Upsala J Med Sci 110: 115–150, 2005

The Duodenal Mucosal Bicarbonate Secretion

Review based on the doctoral thesis
The Duodenal Mucosal Bicarbonate Secretion
Role of Melatonin in Neurohumoral Control and Cellular Signaling

Markus Sjöblom

Department of Neuroscience, Division of Physiology,
Uppsala University, Uppsala, Sweden.

ABSTRACT

A free full-text copy of this article can be found at the web page of Upsala J Med Sci:
http://www.ujms.se

The duodenal lumen is exposed to aggressive factors with a high potential to cause damage to the mucosa. Bicarbonate secretion by the duodenal mucosa is accepted as the primary important defense mechanism against the hydrochloric acid intermittently expelled from the stomach.

The present work concerns both the influence of the central nervous system and the effects of the hormone melatonin on duodenal bicarbonate secretion in anesthetized rats *in vivo* as well as effects of melatonin on intracellular calcium signaling by duodenal enterocyte *in vitro* examined in tissues of both human and rat origin. The main findings were as follows:

Melatonin is a potent stimulant of duodenal mucosal bicarbonate secretion and also seems to be involved in the acid-induced stimulation of the secretion. Stimulation elicited in the central nervous system by the α_1-adrenoceptor agonist phenylephrine induced release of melatonin from the intestinal mucosa and a four-fold increase in alkaline secretion. The melatonin antagonist luzindole abolished the duodenal secretory response to administered melatonin and to central nervous phenylephrine but did not influence the release of intestinal melatonin. Central nervous stimulation was also abolished by synchronous ligation of the vagal trunks and the sympathetic chains at the sub-laryngeal level.

Melatonin induced release of calcium from intracellular stores and also influx of extracellular calcium in isolated duodenal enterocytes. Enterocytes in clusters functioned as a syncytium.

Overnight fasting rapidly and profoundly down-regulated the responses to the duodenal secretagogue orexin-A and the muscarinic agonist bethanechol but not those to melatonin or vasoactive intestinal polypeptide.

Received 26 October 2004
Accepted 11 November 2004

INTRODUCTION

The major functions of the gastrointestinal tract are to distribute to the body sufficient amounts of ingested nutrients and of water and electrolytes, and to expel waste products. To achieve these processes in an adequate manner, this tract has to resist the repeated challenges of ingested noxious, toxic and aggressive agents. It also has to stand firm against potentially harmful endogenous factors, such as hydrochloric acid and digestive enzymes, and prevent them from damaging the mucosa and/or entering the body. The lining of the gastrointestinal tract constitutes the body's largest surface area facing the external environment. The integrity of the mucosa depends on the balance between aggressive luminal factors and mucosal defense mechanisms. Changes in this balance may sooner or later lead to gastrointestinal disorders or diseases. The complex way in which this tube, almost nine meters long in humans, maintains its integrity has challenged and fascinated physiologists for centuries. This work deals with mechanisms that regulate the duodenal mucosal bicarbonate (HCO_3^-) secretion, one important mucosal defense mechanism.

Aggressive factors in the duodenal lumen

The duodenal lumen is frequently exposed to aggressive factors with a potential to cause damage to the mucosa. These factors can be divided into two groups: endogenous and exogenous. The main endogenous aggressive factor is hydrochloric acid (HCl), secreted by the parietal cells in the stomach. The secretory capacity is about three liters of HCl in 24 hours, with a pH of about one (1). In 1910 Karl Schwarz published the first clinical observation that gastric acid was associated with gastric and/or duodenal ulcer disease (2). He noted that acid caused mucosal damage and that this damage could be decreased by luminal neutralization. He formulated the famous dictum *"Ohne saueren Magensaft kein peptisches Geschwür" ("Without acid gastric juice – No peptic ulcer"*).

Another harmful factor is pepsin, an enzyme essential for digestion of proteins. The proenzyme pepsinogen is secreted by the peptic cells in the stomach. When catalyzed by secreted HCl, pepsinogen is cleaved into the active enzyme pepsin.

There are numerous exogenous factors with the potential to increase the sensibility of or cause damage to the intestinal mucosa. Infection with the gram-negative bacteria *Helicobacter pylori (H. pylori)* has a strong correlation with the development of gastroduodenal ulcers (3, 4). When the knowledge of the bacteria became clearer a new dictum was formulated: *"No bacteria – No ulcer"*. Eradication of the bacteria in combination with administration of proton pump inhibitors is effective ulcer treatment. However, duodenal ulcers do occur in non-infected patients. One well known group of substances increasing gastrointestinal damage is the nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit mucosal prostaglandin synthesis. It has further been shown that cigarette smoke (5, 6) decreases duodenal bicarbonate secretion and that ethanol (7, 8) increase the susceptibility of the gastrointestinal mucosa to damage.

Duodenal mucosal mechanisms of protection

The duodenum is the most proximal part of the small intestine, into which the stomach intermittently expels chyme with a high concentration of HCl. A healthy duodenal mucosal epithelium resists this challenge. The physiological basis of this barrier function involves several factors and mechanisms. Duodenal mucosal protection can be divided into three categories: pre-epithelial, epithelial, and sub-epithelial.

Pre-epithelial protection

The pre-epithelial defense mechanism is often referred to as "the first line" of duodenal mucosal defense. The proximal part of the duodenal lumen often attains acidities as high as close to pH 2 (9–11), but in the immediate vicinity of the surface epithelium the pH remains neutral (12). A pH gradient is formed by the secretion of bicarbonate and mucus from the epithelial cells. The viscoelastic mucus gel on top of the epithelial surface and the bicarbonate secreted into the mucus gel thus provide pre-epithelial defense against damage (11, 13). The mucus gel consists of ~5% mucins (glycoproteins) and >90% water (14). The glycoproteins are secreted by exocytosis by the surface epithelial cells and Brunner's glands. Together with water, a gel continuously covering the surface epithelium is formed. The lumen facing part of the mucus gel is loosely adherent to the epithelial surface and the thickness of the gel varies along the gastrointestinal tract (15). The gel provides lubrication for food particles and protects the epithelia from mechanical injury (shear stress). The protective role of the mucus per se is unclear, but it has a low permeability to macromolecules (16) and has been reported to delay back diffusion of hydrogen ions (14). The role of bicarbonate is better clarified, and will be described in more detail in the section "*Regulation of duodenal mucosal bicarbonate secretion*". The pre-epithelial defense can be summarized as the neutralization of acid and inactivation of pepsin at the duodenal mucosal surface.

Epithelial protection

The epithelial defense is often referred to as "the second line". Epithelial cells of the gastrointestinal tract are interconnected via tight junctions, closing the apical spaces between the cells. The duodenal epithelium, when compared with the gastric epithelium, is often referred to as a "leaky" epithelium (11). This is because of its higher permeability to ions, allowing passive transport of electrolytes between the cells.

A characteristic property of the intestinal epithelial cells is that the turnover rate is very high. The average enterocyte only stays alive for two to five days (17). Irritating compounds in the intestinal lumen can decrease this time. With such a high turnover rate it is very important to maintain the barrier function intact, preventing agents from entering the body. This is accomplished by restitution, which is a process of rapid re-epithelialization that occurs within a time-scale of minutes to hours. The maintenance of epithelial integrity is also strongly dependent on cell proliferation. The proliferative zone of the duodenal epithelium is in the crypt region (17). During cell migration, from the crypt region to the villus tip, the duode-

nocytes differentiates and acquire the functional characteristics of a villus cell, such as expression of glucose transporters and brush-border hydrolases. This process of migration and differentiation takes between two and five days (17).

Sub-epithelial protection

The blood flow is an important part of the sub-epithelial protection, since ion transport and intestinal motility are highly energy-consuming processes. The arteries of the proximal duodenum originate from the celiac trunk and divide into the gastroduodenal and pancreaticoduodenal arteries. The superior mesenteric artery supplies the more distal part of the duodenum. To achieve a rich blood supply, the vessels of the gastrointestinal tract have a large number of collaterals. It is well known that the amount of blood supplying the mucosa is regulated at the level of the arterioles, the resistance vessels. Mechanisms of regulation include neural, humoral, metabolic and myogenic factors (18, 19). The blood flow provides the duodenal mucosa with HCO_3^-/CO_2 (20, 21) and transfers absorbed nutrients, water, metabolic end-products and/or toxic substances to the liver.

The enteric nervous system

The enteric nervous system (ENS) plays a crucial part in the regulation of gastrointestinal functions such as ion transport (secretion and absorption), motility and mucosal blood flow. As part of the autonomic nervous system, the ENS is organized in a complex but very sophisticated network and contains as many neurons as the spinal cord (1). A unique feature of the ENS is that it can manage its many functions without input from the brain or spinal cord (22). The ENS is embedded in the gastrointestinal wall and consists of the myenteric plexus, located between the circular and longitudinal muscle layers, and the submucosal plexus, located in the submucosa. In general, the myenteric plexus controls gastrointestinal motility and the submucosal plexus coordinates ion transport and mucosal blood flow, but there is also extensive intercommunication between these plexa.

Although the ENS can function autonomously, the central nervous system (CNS) has a major influence on gastrointestinal functions. The vagal efferents (parasympathetic) project from their nuclei in the medulla oblongata and terminate in ganglia of the myenteric plexus, as described by Kirchgessner & Gershon in 1989 (23). These authors also demonstrated that almost no vagal efferents terminate in the submucosal plexus or at the epithelial cells. Signals from the vagal fibers have to be conveyed in the myenteric plexus. The influence of the sympathetic nervous system is mainly inhibitory. Sympathetic efferent neurons project from the spinal cord, relay in the celiac ganglion and terminate in the myenteric and submucosal plexa, as well as in the mucosa (1).

