli, microbuds and protrusions per μm^2 of membrane (density) was calculated (7, 14). One hundred and fifty photographs were used for calculations and each gland contributed 12 – 15 photos. Statistical differences between experiments were determined by the Kruskal-Wallis test with the Dunn post-test. All the data were expressed as means ± S.E.M. and represented in figures as percentages. The significance level was set at $P < 0.05$. Images were obtained by Quartz PCI (Quartz Imaging, Vancouver, Canada). Measurements and statistical analyses were carried out with Image Tool for Windows (University of Texas Health Science Center in San Antonio), Microsoft Excel 2003 and Graphpad for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

In the specimens serving as controls (*Fig. 1*), by light microscopy (LM) and transmission electron microscopy (TEM), both acinar and canalicular lumina were restricted and the serous cells were full of secretory granules. No profiles of exocytosis were seen.

By LM (*Fig. 1*) and TEM (*Fig. 2*), specimens exposed to melatonin (1 mM) displayed dilated acinar and canalicular lumina. Although they were still well loaded with granules, the serous cells appeared to be less packed with granules after melatonin. Furthermore, the serous cells showed frequently

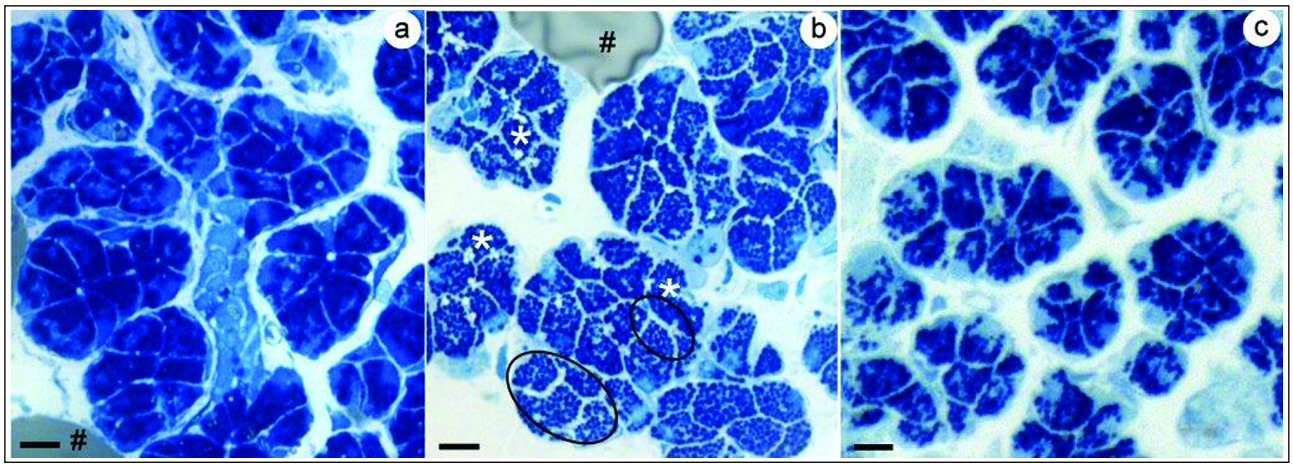

Fig. 1. Light microscope (LM) images. (a) Control parotid tissue, and tissues exposed to (b) just melatonin (1 mM) or (c) melatonin (1 mM) in the presence of luzindole (100 µM). After treatment with just melatonin (b) lumina as well as intercellular canaliculi are dilated, exocytotic profiles are present, and the acinar cells seem less packed with granules. Circle: intercellular canaliculi; Asterisk: exocytotic profiles; (#) adipocytes. Bar: 10 µm.

