Melatonin occurs in large amounts in the intestinal mucosa and is released during a meal. Recent studies of ours reveal that exogenous melatonin evokes the in vivo secretion of protein and amylase from the rat parotid gland. The aim of the present study was to investigate the effect of melatonin on the protein synthesis of the parotid gland of pentobarbitone-anaesthetised rats as estimated by the rate of incorporation of [3H]leucine into trichloroacetic acid-insoluble material of the gland. Compared with the parotid protein synthesis (set at 100%) of those rats exposed to an intravenous infusion of melatonin (25 mg/kg during 1 hour), under muscarinic and α- and β-adrenoceptor blockade, the synthesis in the corresponding glands of saline-treated control rats was less (by 25%). The synthesis was also less when the melatonin administration was combined with the melatonin 2-prefering receptor antagonist luzindole (24%), the non-selective nitric oxide synthase inhibitor L-NAME (18%) and the neuronal nitric oxide synthase inhibitor N-PLA (21%). Almost all the melatonin receptor-mediated effect was due to nitric oxide generation via the activity of neuronal type nitric oxide synthase. The present findings lend further weight to the idea that salivary glandular activity associated with food intake is hormonally influenced and they also suggest clinical implications for melatonin in the treatment of xerostomia. Since melatonin is known to exert anti-inflammatory actions in the oral cavity, the stimulatory effect of melatonin may include the synthesis of proteins of importance for the oral defence.

Key words: luzindole, melatonin, salivary glands, nitric oxide synthase inhibitors, parotid gland, protein synthesis, pineal gland

INTRODUCTION

The secretory activity of salivary glands is usually thought to be solely under nervous control (1, 2). However, recent experiments reveal that the intravenous administration of cholecystokinin and pentagastrin induces both the secretion and synthesis of protein in the parotid gland of the rat through a direct action on the cholecystokinin receptors of the acinar cells (3, 4). Melatonin is not only a pineal hormone, it is also a gastrointestinal hormone. It occurs in large amounts in the intestinal tract, where it is synthesised by the enterochromaffin cells of the mucosa (5).

In response to food intake, the concentration of melatonin increases in the gastrointestinal tract, as well as in the peripheral blood (6), and, upon administration, it causes the secretion of duodenal bicarbonate (7) and pancreatic amylase (8) partly or entirely indirectly via nervous activity. Melatonin also causes the in vivo secretion of protein and amylase from the parotid gland of the rat (9). The secretory effect of melatonin is dose-dependent, it is probably due to a direct action on the acinar cells, is partly dependent on nitric oxide (NO) generation and is abolished by the melatonin 2-receptor-prefering antagonist luzindole (9).

In analogy with the action of the cholecystokinin-receptor agonists, it was hypothesised that melatonin also stimulates the synthesis of salivary secretory proteins. As previously (9), the parotid gland of the rat was used as an experimental model and the rate of incorporation of [3H]leucine into trichloroacetic acid-insoluble material was used to indicate protein synthesis.

MATERIALS AND METHODS

Animals and experimental surgery

This study was based on the results from 64 adult female Sprague-Dawley rats (Charles River, Sulzfeld, Germany). The animals were accustomed to the animal house over a period of 3–4 weeks. They were housed at a constant temperature (21°C) with light-dark cycles of 12 hr (lights on at 6 am and off at 6 pm) and were maintained on a pelleted standard diet and tap water ad libitum. They were fasted overnight and the experiments started in the morning. All the protocols were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and with local ethics committee guidelines.