The intestine also possesses delicate sensory characteristics. The primary afferent neurons sense the mucosal epithelium and the luminal contents. These neurons can be divided into three classes: i) intrinsic, ii) extrinsic and iii) intestinofugal neurons (24). The intrinsic primary afferent neurons project only a short distance and have

their cell bodies and connections in the intestinal wall. The extrinsic primary afferent neurons have their cell bodies in the vagal and dorsal (spinal) ganglia with processes in the epithelium, and carry information to the central nervous system. The intestinofugal neurons have their cell bodies in the gut wall and carry information to prevertebral ganglia.

Regulation of duodenal mucosal bicarbonate secretion

Duodenal mucosal HCO_3^- secretion has a key role in duodenal protection against pulses of HCl and pepsin that are intermittently discharged from the stomach. One of the unique features of the duodenal epithelium is that it secretes bicarbonate at higher rates than the mucosa of more distal parts of the small intestine. The main physiological stimulant of the HCO_3^- secretion is the presence of acid in the duodenal lumen, and the acid-induced HCO_3^- response is mediated by enteric nervous pathways, involving release of vasoactive intestinal polypeptide (VIP) and acetylcholine (11, 13), as well as by E-type prostaglandins (PGs) released from mucosal cells (25).

Several compounds, of both the hormonal and non-hormonal type, have been shown to stimulate duodenal mucosal bicarbonate secretion. VIP is a peptide which very potently stimulates intestinal secretion, and infusion of VIP increases the HCO_3^- transport by the duodenal mucosa in all species tested (11, 26–28). Other mediators stimulating duodenal bicarbonate transport include cholecystokinin (CCK), pancreatic polypeptide and neurotensin (29), glucagons (30), pituitary adenylate cyclase-activating polypeptide (PACAP) (28, 31, 32) and angiotensin II (33).

The roles of PGs and nitric oxide (NO) in the HCO_3^- secretory response to acid have been studied extensively during recent years. Two cyclooxygenase (COX) enzyme isoforms, COX-1 (constitutively expressed) and COX-2 (inducibly expressed), are responsible for PG synthesis. The enzyme responsible for the increase in bicarbonate secretion after acid challenge is the COX-1 enzyme (34). Although PGs increase bicarbonate secretion, it has been shown that inhibition of PG synthesis with indomethacin also increases the alkaline output, by a mechanism closely coupled to induction of duodenal motility (35, 36).

The effects of NO on duodenal alkaline secretion are complex. There have been several reports that systemic (iv) NO synthase (NOS) inhibition with N-nitro-L-arginine methyl ester (L-NAME) increases duodenal mucosal bicarbonate secretion (37–40). Other studies, however, have shown that both luminal L-NAME (41, 42) and iv L-NAME (41, 43, 44) decrease the bicarbonate secretory response to acid. Three isoforms of the NO-synthesizing enzyme have been found: nNOS (neural), eNOS (endothelial) and iNOS (inducible). The nNOS and eNOS isoforms are constitutively expressed and are usually named cNOS. Takeuchi *et al*. recently suggested that cNOS is responsible for NO production following duodenal acidification (34).

The bicarbonate secretion is inhibited by NSAIDs as well as by α2-adrenorecep-

tor sympathetic stimuli. *H. pylori* infected patients with acute or chronic duodenal ulcer disease have impaired alkaline secretion (45), and eradication of the infection at least partly restores the secretion (46). Further, Fändriks *et al*. showed that water extracts from *H. pylori* inhibit duodenal mucosal bicarbonate secretion in the rat (47).

Central nervous control

Influence of the central nervous system on duodenal mucosal bicarbonate secretion is well established. The proximal duodenum is densely innervated with vagal fibers, passing from the medulla oblongata in the CNS and terminating in the myenteric plexus (48). The myenteric plexus and the submucosal plexus are also innervated by the sympathetic nervous system. Whether the alkaline secretion is stimulated or inhibited depends on the input signals to the secretomotor neurons of the submucosal plexus. Electrical stimulation, in the peripheral direction, of the cut vagal nerves in cats (49, 50) and in rats (51, 52) increases the bicarbonate secretion. The stimulatory effects are abolished by peripheral hexamethonium. A further indication that the CNS influences secretion is that sham-feeding increases the duodenal mucosal bicarbonate secretion in humans (53) and dogs (54). Besides exerting neural influence, the CNS can also regulate and control the secretions via release of hormones. β-Endorphin released from the pituitary gland influences duodenal HCO_3^- secretion (55). There are also reports on centrally elicited stimulation of the secretion by some neuropeptides, including thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and bombesin (11, 55–57), as well as by some benzodiazepines (58).

Furthermore, an up to a four-fold increase in secretion has been observed after intracerebroventricular (icv) infusion of the α_1-adrenoceptor agonist phenylephrine (59, 60). This increase was inhibited by the ganglion-blocking agent hexamethonium and by central nervous (but not intravenous) administration of the adrenoceptor antagonist prazosin.

Duodenal enterocyte ion transport

Approximately 90% of the intestinal epithelium consists of enterocytes (61). The knowledge about the intracellular signaling and different ion transporters involved in duodenal enterocyte bicarbonate secretion is increasing, but is still incomplete. Three major messenger systems have been suggested as being implicated in the intracellular control of HCO_3^- transport processes: i) intracellular calcium-induced responses (muscarinic M_3 agonists and CCK_A), ii) cyclic AMP-activated transport (prostaglandin EP_3 agonists, VIP and dopamine D_1 agonists), and iii) cyclic GMP-activated transport (uroguanylin, guanylin and heat-stable entero-toxin).

The duodenal enterocytes possess different mechanisms for acid/base transport possibly reflecting the second messenger system activated. HCO_3^- and CO_2 reach the epithelium via the blood and HCO_3^- is imported at the basolateral membrane by $Na^+(n)$-HCO_3^- cotransport. CO_2 diffuses into the enterocytes and HCO_3^- is formed intracellularly by carbonic anhydrase conversion of $CO_2 + H_2O$ to $HCO_3^- + H^+$. The

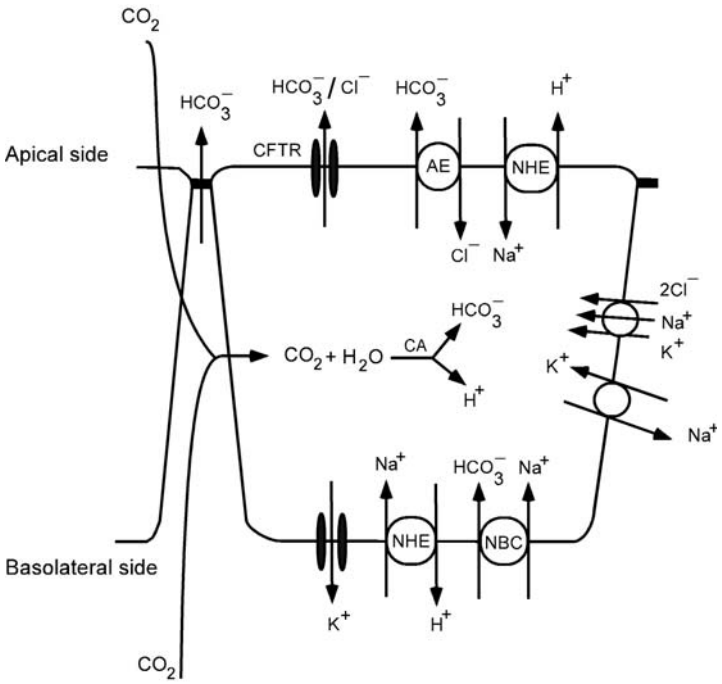

Fig. 1. A schematic illustration of the ion transporters in duodenal enterocytes. The model is based upon *in vitro* and *in vivo* experiments from several species. CFTR = cystic fibrosis transmembrane conductance regulator. AE = anion exchanger. NHE = sodium hydrogen exchanger. NBC = sodium bicarbonate cotransporter.

enterocytes export HCO_3^- at the apical membrane by a Cl^-/HCO_3^- exchanger as well as via an anion conductive pathway. It is suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) is the ubiquitous membrane spanning channel that transports Cl^- as well as HCO_3^- (62–64). An amiloride-sensitive Na^+/H^+ exchanger extrudes acid both at the apical and at the basolateral membrane. Fig. 1 shows a schematic illustration of the HCO_3^- transport by duodenal epithelium.

As the duodenal epithelium consists of both villus and crypt cells it is of great importance to verify the source of the secretion of HCO_3^-. In general, crypt cells are thought to have a secretory function, whereas the cells in the villi are mainly absorptive (65). The earlier hypothesis that intestinal secretions are only of crypt origin while the absorptive functions are restricted to the villi is, however, under re-evaluation. Suppression of carbonic anhydrase (CA) activity with acetazolamide decreases duodenal mucosal HCO_3^- secretion in humans (66), rabbits (20) and the guinea pig (67). In rats, acetazolamide has been reported to decrease bicarbonate secretion (12), but other authors have found no effect (68, 69). Furthermore, the CA isoenzyme II (CA II), associated with alkaline secretion, is located mainly in the villi and not in the duodenal crypts (70).

Melatonin

Melatonin is the major hormone of the pineal gland and was first isolated by Lerner and colleagues (71). Melatonin is derived from the amino acid tryptophan, which is converted into serotonin. Two enzymes then synthesize serotonin into melatonin. The first enzyme, the light sensitive, is N-acetyltransferase (NAT) and the second, the terminal and light insensitive, enzyme is hydroxyindole-O-methyltransferase (HIOMT) (72, 73). In the presence of light, no melatonin is synthesized in the central nervous system.