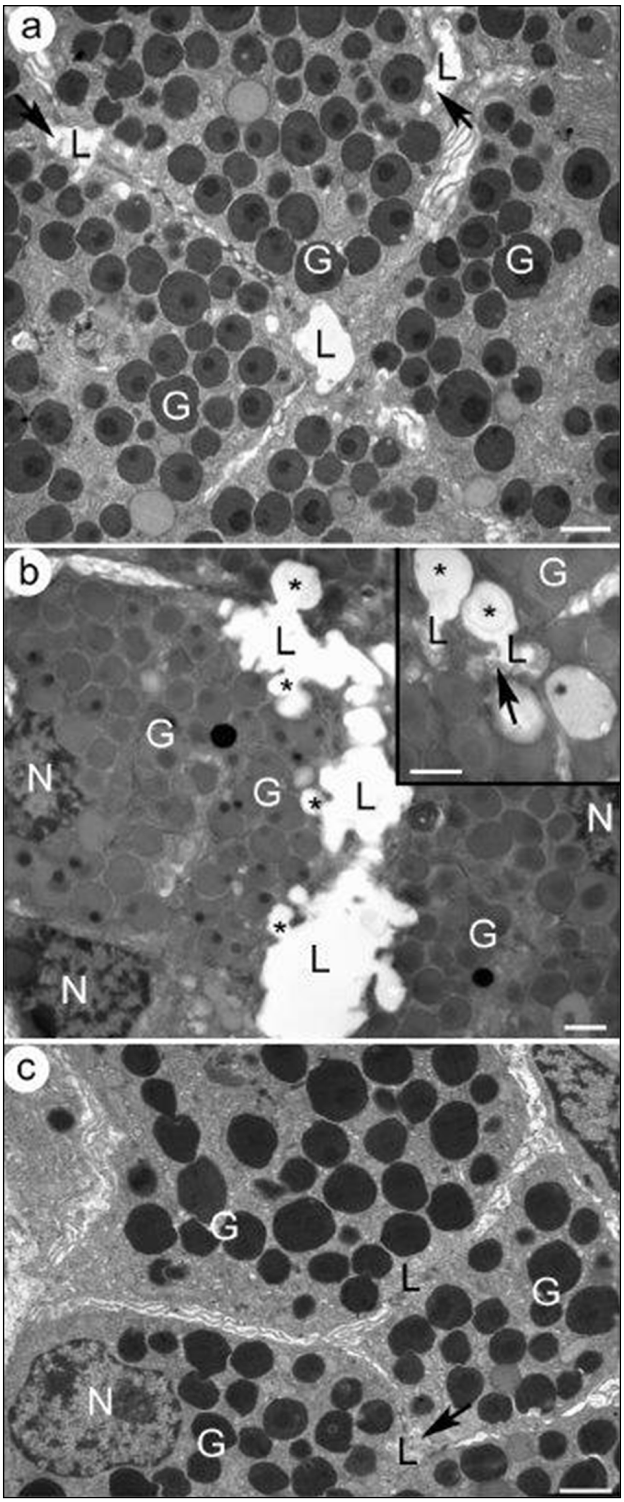

Fig. 2. Transmission electron microscopy (TEM). (a) Control parotid tissue, and tissues exposed to (b) just melatonin (1 mM) or (c) melatonin (1 mM) in the presence of luzindole (100 µM). Lumina are dilated and several exocytotic profiles are visible after just melatonin; inset: transverse-sectioned intercellular canaliculi with exocytotic profiles clearly connected to them. In control tissue as well as in tissue exposed to melatonin + luzindole, no exocytotic profiles of secretory granules are observed. The difference in staining intensity between the granules of the three specimens is ascribed to a difference in staining time. G: secretory granules; L: lumina; N: nuclei. Asterisks: exocytotic profiles. Arrows: microvilli. Bar: 1 µm.

exocytotic profiles which, by TEM, were visible as Ω-shaped profiles connected to the lumen with the size of a single granule.