In each study group, two rats were paired, based on age and body weight, before the experiment began, one to serve as an "experimental animal" and the other to serve as a "control animal". The pairs were treated concomitantly and in the same way, but there was a difference in the type of infusion and pretreatment of drugs when appropriate. Furthermore, the glands removed from each pair of animals were processed and analysed at the same time. The study groups were: (I) melatonin (mean body weight ±S.E.M., 237±12 g; n - number of observations, n=8) and saline (238±7 g, n=8); (II) melatonin (281±16 g, n=5) and melatonin + luzindole (267±9 g, n=5); (III) melatonin (253±16 g, n=5) and melatonin + L-NAME...
(255±6 g, n=5); (IV) melatonin (229±10 g, n=4) and melatonin + N-PLA (229±12 g, n=4); (V) saline (235±12 g, n=5) and saline + luzindole (245±16 g, n=5); and (VI) saline (251±2 g, n=5) and saline + L-NAME (249±7 g, n=5). Between the paired groups of rats in each study group, there were no statistically significant differences with respect to body weights. The rats in study group VI were also used in an earlier study of ours (4).

The animals were anaesthetised with pentobarbitone (50 mg/kg, i.p.). The anaesthetised animals were placed on a thermostatically controlled blanket with a rectal probe fitted, keeping the body temperature at 38°C. After cannulation of the trachea, a venous conduit was provided by a polyethylene catheter fitted into the femoral vein on both sides, one for the injection of drugs and radiolabelled leucine and one for the continuous infusion of melatonin or saline. All the animals were pretreated with phentolamine, propranolol and atropine (1 mg/kg, i.v., of each, to avoid any influence on the α- and β-adrenoceptors, as well as on the muscarinic receptors) about 10 min before the infusion of melatonin or saline and, once again, 40 min after the start of the infusion period. When appropriate, the melatonin receptor antagonist luzindole (2 mg/kg, i.v.), the non-selective NO-synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 30 mg/kg) or the highly selective inhibitor of neuronal type NO-synthase, Nω-propyl-L-arginine (N-PLA, 30 mg/kg), was administered intravenously (10-12), 5 min before the start of the infusion of melatonin or saline. Melatonin (25 mg/kg, dissolved in saline) was infused intravenously in a fixed volume of 0.8 ml for 1 hour (as was also the case for just saline). Thus, for a rat weighing 250 g, the melatonin concentration of the intravenous fluid was 7.8 mg per ml. The choice of drug doses was based on a previous study (9). After the infusion period, there was a resting period of thirty minutes and [3H]leucine (500 µCi/kg in 0.5 ml saline), from Amersham Biosciences (Uppsala, Sweden), was then injected intravenously. Fifteen minutes later, the abdomen was opened, the aorta cut and the animal exsanguinated. The parotid glands on both sides were rapidly removed, washed in saline, pressed gently 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.

Processing of tissues

Each gland was treated separately. After thawing, the glands were homogenised in 1 ml of cold 5 mM NaOH of the supernatant; two aliquots of 250 µl were used. The samples were diluted to 1 ml and, to 100 µl of this solution, 500 µl of 5% trichloroacetic acid was added to precipitate the gland homogenate and the mixture was centrifuged at 3000 g for 5 min. This procedure was repeated twice; at the last centrifugation, the supernatant was virtually devoid of radioactivity. To the final precipitate, 500 µl of Soluene 100 (Perkin Elmer, Boston, MA, USA) was added and left over night; the blank consisted of 500 µl of Soluene 100. Then 8 ml of Optiphase HiSafe 2 (Fisons Chemicals, Leicester, UK) was added and the mixture was analysed in a scintillation counter (LKB Wallac, Perkin Elmer, Wellesley, MA, USA). The tests were conducted in duplicate. The amount of radiolabelled leucine incorporated into the trichloroacetic acid-insoluble material of the parotid gland was expressed as disintegrations per minute per milligram of gland tissue (dis/min per mg gland).

Fifteen minutes after an intravenous bolus injection of radiolabelled leucine, all radioactivity of the serum is confined to the trichloroacetate-soluble fraction (13). Labelled leucine in blood or serum is, however, unlikely to have contaminated the gland homogenates to any degree of importance to the outcome of the present study. The rats were exsanguinated, the glands were washed and excess fluid was removed on a filter paper. Moreover, following the precipitation of the gland homogenate, the final supernatant was virtually devoid of radioactivity. The radiolabelled leucine-incorporation technique is a convenient and well-established way of studying protein synthesis. Even if it does not affect the interpretation of the results of the present study, it is, however, less accurate than the "flooding dose" technique for calculations of the rate of protein synthesis (14).

