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MODULATION OF PANCREATIC ENZYME SECRETION BY MELATONIN AND ITS PRECURSOR; L-TRYPTOPHAN. ROLE OF CCK AND AFFERENT NERVES.

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Melatonin, a pineal hormone, is also produced in the gastrointestinal tract. Melatonin receptors have been detected in the stomach, intestine and pancreas. This indole inhibits insulin secretion but its role in the physiological modulation of exocrine pancreatic function is yet unknown. The aim of this study was to evaluate the pancreatic secretory effect of melatonin and its precursor; L-tryptophan given intraduodenally (i.d.) to the conscious rats with intact or capsaicin deactivated sensory nerves. CCK_1 receptor antagonist; tarazepide, was used in the part of the study to determine the involvement of CCK in the secretory effects of melatonin. The secretory studies were performed on awaken rats surgically equipped with silicone catheters, one of them was inserted into pancreato-biliary duct, the other one - into duodenum. Melatonin (1, 5 or 25 mg/kg) or L-tryptophan (10, 50 or 250 mg/kg) were administered i.d. Samples of pancreatic juice were collected in 15 minutes aliquots. Tarazepide (2,5 mg/kg i.p.) was given to the rats 15 min prior to the administration of melatonin or L-tryptophan. Neurotoxic dose of capsaicin (100 mg/kg s.c.) was used to deactivate afferent nerves and thus to assess the role of these nerves in the melatonin-induced pancreatic enzyme secretion. Administration of melatonin (1, 5 or 25 mg/kg i.d.) or L-tryptophan (10, 50 or 250 mg/kg i.d.) significantly increased pancreatic amylase outputs. Deactivation of sensory nerves by capsaicin or administration of CCK_1 - receptor antagonist; tarazepide, reversed the stimulatory effects of melatonin or L-tryptophan on pancreatic secretory function. Administration of melatonin or its amino-acid precursor to the rats resulted in the significant and dose-dependent rises of melatonin and CCK plasma levels. We conclude that melatonin or its precursor; L-tryptophan stimulates pancreatic enzyme secretion via stimulation of CCK release and activation of duodeno-pancreatic reflexes.

Key words: *melatonin, L-tryptophan, pancreatic enzyme secretion, tarazepide, sensory nerves*

INTRODUCTION

Melatonin, a pineal hormone, is synthethsized from L-tryptophan through a four steps reaction (1-2). Apart from the pineal gland, melatonin is produced also in retina, Harderian gland, cilliary bodies and in enterochromaffin (EC) cells of gastrointestinal tract (g.i. tract) (3-5). The synthesis and secretion of this indoleamine in pineal gland is tightly related to the dark phase of the diurnal light/dark cycle (3, 6) but it is not clear whether g.i. tract originated hormone also shows diurnal rhythm.

The melatonin effects are mediated through the specific high-affinity receptors localized on plasma membrane of target cells (7). Melatonin receptors have been detected in the central nervous system; in the suprachiasmatic *nucleus, pars tuberalis*, in the cerebral and cerebellar cortex and in the g.i. system; namely in the gut or in the pancreatic β-cells (8-10). Recently, nuclear RZR/ROR receptors for melatonin have also been discovered but their physiological significance is unknown (11). Previous data suggest that melatonin scavenges reactive oxygen species (ROS) and prevents their noxious effects in rat pancreatic cells *in vitro* by direct detoxification of hydroxyl radicals (12). Melatonin has been also shown to prevent pancreatic β-cells injury induced by streptozotocin, a compound inducing ROS accumulation in the pancreas (13). It has been reported that this indoleamine reduces glucose-induced secretion of insulin, increases blood glucose level and decreased glucose tolerance (14-15). In addition, it was documented that endocrine function of pancreas (insulin secretion) depends on the rhythmic production of melatonin and is modified by the pinealectomy (16). Interestingly melatonin, originating from intestinal enterochromaffin cells, mediates vagal and sympathetic neural stimulation of the duodenal mucus/HCO^- secretion in response to duodenal acid load (17). Exogenous melatonin as well as this produced endogenously from L-tryptophan, attenuates pancreatic damage induced by caerulein overstimulation or ischemia-reperfusion and this has been attributed to the reduction in lipid peroxidation and reduction in pro-inflammatory cytokine production with concomittent increase of anti-inflammatory cytokines (18).

Pancreatic secretion is regulated by a complex neurohormonal system involving both the central nervous system, the afferent (sensory) and efferent (motor) vagus and sympathetic nerves and enteric nervous system operating in the stomach, the duodenum and in the pancreas (19-22). Cholecystokinin (CCK) that is released from duodenal mucosa I cells stimulates pancreatic secretion by activation of CCK_1 receptors and activation of entero-pancreatic neural reflex (20).