By high resolution scanning electron microscopy (HRSEM), in the specimens subjected to the melatonin treatment (*Fig. 3*), the canaliculi showed, on the cytoplasmic side of their membrane, large protrusions (related to anchored granules) and some small microbuds (80 – 120 nm thickness, related to vesicles and/or recycling membranes) located among the bases of microvilli, visible as holes. A few microbuds were frequently observed on the protrusions. Morphometric calculations related to the μm^2 of membrane showed that the protrusion density was 446% higher (*Fig. 4*) in the melatonin-exposed specimens than in the controls (0.71 ± 0.08 versus 0.13 ± 0.04, $P < 0.001$). The density of microvilli in the presence of melatonin was 57% lower than that in its absence (11.3 ± 1.2 versus 26.0 ± 1.8, $P < 0.001$). With respect to the density of the microbuds, it was 140% higher in the melatonin-exposed specimens than in the controls (10.8 ± 0.8 versus 4.5 ± 0.6, $P < 0.001$). The response to melatonin in the presence of luzindole, as compared to that in its absence, was markedly diminished ($P < 0.001$, protrusion density 0.28 ± 0.05, microvilli density 21.6 ± 1.3 and microbud density 5.8 ± 0.6) and, in fact, not statistically significantly different from that of control specimens (*Fig. 4*). Moreover, the luminal dilatation was absent and the exocytotic profiles were few in number (*Figs. 1c, 2c, 3d*).

DISCUSSION

The present *in vitro* approach revealed morphological changes in the surgical specimens of human parotid glands exposed to melatonin, which are associated with secretory activity. Both the acinar and the intercellular canalicular lumina were dilated and exocytotic profiles of granules were frequently observed. With the emphasis on the cytoplasmic aspect of the canaliculi membrane, morphometric assessments of the melatonin-exposed parotid tissues, compared with the control specimens, showed a conspicuous increase (446%) in the density of protrusions, reflecting anchored granules ready for secretion by the major regulated pathway (20, 21), a decrease (57%) in the density of microvilli, reflecting the cytoskeletal re-arrangement associated with the major regulated secretory pathway and permitting granules to be translocated to the luminal membrane (13, 22), and an increase (140%) in the density of microbuds, reflecting recycling membranes (related to endocytosis) and/or small micro-exocytotic events associated with constitutive and constitutive-like secretion (20, 21, 23). At the same time, the widening of the lumina may indicate some water secretion, while acinar vacuolisation, associated with a rich fluid secretion like that occurring in response to muscarinic agonists, was lacking (7, 22). The overall response to melatonin is reminiscent of that to the β-adrenoceptor agonist, isoprenaline, and to pentagastrin, agonists which preferentially cause the release of proteins with only little or no fluid secretion (7, 22, 24).

Melatonin exerts both receptor-dependent and receptor-independent actions. The melatonin receptors, MT1 and MT2, which belong to the family of cell membrane G-protein receptors, are localised at various sites in the central nervous system and in numerous, if not all, peripheral tissues. The intracellular signalling systems responding to the activation of the melatonin receptors appear to be tissue specific and may include adenyl cyclase, guanyl cyclase, phospholipases C and A_2 and potassium and calcium channels (25). Receptor-independent phenomena of melatonin are the free radical scavenging action, as well as the mobilisation of anti-oxidative enzymes (26, 27). Human parotid acinar cells show diffuse cytoplasmic immunoreactivity to both MT1 and MT2 receptors, as judged by light microscopy (28-30). By electron