The physiological functions of melatonin are numerous. Among other effects, findings have suggested that melatonin may be involved in the regulation of circadian rhythms (74), scavenging of free radicals (75, 76), alleviation of jet lag (77) and (in non-human mammals) seasonal reproductive behaviors (74).

For many years melatonin was thought to be exclusively synthesized by the pineal gland, but it has become well established that active synthesis of melatonin also occurs in extrapineal sources. In 1975, some Russian scientists demonstrated melatonin synthesis in human intestinal enterochromaffin cells (78). Furthermore, Quay & Ma (1976) showed the presence of HIOMT in the duodenal mucosa, and Hong & Pang (1995) provided evidence for NAT activity in this tissue. These results have recently been confirmed (79). The amount of melatonin in the gastrointestinal tract does not depend on the presence of light and is not reduced by pineal glandectomy (80).

Distribution of peripheral melatonin

The major source of melatonin in the body is the gastrointestinal tract (81–83). Huether showed that the total amount of melatonin in the gastrointestinal tract is at least 400 times greater than the amount in the pineal gland at any time of the day and night (81). A similar observation has been made for serotonin (5-HT), of which approximately 95% is found in the alimentary canal (84). Melatonin is also present in several other organs, for example the pancreas, liver, bile, urogenital tract, air way epithelium and the retina.

Melatonin produced by the enterochromaffin (EC) cells in the intestinal mucosa seems to contribute to the circulating blood concentration during the daytime (85), whereas melatonin released from the pineal gland is responsible for the higher concentrations during darkness (86). The pineal gland melatonin is released in a circadian fashion (74, 86), while the melatonin produced in the gastrointestinal tract steadily enters the circulation (87, 88). Most of the melatonin released from the gastrointestinal tract into the portal vein is metabolized in the liver. An interesting phenomenon observed is that when the concentrations of melatonin decrease to a daytime level, the hormone escapes liver metabolism (89). It has also been reported that, in both normal (85, 90) and pineal glandectomized rats (91), a high tryptophan (melatonin precursor) diet drastically increases the blood levels and intestinal levels of melatonin.

Being a non-polar and lipid-soluble hormone, melatonin crosses biological membranes, such as the blood-brain barrier, and acts at melatonin-specific receptors in the central nervous system as well as at such receptors in peripheral tissues. The

half-life of melatonin in the peripheral circulation is ~20–40 minutes, depending on the species (92).

Melatonin receptors

Melatonin acts principally via high-affinity receptors coupled to hetero-trimeric guanine nucleotide-binding regulatory proteins (G-proteins). Three receptor subtypes have been found (93); two of them, the MT_1 and MT_2 melatonin receptors, have been identified in mammals in molecular cloning studies, and a third receptor, named MT_3, has been found though not yet cloned (93, 94). Melatonin receptors are distributed throughout the gastrointestinal tract (82, 83, 95).

The signaling properties of the MT_2 receptor are becoming clearer since the recent development of MT_2-selective ligands. Unfortunately, no high affinity MT_1 receptor ligands have yet been discovered. Ligands with high affinity for MT_1 receptors are required to further clarify the physiological and pathophysiological roles of the biological actions of melatonin. The two receptor subtypes MT_1 and MT_2 have in common that they inhibit cAMP formation and stimulate phosphatidylinositol hydrolysis (93).

The melatonin receptors mentioned in this work are defined according to the nomenclature and classification of the Nomenclature Committee of the International Union of Pharmacology (96). The denomination MT_1 corresponds to that of the recombinant receptor previously termed ML_{1A} or Mel_{1a}. MT_2 refers to the native functional receptors with pharmacological characteristics similar to those of the recombinant receptor MT_2 previously termed ML_{1B} or Mel_{1b}. MT_3 corresponds to the pharmacologically defined melatonin receptor subtype, with unknown gene sequence, previously referred to as ML_2.

AIMS OF THE INVESTIGATION

The general aim of this investigation was to further elucidate the central nervous and the peripheral regulation of the mucosa-protective duodenal mucosal bicarbonate secretion. One intention was to examine the influence of the hormone melatonin on the secretion *in vivo* and to study its effect on duodenal enterocyte intracellular calcium signaling *in vitro*.

More specifically, the following aims were addressed:

- study the effects of intravenous infusion, intraarterial infusion close to the duodenum, and duodenal luminal administration of melatonin and some melatonin agonists/antagonists on duodenal mucosal bicarbonate secretion in anesthetized rats *in vivo*.
- elucidate the neurohumoral pathways mediating the increase in duodenal mucosal bicarbonate secretion elicited by phenylephrine administered icv.
- investigate duodenal mucosal bicarbonate secretion in pineal glandectomized rats *in vivo*.

- study the release of melatonin from the duodenal mucosa.
- examine acid-stimulated duodenal mucosal bicarbonate secretion and the role of melatonin.
- compare basal and stimulated duodenal alkaline secretory rates in fed animals and in animals fasted for a short period (overnight).
- develop a method for isolation of duodenal enterocytes suitable for studies of intracellular signaling.
- elucidate the effects of melatonin on intracellular calcium signaling in duodenal enterocytes.

MATERIALS & METHODS

In vivo experiments

Animals

All experiments on animals were approved by the Uppsala Ethics Committee for Experiments with Animals. Male outbred Sprague-Dawley rats (190–260 g) or F_1-hybrids of Lewis x Dark Agouti rats (200–300 g) were placed in a conditioning unit under standardized temperature and light conditions (21–22°C, 12:12 h light-dark cycle) for at least four days after purchase. The rats were kept in cages in groups of two or more and had access to tap water and pelleted food (Ewos, Södertälje, Sweden) ad libitum.

Anesthesia and general surgery

The rats were deprived of food for 16–20 h before the experiments, but had free access to drinking water. The experiments were started by anesthetizing the animal with 5-ethyl-5-(1'-methyl-propyl)-2-thiobarbiturate (Inactin®), 120 mg/kg body weight intraperitoneally. The animals were anesthetized in the Animal Department by the person who had previously handled them. Subsequently, the rats were tracheotomized with a tracheal tube to facilitate respiration, and the body temperature was maintained at 37–38∞C throughout the experiments by a heating pad controlled by a rectal thermistor probe.

A femoral artery and vein were catheterized with PE-50 polyethylene catheters. For continuous recordings of systemic arterial blood pressure the arterial catheter, containing 20 IU/ml of heparin isotonic saline, was connected to a pressure transducer operating a PowerLab system. The vein was used for injection of some of the drugs and for continuous infusion of Ringer solution ([in mM] 145 Na^+, 124 Cl^-, 2.5 K^+, 0.75 Ca^{2+} and 25 HCO_3^-) at a rate of 1.0 ml/h. The latter infusion was given to compensate for fluid loss and to avoid acid/base changes during the experiments. Blood acid/base balance was checked in 40 µl arterial blood samples taken at the start and end of the experiments. After completion of the operative setup, the abdomen was closed with sutures and the animal was left undisturbed for 40–60 minutes for stabilization of the cardiovascular, respiratory and gastrointestinal functions.

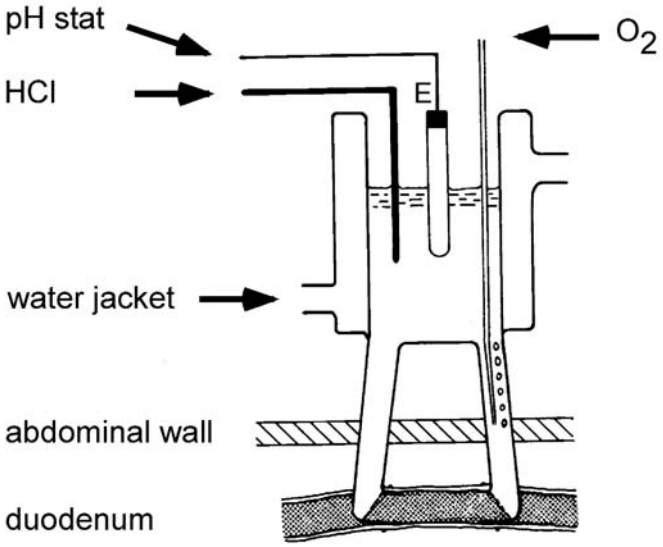

Fig. 2. Rat proximal duodenum was cannulated *in situ* between two glass tubes connected to the same reservoir containing isotonic saline. The mucosal HCO_3^- secreted into the luminal perfusate was continuously titrated under automatic control of a pH-stat system.

Duodenal preparation

The abdomen was opened by a midline incision and the gastric pylorus was ligated with a suture. To prevent bile and pancreatic secretion from entering the intestine, the common bile duct was always catheterized close to its entrance to the duodenum, with a PE-10 polyethylene tubing. For measurement of duodenal mucosal HCO_3^- secretion, a 12 mm segment of duodenum with its blood supply intact, starting 10–12 mm distal to the pylorus and thus devoid of Brunner's glands, was cannulated *in situ* between two glass tubes connected to a reservoir (Fig. 2). Fluid (10 ml of 154 mM NaCl), maintained at 37°C by a water jacket, was rapidly circulated by a gas lift of 100% oxygen. HCO_3^- secretion into the luminal perfusate was continuously titrated with 50 mM HCl at pH 7.4 under automatic control of a pH-stat system.

Intraarterial infusions

To study effects elicited in the duodenal segment and to minimize possible central nervous actions, compounds were administered close to the duodenal segment by intraarterial (ia) infusion. The hepatic artery was cannulated, tied 3–4 mm proximal to its entrance into the liver, and perfused in the retrograde direction at 17 µl/min (Fig. 3). This perfusion results in distribution of the perfusate mainly to the duodenum (via the cranial pancreaticoduodenal artery) and pancreas.