Recent study has shown that melatonin or its precursor; L-tryptophan, stimulates pancreatic enzyme secretion, when given parenterally to the rats (23). Above pancreatic secretory effects of melatonin or L-tryptophan are indirect and probably depend on the CCK release and activation of nervous reflex mechanisms caused by these substances (23). However it has not been documented whether melatonin is able to affect pancreatic enzyme secretion when applied into gastrointestinal lumen.

The aim of this study was: a) to investigate the influence of melatonin or its precursor; L-tryptophan given intaduodenally (i.d.) on pancreatic amylase secretion in the anaesthetized rats with pancreato-biliary fistulas and; b) to assess the involvement of CCK and sensory nerves in the pancreatic secretion modulated by melatonin or L-tryptophan.

MATERIAL AND METHODS

Animals and drugs

Studies were performed on male Wistar rats weighing 300-320 g. Animals were housed in cages under standard conditions, on commercial pellet chow, at room temperature with a 12-hr light: 12-hr dark cycle. Rats were deprived at food 18 h prior to the start of experiment, while drinking water was available *ad libitum*.

The following items were purchased: melatonin, L-tryptophan and capsaicin, were from Sigma Co (St. Louis, MO, USA). Specific CCK-A receptor antagonist; tarazepide was the gifts from Solvay Co (Solvay, Hannover, Germany), and Merck, Scharp and Dohme Labs (West Point, PA, USA). CCK and melatonin radioimmunoassay commercial kits were from DRG International Inc. (Mountainside, WI, USA). Amylase reagent was purchased from Dialab Diagnostic Ges. MBH, (Wien, Austria). Morbital (Pentobarbitalum) was from BIOWET (Puławy, Poland). PE10 and PE50 polyethylene tubing was purchased from Beckton Dickinson (Sparks, MD, USA).

Experimental protocol

All tests started at the same time in the morning. Before the experiment the rats were anaesthetized with Morbital, given i.p. at a dose of 15 mg/kg. Through an upper midline laparotomy, the duodenum was identified and the bile-pancreatic duct was isolated as its entrances to the duodenum. A polyethylene cannula (PE10) was inserted into the common bile-pancreatic duct and was secured with a fine suture. Through this tube the bile-pancreatic juice was continuously collected. A second polyethylene tube (PE10) was placed into the duodenum and its tip secured proximal to the ampulla for reinfusion of previously harvested bile-pancreatic juice (after dilution with saline 1:2) and for application an investigated substances (melatonin, L-tryptophan or tarazepide). The abdominal incision was sutured with a double layer suture and the rats were kept under the heating lamps to maintain the right body temperature (37 °C). At the end of selected tests, the abdominal *vena cava* was exposed and the blood was withdrawn into EDTA containing tubes for determination of plasma melatonin or CCK by specific radioimmunoassay.

Rats with pancreato-biliary fistulas were placed in individual Bollmann cages for the duration of the test. The bile-pancreatic juice (BPJ) samples were collected in small preweighted vials in 15 min aliquots to measure the volume and amylase concentration of each sample. At the start of each test basal secretion was collected for 60 min to allow for stabilization of flow affected by surgical manipulation. Amylase concentration in each sample was measured by enzymatic method, as previously described (21). The results were expressed as total amylase output (IU per 15 min). The duodenal cannula was continously perfused at a rate of 1 ml/h throughout the experiment using 1:2 diluted pancreatic juice (obtained from previous experiments and stored at -20°C). Such duodenal perfusion was required to compensate for the loss of water and electrocytes during experiments and for the maintenance of feedback control exerted on pancreatic secretion by pancreatic proteases in duodenum.

Determination of melatonin and CCK plasma level:

Plasma melatonin or CCK concentrations were measured by radioimmunoassay (RIA) using rat melatonin and CCK radioimmunoassay commercial kits, according to the manufacturer's protocol. The melatonin assay detected melatonin in saliva, serum or plasma and the intra-assay and inter-assay shift were 31.78-50.61 and 29.9-75.2 pg/ml respectively. The lowest detectable level of melatonin that can be distinguished from the zero standard is for plasma < 3.5 pg/ml as read from the standard curve. CCK radioimmunoassay commercial kit detected CCK in plasma or tissue extracts. CCK is extracted from plasma by an ethanol extraction method. The lowest detectable concentration is 0.3 pmol/l. The intra-assay and inter-assay shift were 4.4-20.6 and 4.2-20.6 pmol/L respectively.

The experimental protocol has been approved by the Jagiellonian University Ethical Committee for Animal Experimentation.

The study included three series of experiments (A, B, C).

In series A, the groups of rats with intact sensory were used. Series B included groups of rats with sensory nerves deactivated with capsaicin. In series C; CCK_1 receptor antagonist; tarazepide, was used to determine the involvement of CCK in secretory effects of melatonin or L-tryptophan on exocrine pancreas. Each experimental group of rats consisted of 6-8 fasted animals.