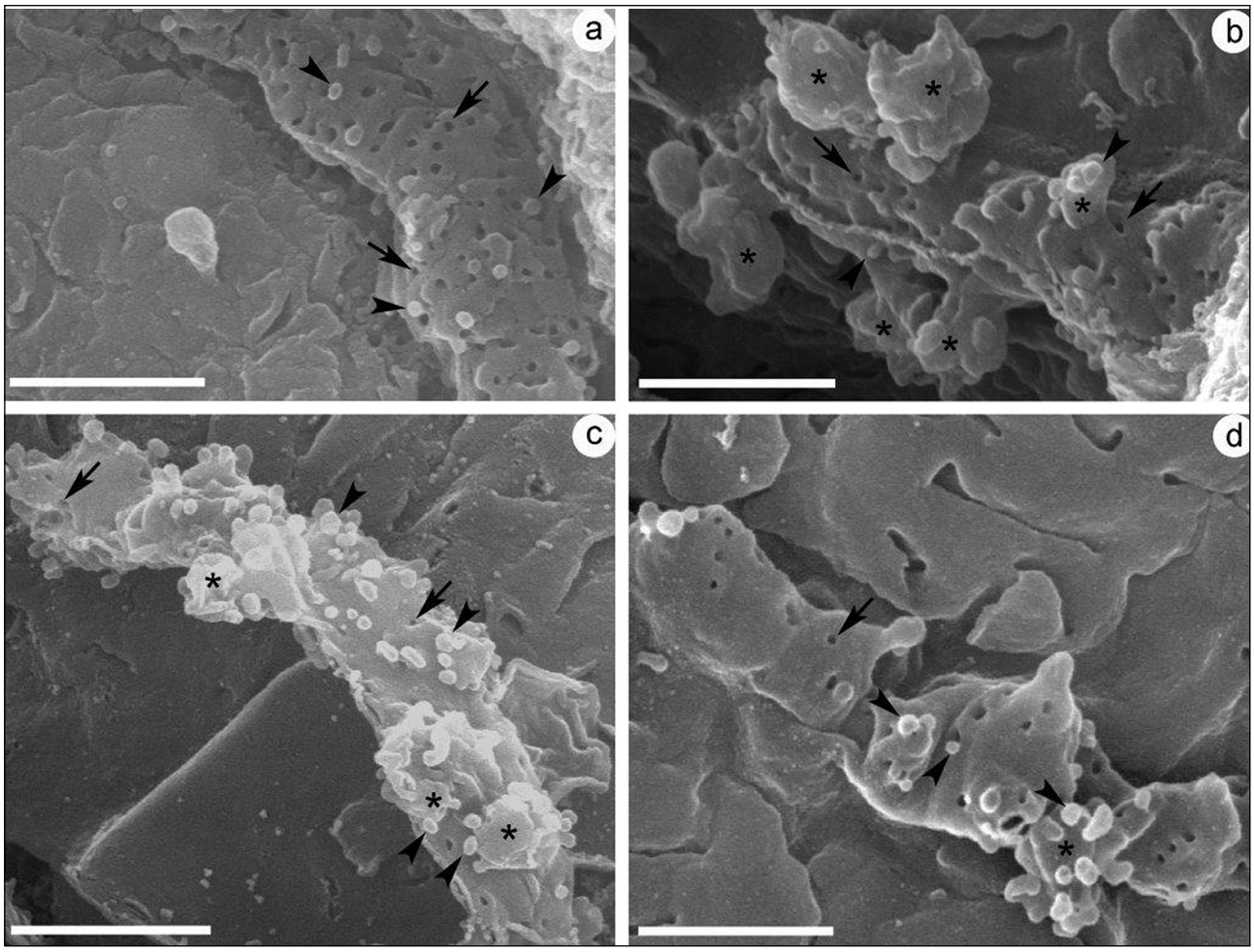

Fig. 3. High resolution scanning electron microscopy (HRSEM) images. (a) Control parotid tissue, and tissues exposed to (b and c) just melatonin (1 mM) or (d) melatonin (1 mM) in the presence of luzindole (100 µM). As seen from the cytoplasmic side of the intercellular canaliculi, just melatonin (b and c) resulted in the appearance of protrusions and microbuds. In control and melatonin + luzindole exposed tissues none or only few protrusions are visible. Microvilli are represented by holes. Arrows: microvilli. Arrowheads: microbuds. Asterisks: protrusions. Bar: 1 µm.