The distribution was checked visually at the start and end of the experiments by ia injection of a small amount of isotonic saline. This procedure changed the bright-

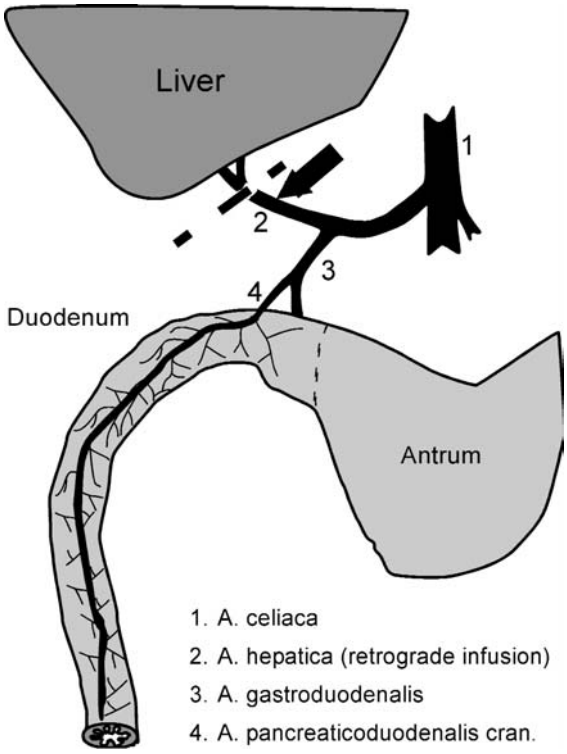

Fig. 3. For intraarterial infusion close to the duodenal segment, the hepatic artery was cannulated, and tied before its entrance into the liver, and the administered drugs were infused in the retrograde direction. [from Flemström *et al*. (121) with permission].

ness of the duodenal segment.

Intracerebroventricular infusions

Compounds were administered by icv infusions in order to study duodenal secretory stimulation elicited in the central nervous system. A metal cannula was inserted into the right lateral cerebral ventricle by using a stereotactic instrument. A skin incision was made over the right parietal bone, and a 1 mm hole was drilled through the bone, 0.8 mm posterior to the bregma and 1.5 mm lateral to the midsagittal suture. A stainless steel cannula was inserted stereotactically and cemented to the skull. Artificial cerebrospinal fluid ([in mM] 151.5 Na^+, 3.0 K^+, 1.2 Ca^{2+}, 0.8 Mg^{2+}, 132.8 Cl^-, 25 HCO_3^-, 0.5 phosphate; pH 7.4) was infused through this cannula at a rate of 30 µl/h. All agents infused icv had been dissolved in artificial cerebrospinal fluid. The location of the end of the cannula within the icv space was tested at the end of most experiments by adding Evans blue solution to the infusate, followed by dissection of the brain.

Pineal glandectomy

We modified a method described by Hoffman & Reiter in 1965 (97). Sprague-Dawley rats were anesthetized by intraperitoneal injection (0.27 ml/kg body weight) of a solution (Hypnorm®) containing fentanyl 0.315 mg/ml, fluanisone 10 mg/ml and midazolam 5 mg/ml, which induced surgical anesthesia for about 30 min. Using the stereotactic instrument, the head of the rat was fixed and the scalp was cut anteroposteriorly along the midline. The skin flaps were reflected and the temporal and occipital muscle masses were scraped free. Three lines were cut with a dental drill equipped with a fissure bar, the bone flap was raised and the dura mater was cut with a sharp needle. A forceps was put beneath the superior sagittal sinus and the pineal gland (white and 0.5–1.0 mm in diameter) was removed. Experiments on the duodenum were not performed until at least one week after pineal glandectomy.

Pituitary glandectomy

Pituitary glandectomy was performed at the Møllegaard Breeding Center by personnel with routine experience of this operation. The pituitary gland in male Sprague-Dawley rats (weighing 190–230 g) was removed by suction with a syringe through the ear. The animals were observed for one week following the operation, and absence of gain in weight (indicating lack of growth hormone incretion) was used to confirm the removal of the pituitary gland. The animals were then transported to Uppsala together with non-operated animals of the same breed for control experiments. To maintain body acid/base balance the rats were always supplied with drinking water adjusted to pH 3 with HCl. Twenty-four hours before the experiments 0.5 mg/kg dexamethasone (Decadron®) was injected intramuscularly to compensate for the loss of endogenous glucocorticosteroids. This injection was necessary to keep the rats alive during the experiments.

Transmucosal electrical potential difference

The duodenal transmucosal electrical potential difference (PD) was measured in some experiments and recorded between the duodenal mucosa and posterior vena cava with a high-input impedance voltmeter via matched calomel half-cells. The half-cells were connected to the animal by means of agar bridges (2 M KCl) with their distal ends located in the luminal solution and the posterior vena cava, respectively.

Section of vagus nerve and sympathetic chain

The common carotid arteries were identified and the surrounding nerves were dissected free from the arteries under light microscopy. In one group only the cervical vagal nerves were cut, at the sub-laryngeal level. In a second group the cervical paravertebral sympathetic chain was cut. In a third group all nerves around the carotid arteries (including vagal trunks and sympathetic chain) were ligated and cut at the sub-laryngeal level.

Melatonin analyses

Arterial blood samples (0.7–1.0 ml) were obtained from the rat tail artery and from the femoral artery. All blood samples were taken between 11 am and 3 pm. The blood samples were left for 30 min at room temperature to coagulate and then centrifuged at 4000 rpm at 4°C for 7 min. The serum was then stored at –20°C until analyzed at Nova Medical AB, Skövde, Sweden, using an ELISA assay (Bühlmann Labs., Allschwil, Switzerland). The detection limit of the assay was 0.05 pmol/ml. The intra-assay and inter-assay coefficients were below 6.6 %.

Melatonin was also determined by high-performance liquid chromatography (HPLC) with electrochemical detection, running Chromeleon™ software (Dionex Corporation, Sunnyvale, USA) on an IBM-compatible computer. Melatonin was separated on a Luna C18 column (5 µm particle size, 150 x 4.6 mm). The isocratically operated chromatographic system was perfused with the following mobile phase: 0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, 30 % methanol, pH 3.7, at a flow rate of 1.0 ml/min. The electrochemical detector potential was adjusted to +900 mV. The total runtime was 15 min. Melatonin was eluted at 12 min and 57 sec and 6-fluorotryptamine was eluted at 5 min and 58 sec. Samples of 2.0 ml of re-circulating luminal perfusate, from the chamber illustrated in Fig. 2, were taken and 1.0 ng of the internal standard 6-fluorotryptamine was added. The samples were then filtered through an Acrodisc® LC 13 mm syringe filter with a 0.2 µm PVDF membrane (Pall Gelman Laboratory, USA) and freeze-dried. The residues were dissolved in 230 µl HPLC mobile phase. Duplicate 100 µl samples of the solution were injected into the chromatographic system (Injector Mod. 7725i, 100 µl loop, Rheodyne Inc., San Francisco, USA). Melatonin concentrations were calculated on the basis of comparison with the internal standard. The melatonin detection limit of the HPLC system was 0.5 ng. The calibration curves for melatonin and 6-fluorotryptamine showed linear responses over the studied ranges. Triplicates of melatonin standards (0.5, 1.0, 10, 100 and 1000 ng) were injected into the HPLC system and the calibration curve equation obtained was $y = 3.25x + 0.42$, $r^2 = 0.99$. The internal standard was also injected into the HPLC system in triplicates. 6-fluorotryptamine (0.05, 0.5, 5.0 and 50 ng) yielded the calibration curve equation $y = 18.6x + 0.075$, $r^2 = 0.99$.

Statistical analysis

Descriptive statistics are expressed as means ± SEM, with the number of experiments given in parentheses. Rates of alkaline secretion by the duodenum are expressed as microequivalents of base (HCO_3^-) per centimeter of intestine per hour ($\mu Eq \bullet cm^{-1} \bullet h^{-1}$). The secretion and mean arterial blood pressure (MAP) were monitored continuously and recorded at 10-min intervals. The statistical significance of data was tested by repeated measures analysis of variance. To test differences within a group a one-factor repeated measures ANOVA was used, followed by Fishers's PLSD post hoc test. Between groups the results of HCO_3^- secretion with drug administration were compared with the secretory rates obtained with control animals infused with vehicle alone or with other compounds. For this comparison, a

two-factor repeated measures ANOVA followed by a one-way ANOVA at each time point was used. If the ANOVA was significant at a given time point, a Fisher's PLSD post hoc analysis was used. All statistical analyses were performed on an IBM-compatible computer using StatView 5.0 software. P values of <0.05 were considered significant.

In vitro experiments

Human biopsies

Biopsy specimens from the duodenum were obtained from patients undergoing upper endoscopy at the Gastroenterology Unit, Uppsala University Hospital and found to have endoscopically normal duodenal and gastric mucosae. Results obtained from 17 biopsy specimens from 8 patients are presented. The project was approved by the Ethics Committee of the Medical Faculty at Uppsala University, and all subjects provided written informed consent. The specimens were taken between 9 am and 10 am with Radial Jaw (Large Capacity with Needle) single-use biopsy forceps and immediately transported to the laboratory at the Biomedical Center, Uppsala, Sweden.