Series A. The effects of intraduodenal (i.d.) administration of melatonin or L-tryptophan on pancreatic secretion.

For this series melatonin was dissolved first in ethanol to obtain a stock solution and then diluted in physiologic saline to appropriate concentration. L-tryptophan was dissolved in physiologic saline containing a drop of 0.1 HCl. Melatonin or L-tryptophan solutions were given in a volume of 0.5 ml to the rats as a bolus intraduodenal (i.d.) injection after stabilization of basal pancreatic secretion.

In this series the following study groups, each consists of 6-8 animals, were employed including: 1) controls (receiving bolus i.d. injection of vehicle saline) and; 2) Melatonin (1, 5 or 25 mg/kg) or L-tryptophan (10, 50 or 250 mg/kg) given i.d. to the rats with intact sensory nerves.

Series B was designed to determine the involvement of sensory nerves in pancreatic secretory response to i.d. melatonin or its precursor; L-tryptophan.

In this series of the study the rats with sensory nerves activated by capsaicin were used. Capsaicin deactivation was attained using a total dose of 100 mg/kg of this neurotoxin given s.c. 10 days before the tests, as described previously (21). In these tests selected dose of melatonin (5 mg/kg i.d.) or L-tryptophan (50 mg/kg i.d.) was given after maintaining of basal secretion of pancreatic juice. In the control tests vehicle saline instead of melatonin or L-tryptophan was administered into the duodenum.

Series C was used to assess the effects of inraduodenal infusion of CCK_1-antagonist tarazepide on pancreatic secretory response to melatonin or L-tryptophan.

Tarazepide was suspended in 5 ml of 1% solution of methyl cellulose using short- lasting sonification and at least 30 min stirring with a magnetic stirrer. Tarazepide was given i.d. as a bolus injection 15 min prior the administration of melatonin or L-tryptophan in a volume of 0.5 ml.

The several study groups, (6-8 animals in each single group) were employed including: 1) Vehicle (0.5 ml of methyl cellulose) injected intraduodenally (i.d.) as a bolus, 2) Tarazepide (2.5 mg/kg) given intraduodenally (i.d.); followed 15 min later by i.d. injection of vehicle; 3) Tarazepide (2.5 mg/kg id) followed 15 min later by selected dose of melatonin (5 mg/kg i.d.) or L-tryptophan (50 mg/kg i.d.).

Statistical analysis

Comparison of the differences between the mean values of various groups of experiments was made by analysis of variance and the Student's t test for unpaired data. Differences with a p value of < 0.05 were considered statistically significant. Results are expressed as means ± SEM.

RESULTS

The effects of in traduodenal (i.d.) administration of melatonin or L-tryptophan on pancreatic secretion.

During reinfusion of biliary-pancreatic juice into the duodenum basal secretion of amylase was relatively well sustained and averaged about 400 ± 42 IU/15 min (*Figs 1-6*). Intraduodenal administration of melatonin at doses of 5 or 25 mg/kg resulted in the dose-dependent increase of pancreatic amylase secretion, reaching about 2535 ± 210 IU/15 min at dose 25 mg/kg i.d of melatonin. The lowest dose of this indoloamine (1 mg/kg i.d.) failed to affect significantly pancreatic enzyme secretion (*Fig. 1*).

Application of melatonin precursor; L-tryptophan also resulted in the significant and dose-dependent augmentation of amylase output reaching 1097 ± 95 IU/15 min at 250 mg/kg dose of L-tryptophan (*Fig. 2*).

The volume of pancreatic juice averaged about 0.25 ml/15 min and was not siginificantly affected by melatonin or L-tryptophan i.d. administration.

Effect of sensory nerves deactivation on pancreatic secretion modulated by melatonin or its precursor; L-tryptophan.

Deactivation of sensory nerves with capsaicin (CD) completely reversed the stimulatory effects of melatonin (5 mg/kg i.d.) or L-tryptophan (50 mg/kg i.d.) on pancreatic enzyme secretion (*Figs 3* and *4*). In the rats with sensory nerves deactivated with capsaicin basal secretion of enzyme was not significantly different from those obtained in animals with intact sensory nerves (*Figs 3* and *4*).

Effect of in raduodenal (i.d.) infusion of tarazepide on pancreatic secretion modulated by melatonin or L-tryptophan.