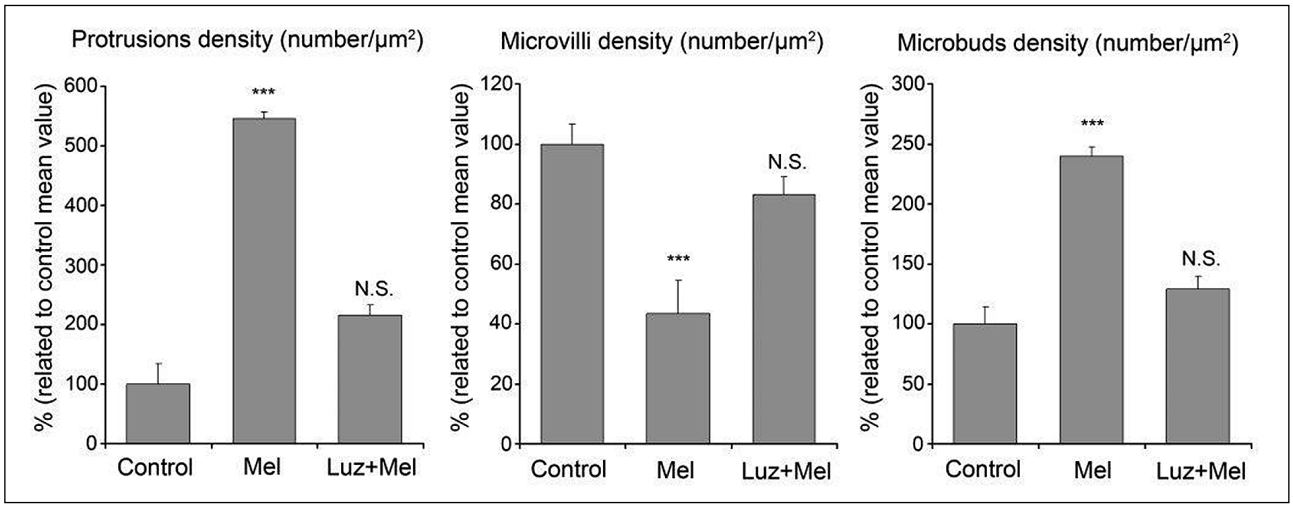

Fig. 4. Percentage comparisons. Effects of 1 mM of melatonin (Mel) or 1 mM of melatonin combined with 100 µM of luzindole (Luz + Mel) on the density of microvilli, microbuds and protrusions. Mean density of the control values was set at 100%. The columns represent means and bars + S.E.M. *** $P<0.001$, statistically significant with respect to control. N.S.: not significant compared with control.

microscopy, using the immunogold staining technique, the distribution of the two subtypes of receptor was localised in both the cell membranes and the subcellular structures. In comparison with the MT1 receptors, the reactivity to the MT2 receptors was

weaker and less frequently observed (30). Evidently, the exocytotic events presently observed involved melatonin receptors, as judged by using luzindole. Although luzindole is usually regarded as an MT2-receptor blocker, it also displays a certain affinity for the MT1 receptor (31, 32). For this reason, the possibility cannot be excluded that the present exocytotic events also involved the stimulation of the MT1 receptors to some extent. Albeit not of statistical significance, the figures for the protrusion density of the specimens exposed to melatonin combined with luzindole were higher than the corresponding figures for the control specimens, which may be related to the concentration with respect to agonist and antagonist, as discussed in some other *in vitro* preparations testing the two substances (16, 33, 34). It should, however, be mentioned that receptor-independent effects are reported in response to high doses of melatonin, mainly in connection with its anti-oxidant activity (35, 36). The anti-inflammatory action of melatonin demonstrated in the rat parotid gland may be one such example (37).

Interestingly, melatonin at low concentrations inhibits the *in vitro* release of vasopressin from the rat hypothalamo-neurohypophysial system by acting on MT1 receptors. In contrast, a high pharmacological concentration of melatonin stimulates the release of vasopressin through mechanisms independent of MT1 and MT2 receptors, since the secretory response to melatonin is unaffected by luzindole or the MT2 receptor selective antagonist 4-P-PDOT (38). The fact that, in the current *in vitro* preparation, luzindole markedly affected the melatonin-evoked response makes it likely that melatonin would also act directly on the melatonin receptors of salivary secretory cells in humans *in vivo*. In the rat parotid gland, melatonin causes the *in vivo* secretion of amylase/proteins, most probably by direct action on the melatonin receptors of the acinar cells (6). In contrast, melatonin evokes duodenal bicarbonate secretion (39) and pancreatic amylase secretion (40) indirectly, by acting *via* enteric nerves (bicarbonate secretion) or vago-vagal reflexes (amylase secretion).