Rat tissue preparation

The experiments were begun before 9 am, and to avoid possible stimulatory effects of anesthetics on intestinal mucus release, the rats were decapitated. A 3-cm segment of duodenum, starting 2–3 mm distal to the pylorus, was promptly excised via an abdominal midline incision and freed from mesentery. The segment was opened along the antimesenteric axis and the luminal surface was rinsed with a normal respiratory medium (NRM) ([in mM] 114.4 Na^+; 5.4 K^+; 1.0 Ca^{2+}; 1.2 Mg^{2+}; 121.8 Cl^-; 1.2 SO_4^{2-}; 6.0 phosphate; 15.0 HEPES; 1.0 pyruvate; and 10 glucose plus 10 mg/l phenol red, 0.1 mg/ml gentamicin and 2.0% fetal calf serum). The pH was adjusted to 7.40 immediately before use and the temperature was maintained at 37°C. The sheet of duodenal wall was then put on a precleaned glass slide (lumen side up) and the mucosa was gently scraped-off. The depth of mucosal tissue removed by the scraping procedure (and used for experiments) was tested by morphological examination of the remaining tissue (fixed in 10% neutral buffered formalin and stained with hematoxylin-eosin). The duodenal remnant contained some crypt bases and all submucosa containing Brunner's glands. Cells originating from the latter glands were thus excluded from the studied preparations.

Isolation of enterocytes in clusters

The scraped-off rat mucosa or the human biopsy specimens were then cut into pieces 0.3–0.8 mm in diameter which were dispersed and briefly shaken in NRM solution also containing 0.5 mM dithiothreitol (DTT). After sedimentation for 2–3 min, the supernatant was removed and the tissue fragments (in the sediment) washed three times in NRM solution (not containing DTT). Following brief gassing

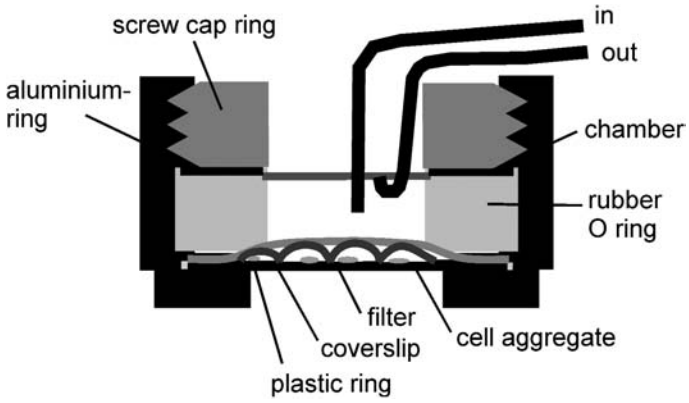

Fig. 4. Schematic illustration of the temperature-controlled perfusion chamber.

with 100% O2, the tissue fragments (15–20 µl) were then exposed to mild digestion for 3 min by inoculation in 10 ml NRM solution containing 0.1 mg/ml collagenase type H (Sigma) and 0.1 mg/ml dispase II (Mannheim). Digestion was performed at 37°C in a horizontal shaking water bath, and was stopped by adding DTT (to a concentration of 0.3 mM) and the solution was centrifuged (3 min at 1,000 g). The pellet was washed three times by suspension in 10 ml DME/F12 (with 15 mM HEPES and 2.5 mM glutamine) followed by centrifugation (3 min at 1,000 g). HCO_3^- (1 mM), gentamicin (0.01 mg/ml) and fetal calf serum (2.0%) were always added to the DME/F12 and the pH was adjusted to 7.40. The preparatory procedure yielded clusters (10–100 cells) of interconnected duodenal enterocytes as well as smaller amounts of single cells. The clusters were composed predominantly of cells with morphological characteristic of crypt cells. The viability after the preparation was tested by trypan blue exclusion (>95%). The final pellet was suspended in ~1.0 ml of DME/F12 (with the same additives) solution and immediately put on ice, a procedure found to increase the viability of the enterocyte clusters compared with keeping the cells at 37°C.

Cell loading and calcium measurements with fura-2

For measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$), 70 µl of the cell cluster suspension was loaded at 37°C with fura-2 acetoxymethyl ester (2 µM) for 20–30 min in an electrolyte solution ([in mM] 141.2 Na^+; 5.4 K^+; 1.0 Ca^{2+}; 1.2 Mg^{2+}; 146.4 Cl^-; 0.4 phosphate; 20.0 TES; and 10 glucose; pH 7.40) that has been found appropriate for studies of cell aggregates from other tissues (98). Probenesid (1 mM), pluronic F-127 (0.02%) and fetal calf serum (2.0%) were present during the loading procedure. The fura-2-loaded cell aggregates were spun down and placed on an uncoated, precleaned circular glass coverslip (Ø 25 mm) at the bottom of a temperature-controlled (37°C) perfusion chamber (Fig. 4) and fixed on top of the coverslip by a uniformly sized pore polycarbonate membrane filter. The covering filter and the cell preparation were perfused (1 ml/min) with the electrolyte

solution and receptor ligands to be tested were added by inclusion in the perfusate.

Changes in $[Ca^{2+}]_i$ in the fura-2-loaded cells were measured by the dual-wavelength excitation ratio technique by exposure of the cells to alternating 340 and 380 nm light with the use of a filter changer under the control of an InCytIM-2 system (Intracellular Imaging) and a dichroic mirror (DM430, Nikon). Emission was measured through a 510 nm barrier filter with an integrating CCD camera. Calibration of the fluorescence data was accomplished *in vitro* according to the method described by Grynkiewicz *et al*. in 1985.

Data analyses

All statistical analyses were performed on an IBM-compatible computer using StatView 5.0 software. When appropriate, statistical significance was calculated using Student's *t*-test. Non-linear curve-fitting of the data was achived by use of SigmaPlot for Windows 4.01.

RESULTS AND DISCUSSION

The gastrointestinal epithelium is the largest surface area in the body. Facing the external environment, the epithelium is repeatedly challenged by aggressive factors of both exogenous and endogenous origin. Today, the duodenal mucosal bicarbonate secretion is accepted as the primary defense mechanism against the HCl that is intermittently expelled from the stomach. The secretory rates of HCO_3^- are higher in the duodenum than in the stomach and in other, more distal parts of the small intestine (11). The investigations described in this study have focused on the central nervous regulation on duodenal mucosal bicarbonate secretion as well as the effects of melatonin on this secretion. On the basis of results from *in vivo* and *in vitro* studies, physiological processes of potential importance for regulation of the protective alkaline secretion by the duodenal mucosa are suggested.

The essence of the results will be discussed below, but the "take-home" message of this work is that intestinal melatonin is an important mediator in the CNS- and HCl-elicited stimulation of duodenal mucosal bicarbonate secretion. This in turn suggests that melatonin may be involved in duodenal mucosal protection against acid. Intestinal melatonin most probably originates from the enterochromaffin cells, and the released melatonin activates adjacent enterocytes to secrete HCO_3^-. With calcium as an intracellular and intercellular messenger, the duodenal enterocytes form a secretory functional syncytium. Further, the sensitivity to some peripheral stimulators of duodenal mucosal HCO_3^- secretion depends markedly on the feeding status of the animals.

The physiological relevance of bicarbonate secretion

Evidence for HCO_3^- secretion originating from the duodenal mucosa was first reported a century ago in a thesis from Pavlov's laboratory in St. Petersburg (99). Since that time, both *in vitro* and *in vivo* studies have shown that the duodenal

mucosa secretes HCO_3^- at high rates (11). In the normal situation, when the duodenal mucosa is healthy, bicarbonate enters the continuous layer of viscoelastic mucus gel on top of the epithelial surface and maintains the pH in its cell-facing portion at neutrality in spite of high acidities in the duodenal lumen (12, 13, 16). When the bicarbonate secretion is inhibited by NSAIDs, or when the secretory neurohumoral regulation is malfunctioning, as in *H. pylori*-infected patients with acute and chronic duodenal ulcer disease, the acid may acidify the epithelial surface and cause mucosal damage. It should be noted that bicarbonate secreted from the duodenal epithelium is not solely responsible for neutralizing the gastric acid expelled into the intestine. It serves as an epithelial protector and together with bicarbonate-rich juices from the liver and pancreas it inactivates proteolytic enzymes, such as pepsin, and neutralizes the gastric acid. Overall, acid-stimulated mucosal HCO_3^- secretion probably accounts for ~40% of the neutralization of the gastric acid load to the duodenum; pancreatic and biliary HCO_3^- accounting for the remaining bulk neutralization (100, 101).

Melatonin as an intestinal hormone

In humans and other mammals, including rodents, melatonin secretion from the pineal gland peaks at darkness (night), independently of species differences in day or night activity (73). Melatonin is synthesized from tryptophan, with serotonin as an intermediate precursor, and is released from the pineal gland into the circulation. Being a non-polar and lipid-soluble hormone, melatonin crosses the blood-brain barrier and acts at melatonin-specific receptors in the CNS as well as at such receptors in peripheral tissues. Importantly, melatonin is also produced by the EC cells in the intestinal mucosa (102) and the total amount of melatonin in the alimentary tract is considerably higher (>400) than that in the CNS (81). It should also be noted that EC cells are in close contact with fibers from the autonomic nervous system (103). The physiological role of the intestinal source of melatonin has not been fully established. Like the EC cell products guanylin (104) and serotonin (84), intestinal melatonin may have a role in the reaction between the mucosa and the luminal contents.

Luminal perfusion of melatonin induced high rates of duodenal mucosal HCO_3^- secretion (Fig. 5.) (105) and such an effect was also observed after iv or close ia infusion (60). This may be in line with the proposal that melatonin acts as an intestinal intraluminal hormone, exerting actions in intestinal segments distal to the sites of release (88). It should be noted in this context that the continuous discharge of bile into the duodenum that occurs in the rat is probably a source of intestinal intra-luminal melatonin (106, 107). At least during conditions of intestinal paralysis, the mucus layer on the surface of the duodenal mucosa provides a physical barrier to the migration of macromolecules and some secretagogues, including prostaglandins, to the epithelial surface (16). The high rates of mucosal HCO_3^- secretion induced by melatonin would suggest that the mucus layer does not significantly inhibit the migration of melatonin from the luminal fluid to the epithelial surface (105).