Administration of tarazepide (2.5 mg/kg id) did not produce any significant change of basal secretion of amylase in the rat with pancreato-biliary fistulas (*Figs 5, 6*). The increases of amylase outputs produced by melatonin (5 mg/kg

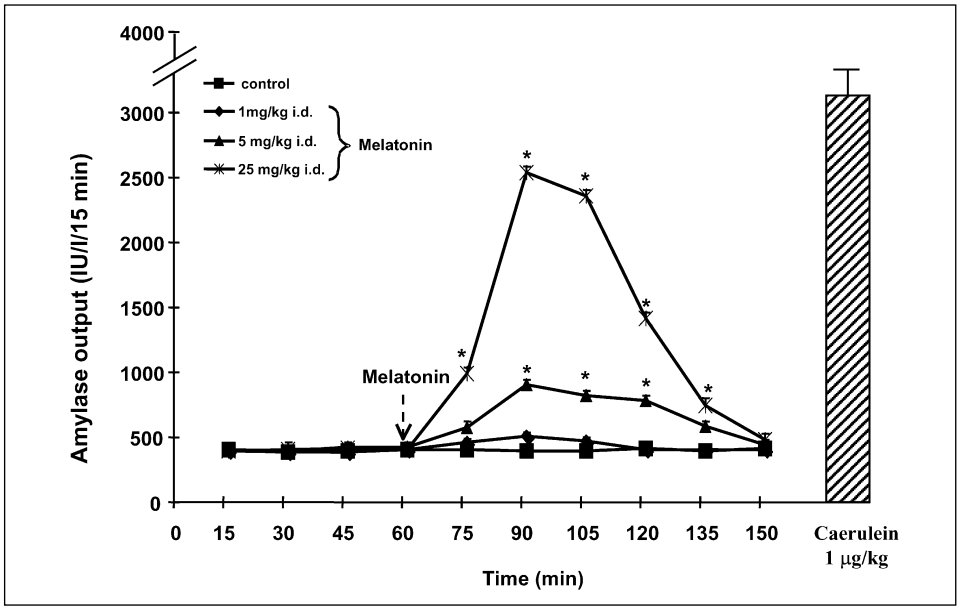

Fig. 1. Time course of pancreatic amylase secretion following the i.d. administration of melatonin (1, 5 or 25 mg/kg) at time 60 min. Control = amylase secretion in rats injected i.d. with vehicle saline instead of melatonin. The results are the means ± SEM of separate experiments, each performed on 6-8 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

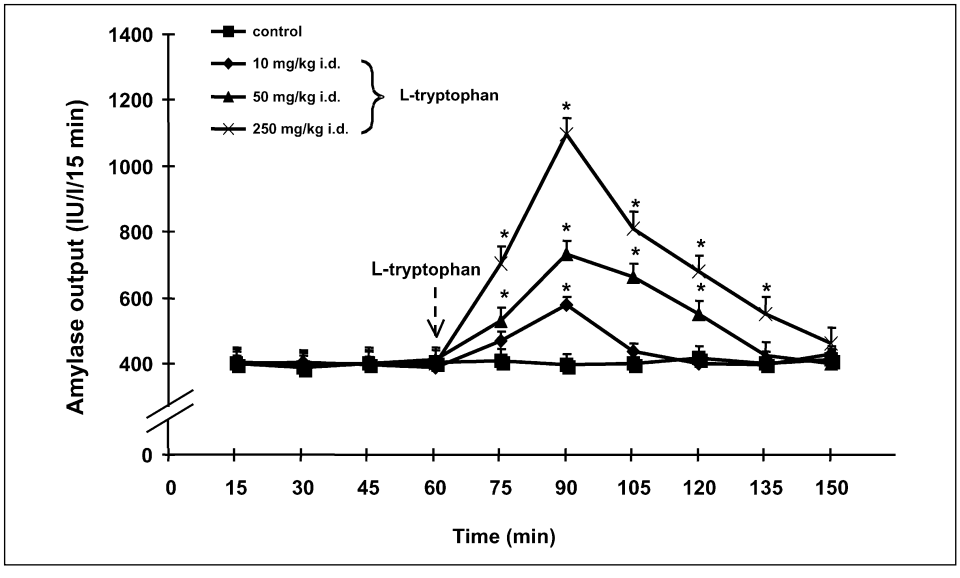

Fig. 2. Time course of pancreatic amylase secretion following the i.d. administration of L-tryptophan (10, 50 or 250 mg/kg). Control = amylase secretion in rats injected i.d. with vehicle saline instead of L-tryptophan. The results are the means ± SEM of separate experiments, each performed on 6-8 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