Large variations in the *in vivo* concentration of melatonin can be anticipated at the receptor sites between various effector organs (36, 41). Although the present *in vitro* concentration of melatonin - the same as in some other *in vitro* studies (15, 16) - provided reproducible data, indicating a potential role for melatonin in the protein secretion of the acinar cells of the human parotid gland, it was much higher than the concentration that can be anticipated in physiological conditions (42). Consequently, it is not possible to determine from the present experiment whether melatonin within physiological concentrations would affect salivary protein secretion. In physiological conditions, melatonin is likely to interact positively with both traditional and non-traditional autonomic transmitters, as well as with gastrointestinal hormones, like gastrin and cholecystokinin, to achieve the most purposeful glandular response. For this reason, the blood level of melatonin needed to contribute to glandular secretory activity can be expected to be lower than the blood level of melatonin necessary to elicit the corresponding response when acting alone. Furthermore, it is important to consider the influence of the circadian rhythm of blood melatonin mainly associated with pineal gland secretion (41), with low blood levels of melatonin during the day and about ten times higher blood levels of melatonin during the night, with a plateau at 02 – 05 h (e.g. 42), combined with the fact that food intake in humans usually occurs during the day in contrast to nocturnal laboratory animals like rodents. In addition, as it has been suggested that a melatonin synthesis occurs in salivary duct cells (43), a paracrine secretion of melatonin that acts on the acinar cells, as suggested by Acuna-Castroviejo *et al.* (36), may have to be taken into account when considering the physiological concentration of melatonin required to contribute to salivary secretory phenomena.

In salivary glands, exocytosis and protein secretion with little or no accompanying fluid secretion, like that in response to isoprenaline and vasoactive intestinal peptide, for example, is associated with the mobilisation of the intracellular cyclic AMP pathway (44, 45). Protein secretion, in response to these agonists, as well as to pentagastrin and melatonin, is partly dependent on the generation of nitric oxide by the activity of NO synthase of the neuronal type but of parenchymal origin, as shown in the rat parotid gland (5, 6, 46). An intracellular interaction between the NO/cyclic GMP and cyclic AMP pathways has been suggested (47). The intracellular pathways that are mobilised in the acinar cells of salivary glands in response to gastrointestinal hormones/hormone analogues, such as melatonin and pentagastrin, remain to be elucidated.

Based on previous *in vivo* studies of the rat parotid gland (4-6), it has been suggested that the secretion of saliva in response to food intake is regulated, as is the case for many digestive glands, not only by nerves but also by hormones and, from various locations, by the traditional cephalic phase and, in addition, by a gastric phase (gastrin) and an intestinal phase (melatonin and also cholecystokinin acting, like gastrin, *via* cholecystokinin-A receptors). The currently demonstrated exocytosis in the human parotid acinar cells, combined with the previously demonstrated pentagastrin-evoked exocytosis and protein secretion in this type of human gland tissue (7), is in agreement with an idea of this kind. In addition, the presence of melatonin receptors in the duct cells of human salivary glands may suggest a regulatory role for melatonin in electrolyte secretion, as pointed out by Arias-Santiago *et al.* (28). In analogy with data obtained on the rat parotid gland, the three hormones are, moreover, potential contributors to the synthesis of salivary secretory proteins in human salivary glands (48, 49). In future experiments, proteomics applied to the saliva or to the gland tissue incubate will probably reveal how hormones, including melatonin, by their actions, influence the protein/peptide profile of the saliva (50). It would, for example, be of interest to investigate whether, in any way, the protein composition of the salivary secretion follows the circadian rhythm of melatonin or whether it is influenced by the intake of melatonin as medication (51).

Studying the impact of gastrointestinal hormones on salivary gland functions may offer new approaches to the treatment of salivary gland dysfunctions and dry mouth. In addition, *via* its melatonin receptors, the salivary gland may actively take up, transport and store circulating melatonin in the secretory granules (30, Isola M, unpublished observations). The elevated blood levels of melatonin during the night may hypothetically serve to load the salivary glands, as has been suggested for the intestinal tract (52). Once secreted into the oral cavity, melatonin may exert a number of beneficial effects on oral health (53). Interestingly, both L-tryptophan as a source of endogenous melatonin and exogenous melatonin have been found to exert oesophagoprotective effects in an animal model focusing on non-erosive oesophageal lesions (54).

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