Drugs

The melatonin, atropine sulphate, luzindole, L-NAME and propranolol hydrochloride came from Sigma Chemicals (St Louis, MO, USA). The phentolamine mesylate was obtained from Novartis Pharma AG (Basel, Switzerland). The N-PLA was purchased from Tocris (Bristol, UK). The pentobarbitone was obtained from Apoteksbolaget, Stockholm, Sweden.

Statistics

For each rat, a mean value of dis/min per mg gland was calculated for left and right glands and it was used for statistical analyses. The statistical significance of difference was calculated using Student's paired t-test and, with respect to dis/min per mg gland, comparisons were based on log values. Probabilities of less than 5% were considered significant. The values presented are means ± S.E.M.

RESULTS

With respect to gland weights, there were no statistically significant differences between the paired groups of rats in each study group (see legends of Figs. 1 and 2).

Effect of melatonin

In the rats exposed to melatonin (25 mg/kg i.v., infused over a period of 1 hour), the rate of incorporation of [3H]leucine into the trichloroacetic acid-insoluble material of the parotid glands (set at 100%) was higher than in the corresponding glands of saline-treated control rats, the difference being 25%, (Fig. 1).

Effect of luzindole on the melatonin-induced response

The rate of parotid protein synthesis was 24% lower in the presence of the melatonin 2 type-prefering receptor antagonist luzindole (2 mg/kg, i.v.) than in its absence (Fig. 1). Luzindole did not affect the protein synthesis in those rats subjected to the saline infusion (Fig. 2).

Effect of the non-selective nitric oxide-synthase blocker L-NAME on the melatonin-induced response

In the presence of L-NAME (30 mg/kg, i.v.), the protein synthesis was 18% lower than in its absence (Fig. 1). L-NAME had no effect on those rats solely subjected to saline infusion (Fig. 2).

Effect of the neuronal type nitric oxide-synthase blocker N-PLA on the melatonin-induced response

The protein synthesis was 21% lower in the presence of N-PLA (30 mg/kg, i.v.) than in its absence (Fig. 1).
The main part of the protein synthesis of the rat parotid gland is directed towards producing secretory products and the rapid incorporation of radiolabelled leucine into the trichloroacetic-acid insoluble material of the gland is thought to reflect this synthesis (13). In salivary glands, the increased rate of protein synthesis is due to a direct effect of the agonist, temporally dissociated from and independent of the agonist-evoked secretory activity (15).

The present study provides further evidence in favour of the idea that melatonin affects parotid glandular activity. The rate of protein synthesis increased in the gland in response to melatonin and the effect was mediated by melatonin receptors. Although luzindole displays a higher affinity for melatonin 2 receptors than for melatonin 1 receptors, the involvement of melatonin 1 receptors cannot be excluded, as luzindole is not a selective melatonin 2 receptor blocker (16, 17). Both types of melatonin receptor occur in parotid gland tissue, as shown by immunoblotting (9). Further, human parotid gland tissue displays ultrastructural changes reflecting secretory activity upon exposure to melatonin 

The increase in protein synthesis in response to melatonin in the present study (by 35% compared with the saline-treated rats) was higher than that reported in response to the infusion of pentagastrin acting on cholecystokinin receptors (17%), an increase which was entirely NO dependent due to neuronal type NO-synthase activity. However, in addition, the endogenous
stimulation of the cholecystokinin receptors helps to maintain the "basal" protein synthesis (by 20%), which is independent of NO generation (4). The activation of β-adrenoceptors by the stimulation of the sympathetic innervation or by isoprenaline infusion may increase the rate of protein synthesis in the parotid gland 2- to 3-fold compared with the basal synthesis, the major part of the increase being NO dependent (20, 21).