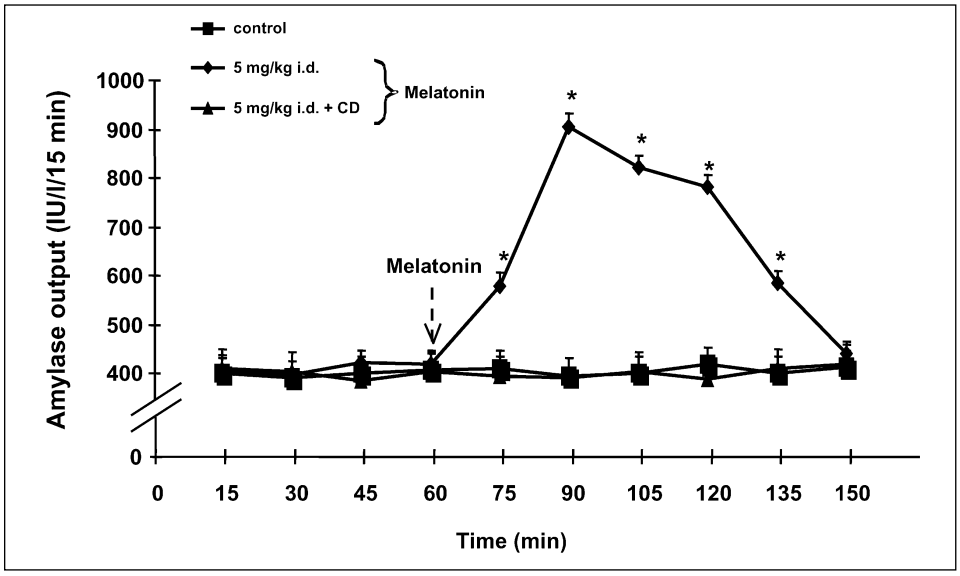

Fig. 3. Amylase responses to melatonin (5 mg/kg i.d.) in rats with intact sensory nerves and sensory nerves deactivated with capsaicin (CD). Control = amylase secretion in normal rats injected i.d. with vehicle saline instead of investigated substances. The results are the means ± SEM of separate experiments, each performed on 4-5 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

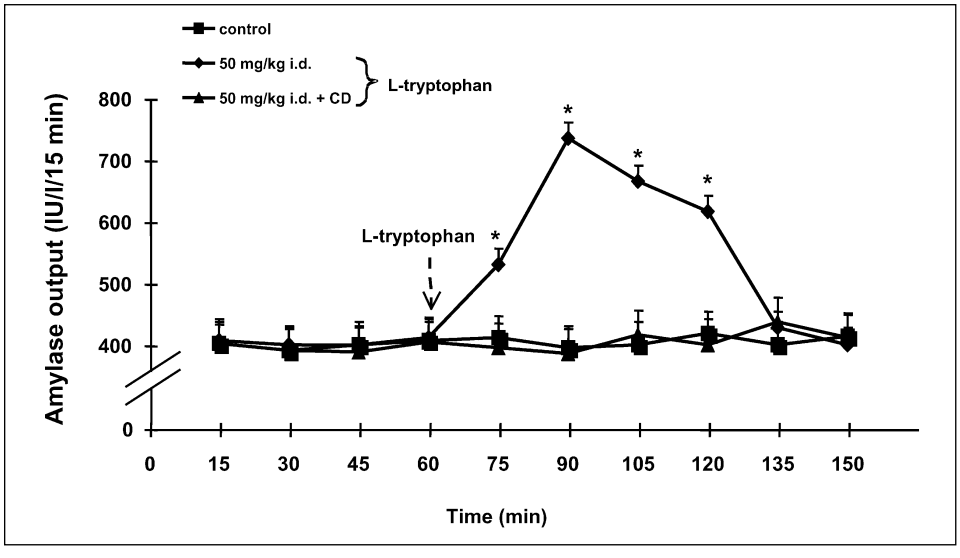

Fig. 4. Amylase responses to L-tryptophan (50 mg/kg i.d.) in rats with intact sensory nerves and sensory nerves deacivated with capsaicin (CD). Control = amylase secretion in normal rats injected i.d. with vehicle saline instead of investigated substances The results are the means ± SEM of separate experiments, each performed on 4-5 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