We demonstrate for the first time that melatonin and melatonin receptor agonists

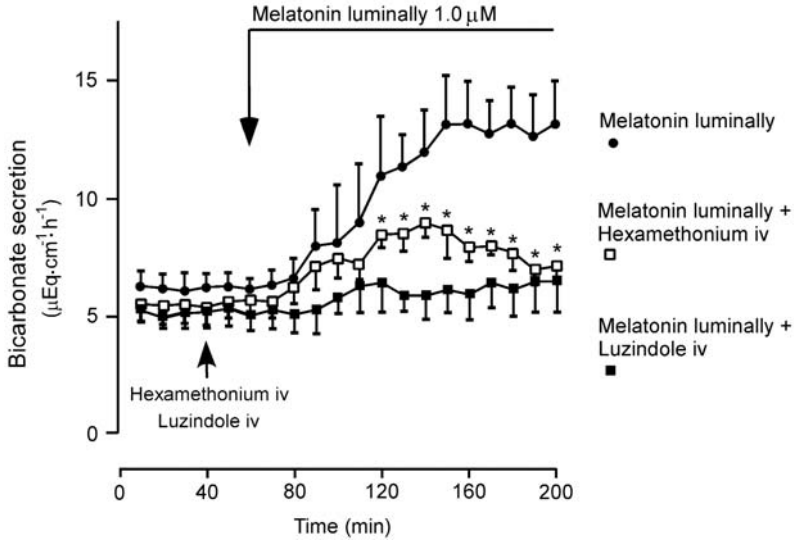

Fig. 5. Perfusion of the duodenal lumen with melatonin increased duodenal bicarbonate secretion. This secretory response was abolished by pretreatment with luzindole and significantly inhibited by pretreatment with hexamethonium. [from Sjöblom *et al*. (105) with permission].

increase the duodenal HCO_3^- secretion in rats (60). The secretagogues were administered by ia infusion close to the duodenal segment, a procedure that would minimize central nervous actions. Considerably higher doses were required for stimulation when the hormone was given iv, strongly indicating that the stimulation by melatonin is elicited within the duodenum and is not mediated by a primary central nervous action. The secretory responses were inhibited by iv infusion of the predominantly MT_2-selective melatonin receptor antagonist luzindole (18-fold selectivity MT_2>MT_1).

Luminal melatonin is a potent stimulator of the HCO_3^- secretion by the duodenal mucosa (105). When rats were pretreated with iv luzindole, the effects of luminal melatonin were efficiently abolished (Fig. 5). The ganglion-blocking agent hexamethonium (a nicotinic receptor antagonist) reduced the magnitude of the stimulatory effect of luminal melatonin on HCO_3^- secretion (105). It should be noted that the HCO_3^- secretory rate always remained significantly higher than that in untreated controls. Stimulation of HCO_3^- secretion by local intestinal melatonin seems to be in line with the finding that melatonin increased the intracellular Ca^{2+} (Fig. 6) in isolated duodenal enterocytes (108). Taken together, these observations suggest an action of melatonin on receptors at duodenal enterocytes as well as on such receptors in the ENS. Neither luzindole at a dose that inhibited the stimulation by exogenous melatonin, nor another melatonin receptor antagonist (4-P-PDOT) affected spontaneous (basal) HCO_3^- secretion. This suggests that endogenous melatonin has no effect on basal secretion.

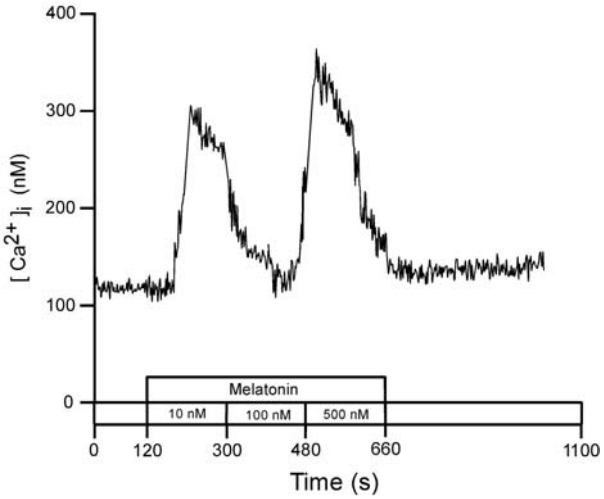

Fig. 6. Isolated human duodenal enterocytes in clusters increased their intracellular calcium concentration after perfusion with melatonin. [from Sjöblom *et al.* (108) with permission].

It should be pointed out that the doses of melatonin required for stimulation of duodenal HCO_3^- secretion seem to be much lower (>100-fold) than those tested in animal models of depressive disease (109) or used in humans for treatment of sleep disturbances or depression (110).

In spite of the considerably smaller total amounts of melatonin produced in and released from the pineal gland during the daytime, melatonin from the CNS may be important in the night-time control of the duodenal alkaline secretion and mucosal protection. The experiments in this study were started at around 9 am and were performed during the daytime when the pineal gland release of melatonin is low. They do not exclude the possibility of an increase in protective HCO_3^- secretion induced by the higher levels of melatonin that occur in darkness.

Recent studies in rats have shown that during the dark-phase, compared with the daylight phase, the frequency of duodenal and jejunal migrating motor complexes was increased by 20% and that this was abolished by the melatonin antagonist S20928 (111, 112). These authors concluded that pineal gland melatonin is involved in the dark-phase physiological control of the pre- and postprandial changes of intestinal motility.

Acid-induced secretion

The HCO_3^- secretion and in particular the secretory response to acid, as stated previously, is the principal mechanism in duodenal mucosal protection against acid expelled from the stomach. In the presence of a low pH in the duodenal lumen, ~pH 5 in rats (12) and ~pH 3 in humans (113), neural reflexes and mucosal production of prostaglandins are stimulated.

The results show that the melatonin antagonist luzindole decreases the HCO_3^-

response to acid (105). This suggests that melatonin is involved in mediating the increase in alkaline secretion induced by the presence of acid in the duodenal lumen.

It is reported that the surface epithelium and its close luminal vicinity are neutral even when the pH in the duodenal lumen is close to 2 (12, 39). This raises the intriguing question of how acid is sensed by the secreting epithelium. One hypothesis is that there are acid-sensitive neural receptors or cell filaments protruding into the surface gel that sense the luminal pH. Holm *et al*., on the other hand, have recently proposed that the stimulation of alkaline secretion may not be due to H^+ itself, but rather to the rapidly diffusible CO_2 generated within the mucus gel during the reaction between secreted HCO_3^- and H^+ ions (114).

There is some uncertainty to which extent cells in the villus tip actually secretes bicarbonate. Furthermore, at least part of the duodenal alkaline secretion originates from the villi, but the major bicarbonate output is from the crypt region, findings in line with the general theory that crypt cells have a secretory function whereas cells in the villi are mainly absorptive (65).

On the basis of recent studies of intracellular pH (pH_i) in apical villus cells *in situ*, it has been suggested as an additional mucosal protective mechanism that an acidic pHi facilitates basolateral uptake of base (HCO_3^-), increasing intracellular neutralization (115). This may be an important defense mechanism for the cells in the villus tip covered by a thin and loosely adherent mucus gel. Further evidence that would support the intracellular buffering mechanism is that CA II is located mainly in the villi and not in the duodenal crypts (70). This suggests that H^+ ions that enter the enterocyte can directly, together with HCO_3^-, be converted into water and CO_2. Concerning the deeper part of the villi and the crypt region secretion of bicarbonate probably plays a crucial role in the protection against the acid.

Central nervous influence of bicarbonate secretion

Intracerebroventricular infusion of the α_1-adrenoceptor agonist phenylephrine has previously been shown to increase the duodenal secretory rate in rats (59). In that study the increase in secretion was abolished by intravenous pretreatment with the ganglion-blocking agent hexamethonium and by icv (but not iv) administration of the adrenoceptor antagonist prazosin.

Centrally elicited stimulation of the secretion has also been observed after administration of some neuropeptides, including TRH (57), CRH (55) and bombesin (56), and of some benzodiazepines (58). Bilateral ligation of the vagal trunks at the sublaryngeal level inhibits the stimulation of duodenal (57) and pancreatic (116) secretion induced by TRH given icv. Truncal vagotomy alone also abolishes the responses to icv bombesin and icv or iv administration of benzodiazepines, but identical vagotomy does not affect the response to icv phenylephrine.