The melatonin release may display a circadian rhythm in certain animals including the rat, as it is high during the night and low during the day (6). Even if the pineal gland is usually said to be responsible for the nocturnal surge of blood melatonin, pinealectomised rats still display elevated blood levels of melatonin during the night, albeit to a lesser degree (22). The persisting elevated blood levels of melatonin during the night, as well as the levels during the daytime, are attributed to the release from the gastrointestinal tract (5). In the present study, the experiments were performed in the daytime and the animals had been fasted over night. Evidently, the amount of circulating endogenous melatonin was not sufficiently high to affect the protein synthesis in those rats subjected to only saline infusion, as luzindole did not lower the on-going rate of "basal" protein synthesis of the gland.

Over the years, a great deal of attention has been paid to the cephalic (nervous) phase of salivary gland regulation, elicited by food in the mouth. Recent studies of ours (3, 4) emphasise the stomach (gastrin) and the intestines (cholecystokinin) as additional locations for the origin of regulatory mechanisms of salivary secretory activity. Melatonin of intestinal origin may therefore be a further example of the intestinal influence on salivary glands. The blood levels of not only gastrin and cholecystokinin but also melatonin increase in response to a meal (6, 23, 24). High nocturnal melatonin levels over a period in which the main food intake of the rat takes place may thus not only influence the composition of the saliva but also contribute to the postprandial re-synthesis of salivary proteins. In pigs, a 5-fold increase occurs in response to food intake (25). In humans, elevated levels of melatonin are demonstrated in the saliva after supper (26).

Melatonin may have a number of clinical implications for improving oral health. Since it influences the protein composition of the saliva (9) and stimulates salivary gland protein synthesis, it may be of importance for the treatment of xerostomia. Further medicinal qualities may be found in the treatment of caries, periodontitis, oral mucosal infections, xerostomia. Further medicinal qualities may be found in the treatment of caries, periodontitis, oral mucosal infections, xerostomia. Further medicinal qualities may be found in the treatment of caries, periodontitis, oral mucosal infections, xerostomia. Further medicinal qualities may be found in the treatment of caries, periodontitis, oral mucosal infections, xerostomia. Further medicinal qualities may be found in the treatment of caries, periodontitis, oral mucosal infections, xerostomia.

In the future, analyses of gland and saliva proteins with mass spectrometry will reveal the effect on the synthesis and secretion of individual proteins in response to various agonists, including the gastrointestinal hormones (33).

The dose of melatonin used was of the same magnitude as in some other studies (e.g. 34, 35), although the route and time period of administration may vary (intravenous bolus injection or local application as in the duodenum). Exogenous melatonin is rapidly and extensively metabolised in the liver, accumulated by certain tissues, such as the gastrointestinal tract, and also excreted via the bile and the saliva (5, 27). As a result, there is no simple linear relationship between the infused dose of melatonin and the concentration of the drug in the blood or in various tissues (36). In rats, the nocturnal levels of melatonin in the blood are 10 times higher than those in the daytime (36). Interestingly, an intraperitoneal bolus dose of 25 mg/kg applied to rats during the daytime increases the blood concentration of melatonin 25-fold (34). It might be expected that the continuous intravenous infusion of such a dose over a period of one hour, as in the present study, would result in less elevated blood levels of melatonin (36). Nevertheless, the dose-regimen presently applied probably exposed the parotid gland cells to supraphysiological blood concentrations of melatonin. The protocol used provided, however, reproducible data, indicating a potential role for melatonin in the regulation of glandular activities. Under physiological conditions, melatonin most likely interacts synergistically with a number of autonomic transmitters and hormones (e.g. cholecystokinin and gastrin) to attain the most purposeful gland response. In such cases, the blood level of melatonin required to contribute to the physiological response can be expected to be lower than that required to evoke a response on its own. Consequently, what should be categorised as physiological or pharmacological dose levels of melatonin is not easily defined (37).

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