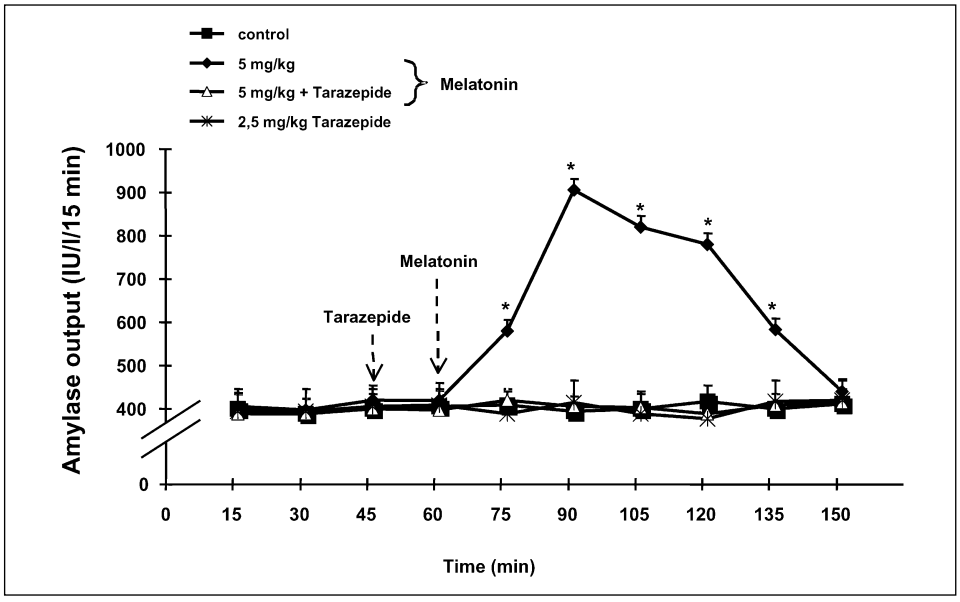

Fig. 5. Amylase responses to melatonin (5 mg/kg i.d.), tarazepide (2.5 mg/kg i.d.), or combination of above. Control = amylase secretion in rats injected i.d. with vehicle saline instead of investigated substances. The results are the means ± SEM of separate experiments, each performed on 6-8 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

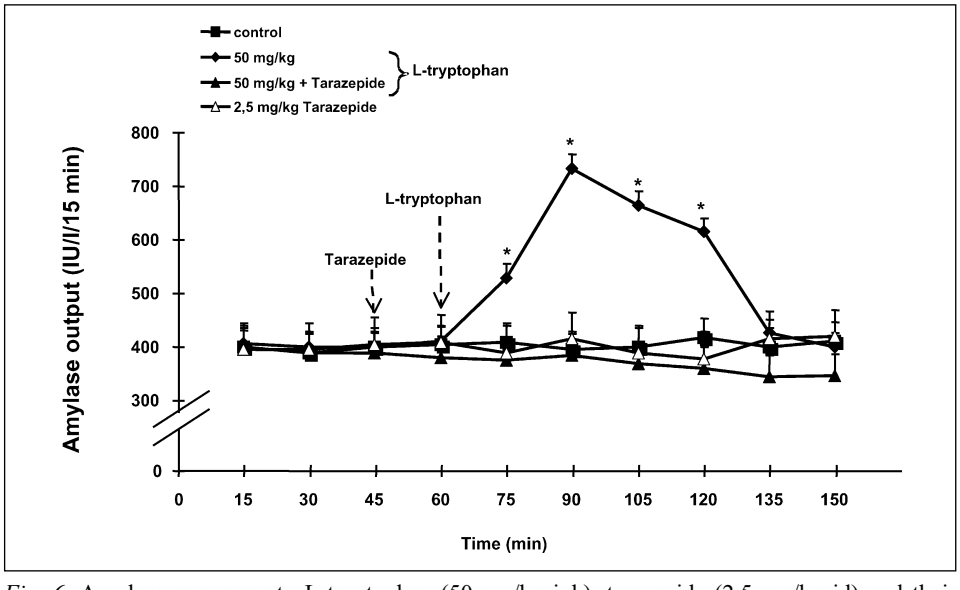

Fig. 6. Amylase responses to L-tryptophan (50 mg/kg i.d.), tarazepide (2.5 mg/kg id) and their combination. Control = amylase secretion in rats injected i.d. with vehicle saline instead of investigated substances. The results are the means ± SEM of separate experiments, each performed on 5-6 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

i.d.) or L-tryptophan (50 mg/kg i.d.) were completely abolished by pretreatment of the rats with tarazepide (2.5 mg/kg i.d.) (*Figs 5* and *6*).

Plasma melatonin and CCK activities.

Plasma melatonin immunoreactivity measured in the morning in intact rats averaged 50 ± 6.2 pg/ml (*Fig 7*). Administration of melatonin, given at doses of 1, 5 or 25 mg/kg i.d. resulted in the dose-dependent rise of plasma melatonin concentrations, reaching the highest value at the dose of 25 mg/kg of i.d. melatonin (*Fig. 7*). Application of melatonin precursor; L-tryptophan also resulted in the significant and gradual augmentation of melatonin plasma level reaching 300 ± 35 pg/ml at 250 mg/kg dose of L-tryptophan (*Fig. 7*).

Under basal conditions plasma level of CCK reached 15.4 ± 1.8 pg/ml. A single application of melatonin at graded doses (1, 5, or 25 mg/kg i.d.) produced dose-dependent rises in plasma CCK level, achieving the highest value (49 ± 3.5 pg/ml) at a dose 25 mg/kg of melatonin. Pretreatment of the rats with increasing doses of L-tryptophan (10, 50, or 250 mg/kg *ip*) also resulted in a significant and dose-dependent increment in plasma CCK immunoreactivity above the level detected in control animals, reaching 50.4 ± 4.1 pg/ml at dose 250 mg/kg of L-tryptophan (*Fig. 8*).