We found that icv phenylephrine stimulated the duodenal bicarbonate secretion (Fig. 7) (60). Sectioning all nerves around the carotid arteries, in contrast to sympathetic chain ectomy alone or truncal vagotomy alone, markedly inhibited the duodenal secretory response to icv phenylephrine (Fig. 8). Differences between effects of

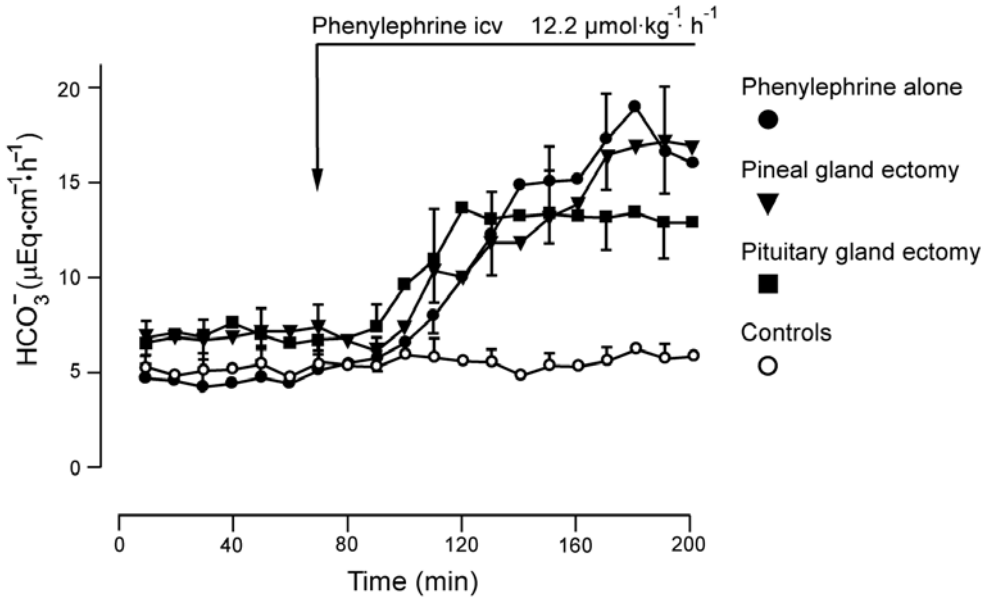

Fig. 7. Bicarbonate secretion increased significantly after administration of phenylephrine icv. Neither pineal nor pituitary glandectomy inhibited the secretory response to icv phenylephrine. [from Sjöblom *et al*. (60) with permission].

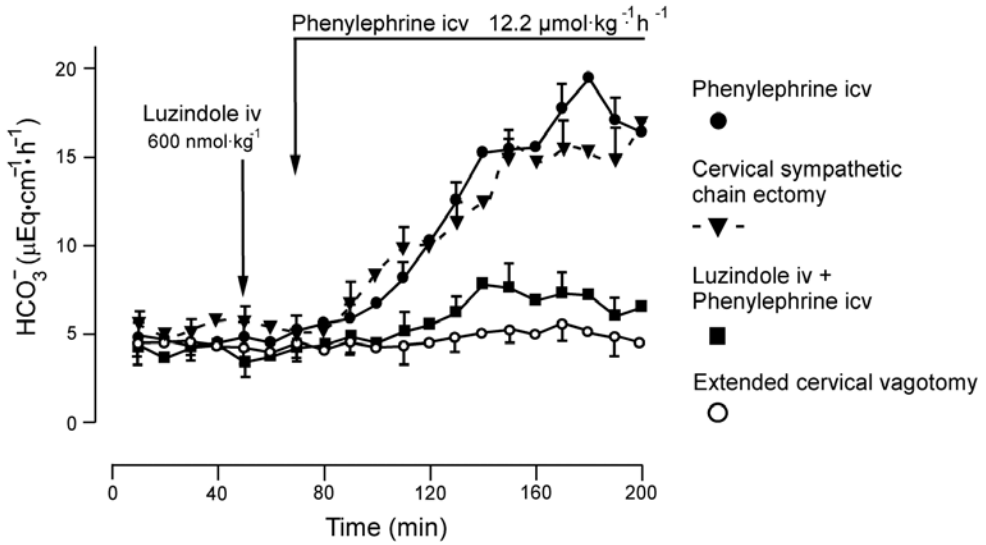

Fig. 8. Pretreatment with iv luzindole and section of both the vagal trunks and the sympathetic chains (at the sub-laryngeal level) significantly inhibited the duodenal bicarbonate secretion occurring in response to icv phenylephrine while cervical sympathectomy did not influence this response. [from Sjöblom *et al*. (60) with permission].

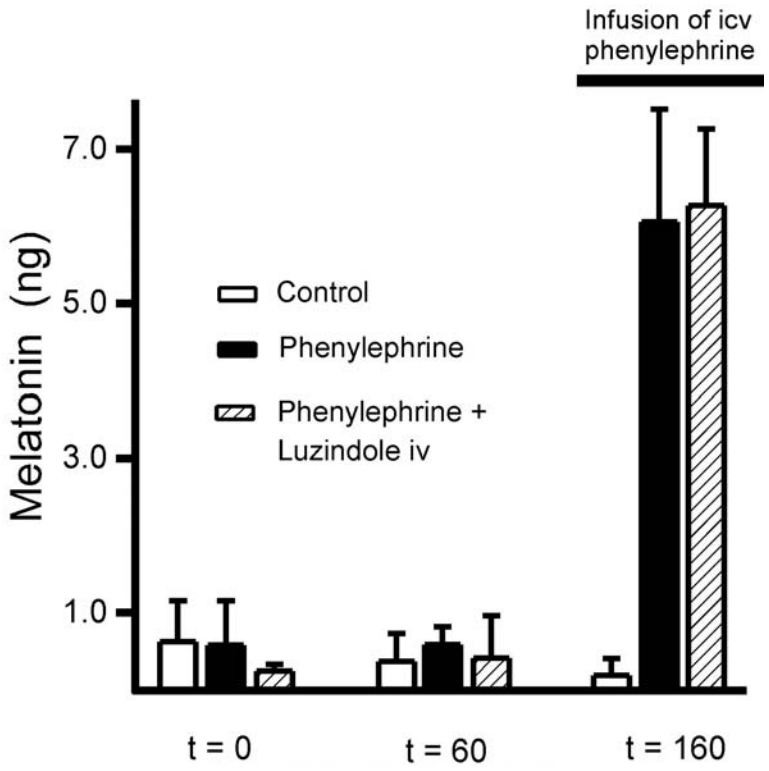

Fig. 9. When the duodenal mucosal bicarbonate secretion was stimulated with icv phenylephrine the total amount of melatonin in the luminal perfusate increased more than 10-fold. Animals pretreated with luzindole and given phenylephrine icv did not increase their alkaline secretion, but released the same amount of melatonin from the proximal duodenum. t = experimental time. [from Sjöblom *et al.* (119) with permission].

truncal vagotomy alone and of extended peri-carotid nervectomy have been observed previosly in studies of duodenal distension-secretory interactions (35). These differences may reflect intercommunications between the vagal and sympathetic neural pathways at the cervical level (117) and the anatomical mixing of pathways (118). Phenylephrine possibly mediates duodenal bicarbonate secretion by a different central mechanism than the other aforementioned neuropeptides and drugs.

Our results demonstrate that the melatonin receptor antagonist luzindole is a potent inhibitor of the duodenal secretory response to icv phenylephrine (60, 119). Central nervous melatonin had no effect on the secretion. It was also established that the basal HCO_3^- secretion in both pineal glandectomized and pituitary glandectomized animals was the same as that in untreated controls (Fig. 8) (60). Further, there were no differences between pineal and pituitary glandectomized rats and rats with these glands intact in respect to the secretory response to icv phenylephrine. Exclusion of a role of pituitary hormones was further confirmed by the finding that iv infusion of neither CRH, ACTH nor MSH affected the duodenal HCO_3^- secretion

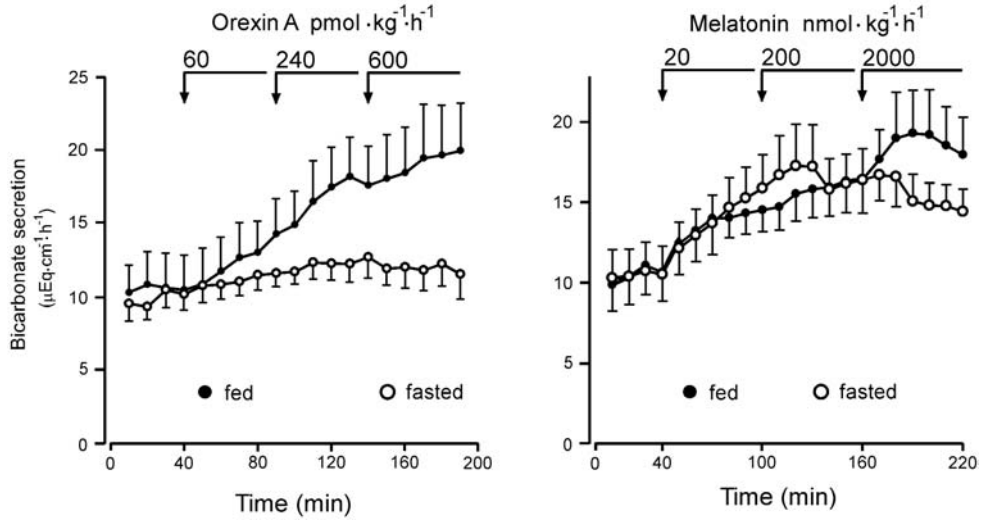

Fig. 10. Orexin-A (left) stimulates the duodenal mucosal bicarbonate secretion in fed animals but not in those fasted overnight. The hormone melatonin (right) is a stimulant of duodenal mucosal HCO_3^- secretion. No significant differences between the fed and fasted animals were observed. [from Flemström *et al*. (121) with permission].

in intact animals.

The release of melatonin from the duodenal mucosa into the luminal perfusate after icv administration of phenylephrine was investigated (119). Compared to control animals, phenylephrine induced an approximately 10-fold intraluminal increase in the melatonin level (Fig. 9). Pretreatment with luzindole almost abolished the marked increase in bicarbonate secretion induced by icv phenylephrine, but did not inhibit the luminal release of melatonin. The blood concentration of melatonin showed a tendency to an increase in pineal glandectomized rats after icv infusion of phenylephrine compared with that in such animals infused icv with vehicle alone (60). The tendency did not attain statistical significance. The combined results strongly suggest that melatonin is released from the intestinal mucosa after icv stimulation with phenylephrine.