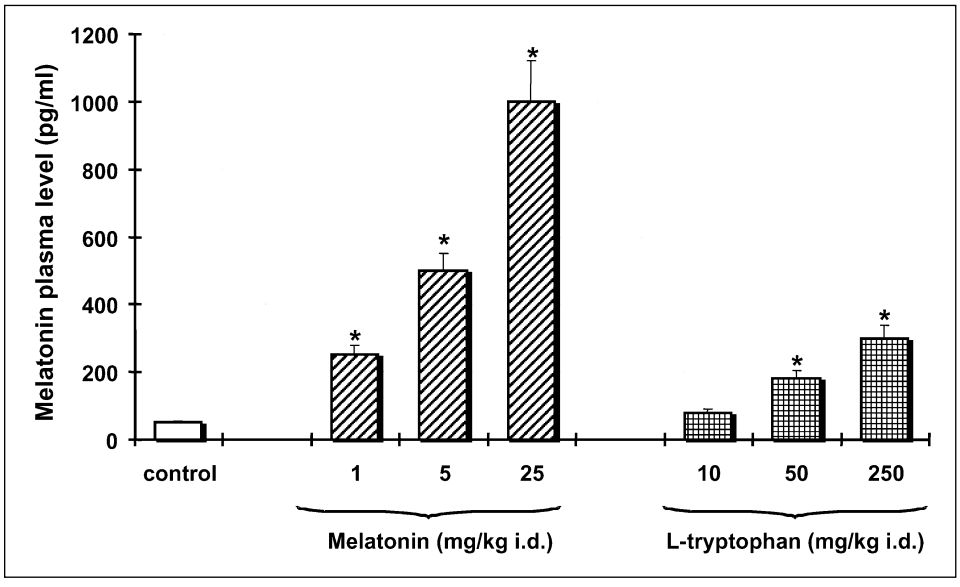

Fig. 7. Effect of i.d. administration of melatonin (1, 5 or 25 mg/kg) or L-tryptophan (10, 50 or 250 mg/kg) on melatonin plasma levels. Means ± SEM of separate experiments, each performed on 6-8 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

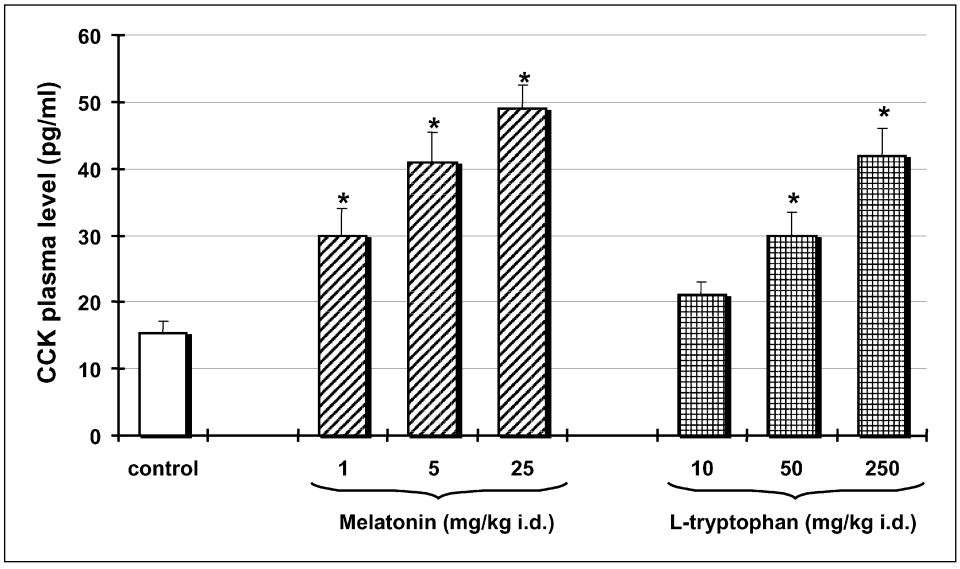

Fig. 8. Effects of i.d. administration of melatonin (1, 5 or 25 mg/kg) or L-tryptophan (10, 50 or 250 mg/kg) on CCK plasma levels. Means ± SEM of separate experiments, each performed on 6-8 rats. Asterisk (*) indicates significant ($p<0.05$) increase above the control value.

DISCUSSION

The major findings of the present study is demonstration for the first time that: 1) intraduodenal administration of melatonin or its precursor; L-tryptophan stimulates pancreatic enzyme secretion and, 2) deactivation of sensory nerves with capsaicin or pretreatment of the rats with CCK_1 receptor antagonist; tarazepide, abolished above stimulatory effect of melatonin or L-tryptophan on exocrine pancreas.

Previous studies have shown that parenteral administration of melatonin or L-tryptophan resulted in a significant increase of pancreatic amylase secretion *in vivo*, however both tested substances failed to affect amylase release from *in vitro* isolated pancreatic acini (23). As we have shown above secretory effects of melatonin or its precursor; L-tryptophan, on the pancreas depended upon the stimulation of CCK release by these substances (23).

Our present data confirm and reinforce the hypothesis that CCK is involved in the stimulatory effect of melatonin or L-tryptophan on the pancreas. CCK is a major hormone responsible for postprandial stimulation of pancreatic enzyme secretion (19). According to Li and Owyang, low, physiological doses of CCK, stimulate the exocrine pancreatic secretion *via* an indirect mechanism depending on the activation of the vagal afferent nerves. The direct stimulation of CCK

receptors on pancreatic acinar cells was observed after administration of high, pharmacological doses of cholecystokinin (20), indicating that these cells are equipped with CCK-receptors but they are not activated physiologically when the neural reflex stimulation of acinar cells takes place. Kirchgessner and Liu also suggested that CCK could bind to its receptors located on vagal afferent fibers, which in turn elicit a vago-vagal reflex stimulation of pancreatic secretion (27). In the present study we have shown that intraduodenal administration of melatonin, and to a lesser extent also L-tryptophan, produced significant and dose-dependent rises of plasma CCK levels. In dogs, tryptophan is considered as one of the most important pancreatic secretagogues when it was given intraduodenally or intravenously (24-25). However application of tryptophan into the ileum caused a dose-dependent decrease in the pancreatic secretory response to hormonal stimulation ("ileal brake") (26). Previous *in vitro* study has shown that tryptophan is able to inhibit CCK-induced amylase release from isolated pancreatic acini, the effect that was found to be dependent, at least in part, on the modulation of Ca^{2+} influx into acinar cells (28). Recent study has also shown that melatonin modulates a daily rhythm of CCK action with intestinal motility. This modulation was completely lost by application of melatonin receptor antagonist S 20928 (29). It appears that the action of CCK on intestinal motility follows a biological rhythm related to the light-dark cycle similar to that of melatonin.

Our present study in agreement with previous findings (23) shows that the blockade of CCK_1 receptors by tarazepide completely abolished the stimulatory effects of luminal application of melatonin or L-tryptophan on exocrine pancreas. This observation confirms and reinforces our previous hypothesis that CCK is strongly implicated in the pancreatic secretory effect of melatonin or its precursor (23).

Zabielski *et al.* demonstrated that tarazepide applied to the conscious neonatal calf inhibits CCK-8-stimulated secretion of postprandial pancreatic bicarbonate and protein secretion (22). These investigators also have reported that intraduodenal administration of tarazepide produced a significant and a dose-dependent increase of this CCK_1 receptor blocker in peripheral blood. These findings suggest that tarazepide may act on CCK_1 receptors, localized not only in duodenum but also in the pancreatic acini. We have previously reported that tarazepide or L-364,718, another CCK-1 antagonist, given to rats with pancreato-biliary fistulae inhibited pancreatic secretion stimulated by intraperitoneal application of melatonin or its precursor; L-tryptophan (23).

Sensory nerves are implicated in neural-reflex stimulation of pancreatic exocrine secretion by CCK and deactivation of these nerves with capsaicin diminishes the pancreatic secretory response to food (30). It has been reported recently that melatonin reduces neurotransmission in the neurons of the enteric nervous system (31). In our previous paper we have shown that deactivation of sensory nerves by capsaicin reversed the stimulation of pancreatic amylase secretion caused by parenteral application of melatonin (23).

Our present study confirms this observation and shows that increase of pancreatic enzyme secretion in response to luminal administration of melatonin or L-tryptophan depends on the stimulation of CCK release and activation of sensory nerves. It is likely that melatonin produced in the gut is released into the gastrointestinal lumen to stimulate CCK release, which in turn activates entero-pancreatic reflex stimulation of pancreatic enzyme secretion. Since the release of melatonin is correlated to the food ingestion (4-5), it could be presumed that this indole could take a part in the postprandial stimulation of pancreatic exocrine secretion.

Our observation remains in agreement with previous studies showing that intraperitoneal administration of melatonin or its precursor; L-tryptophan, produced an increase of melatonin plasma levels (23). This findings confirms previous notion that the pineal gland is not the main source of melatonin and that the gut is also able to release a considerable amount of melatonin following oral administration of L-tryptophan (32). The relationship between pineal and intestinal melatonin has not been investigated.

In conclusion; our present study demonstrates, that melatonin as well as that its precursor; L-tryptophan, given into the gut lumen stimulate pancreatic amylase secretion. The mechanism of this stimulatory action on the pancreas depends on the release of CCK and activation of afferent nerves to trigger entero-pancreatic reflexes.

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