Fasting influence on secretion

Ever since Pavlov presented his classical work at the end of the 19th century, most experimental studies of gastrointestinal physiology and pathophysiology in intact animals have been conducted after an overnight fasting period (120). The presence of food itself has considerable effects on intestinal functions (24). We therefore examined the question whether the fasting procedure *per se* influenced the duodenal alkaline secretory response to some secretagogues.

It was established that feeding induced or very markedly potentiated the response of the duodenal HCO_3^- secreting epithelium to some stimuli but not to others (121). The most pronounced difference was noted after administration of orexin-A. Orex-

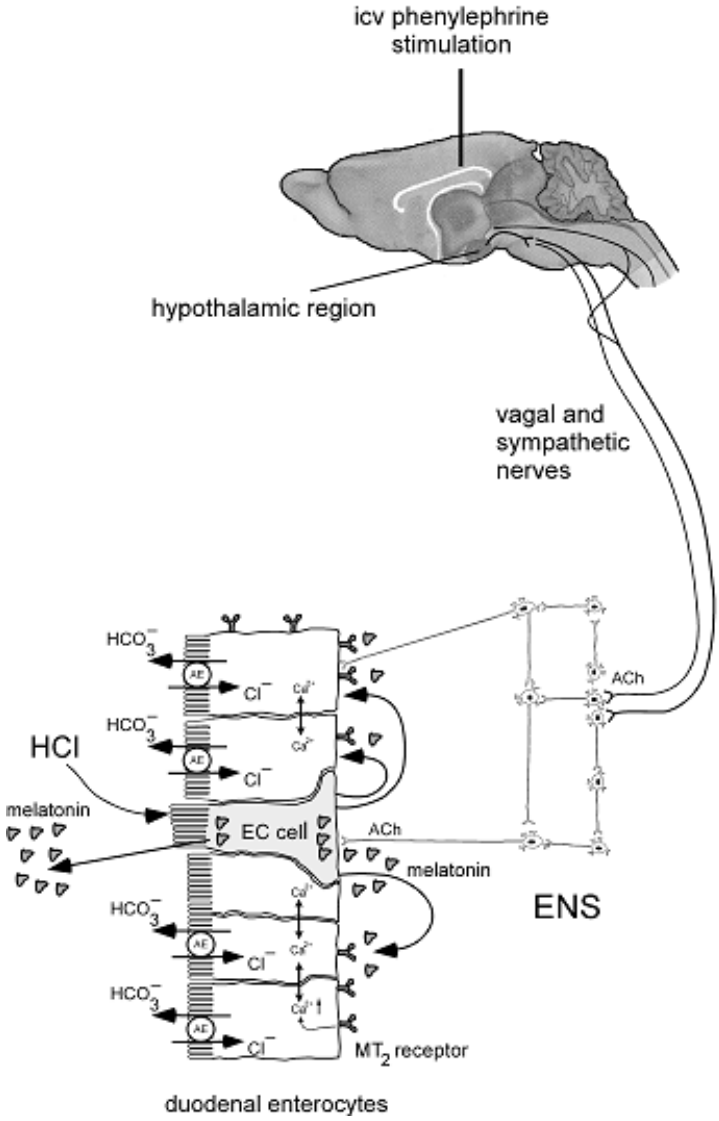

Fig. 11. A model illustrating the proposed role of melatonin in the regulation of duodenal mucosal bicarbonate secretion. The intracerebroventricularly infused phenylephrine binds to α_1-adrenoceptors in the hypothalamus. This activates the paraventricular nucleus, in the hypothalamus, which has substantial projections to the dorsal motor nucleus of the vagus nerve in the medulla oblongata. The vagal nerves, and cervical sympathetic fibers, then project to the enteric nervous system (ENS). The activation of the myenteric plexa, via nicotinic receptors, directly or indirectly via the submucosal plexa, innervates enterochromaffin cells in the intestinal mucosa to release melatonin. The melatonin has paracrine secretory actions at adjacent duodenal enterocytes. Melatonin also activates secretomotor neurons in the ENS, also leading to bicarbonate secretion. Binding of melatonin to the duodenal enterocytes increases intracellular calcium. The increase in calcium concentration activates the electroneutral HCO_3^-/Cl^- exchanger. Duodenal enterocytes intercommunicate with adjacent enterocytes to form a secretory functional syncytium. [from Sjöblom et al (119) with permission].

ins (A and B) were originally discovered in the CNS as peptides that increased the appetite for food in animals (122). Both orexins are also found in neurons and in neuroendocrine cells of the intestine (123, 124), and orexin immunoreactivity is co-localized with VIP and choline acetyltransferase (125). Both OX_1 and OX_2 receptors are thus expressed throughout the intestine in different cell types (125). OX_1 receptors are expressed mainly in neurons, while OX_2 receptors are expressed mainly by endocrine cells. The roles played by orexins in the gastrointestinal tract are not well understood. These peptides have been reported both to increase (123) and to reduce (126) the motility in the small intestine. Orexins thus probably act at several levels and some of their different actions are very probably mediated via other neurohumoral systems in the intestine.

Orexin-A caused a robust increase in the HCO_3^- secretory rate in fed animals, but did not affect that in animals fasted overnight (Fig. 10) (121). Similarly, fasting reduced the secretory sensitivity to the muscarinic agonist bethanechol by a dose-factor of ~100. In contrast, the HCO_3^- secretory responses to melatonin (Fig. 10) and VIP were not affected by overnight fasting. This demonstrates that feeding does not cause a general increase in the responsiveness of secretory peptides, but has a more selective action. The mechanisms by which feeding promotes responses to orexin and bethanechol are not clear. However, fasting may inhibit orexin and muscarinic responses by receptor desensitization or by changing the receptor density.

Enterocyte calcium signaling

Normally cells of various types keep their intracellular calcium concentration ($[Ca^{2+}]_i$) at a constant resting level (around 100 nM) (127). Upon receptor stimulation, extracellular influx or the release of calcium from intracellular storages can increase the intracellular calcium concentration within a very short time. This activation is the first step that finally leads to cellular events. One of the goals in cellular physiology is to understand how intracellular signaling systems regulate different cellular processes. As in other cells and tissues, agonist-induced $[Ca^{2+}]_i$ signaling is probably of utmost importance in control of various aspects of enterocyte function, but very few studies of $[Ca^{2+}]_i$ signaling in enterocytes have been reported. Small intestinal enterocytes *in situ* are programmed to a very restricted life span (2–5 days in rodents) (17). In addition, enterocytes *in situ* rapidly respond to irritating compounds in the intestinal lumen by apoptosis and expulsion. Very probably reflecting these physiological characteristics, small intestinal enterocytes appear more difficult than, for instance, gastric parietal cells or pancreatic β-cells to maintain viable after isolation (128). The results demonstrate that clusters of freshly isolated enterocytes from the proximal small intestine can be kept viable, providing a suitable model for studies of agonist-induced $[Ca^{2+}]_i$ signaling (108). The viability of the enterocytes in clusters, as studied by trypan blue exclusion, was good (>85% after six hours). It may be compared with the viability (10% after 2–4 hours) reported in studies of intracellular pH in acutely isolated villus tips from rat duodenum (129). The findings show further, for the first time, that melatonin has a direct

action on duodenal epithelium (108). Melatonin increases $[Ca^{2+}]_i$ in duodenal enterocytes from both rats and humans. Low concentrations of melatonin, with EC50 17.0 ± 2.6 nM, and of agonists 2-iodomelatonin and 2-ibmt, increased enterocyte $[Ca^{2+}]_i$. The receptor antagonists luzindole (MT_2>MT_1) and DH97 (90-fold selectivity MT_2>>MT_1; Teh & Sugden 1998) abolished the responses to melatonin.

In the main type of melatonin-induced signaling pattern, $[Ca^{2+}]_i$ spiked rapidly and then slowly returned to baseline or almost baseline values (Fig. 6). In a smaller number of cells, $[Ca^{2+}]_i$ tended to remain at a plateau level. The magnitude of the initial rise in $[Ca^{2+}]_i$ was dependent on the perfusate concentration of melatonin in some enterocytes. In other experiments, there was a rapid down-regulation of the response, similar to the desensitization observed with CCK-8 in duodenal enterocytes in primary culture (130). Interestingly, there is a dose-dependent increase in mucosal HCO_3^- secretion as well as apparent desensitization of the response when melatonin is administered to rat duodenum *in situ* (60). The latter occurs during infusion of a relatively high dose (2000 nmol•kg-1•h-1) of the compound. The similarity suggest a role of $[Ca^{2+}]_i$ in mediating melatonin-induced stimulation of the secretion.

Perfusion with calcium-free solutions abolished the plateau phase but not the initial increase in $[Ca^{2+}]_i$ in rat duodenal enterocytes. A biphasic Ca^{2+} response to agonists is characteristic of many non-excitable cell types and a substantial amount of evidence indicates that the initial spike in $[Ca^{2+}]_i$ is the result of release of Ca^{2+} from an intracellular storage site(s), whereas the later sustained phase is due to the influx of Ca^{2+} across the cell membrane. In duodenal enterocytes in primary culture, carbachol (acting at muscarinic M3 receptors) induced biphasic $[Ca^{2+}]_i$ responses (130), similar to those observed with melatonin. The sustained phase of the rise in $[Ca^{2+}]_i$ was, as found here with melatonin, attributable to extracellular Ca^{2+}.

Another interesting type of $[Ca^{2+}]_i$ response to melatonin was observed in the responding preparations. The initial transient increase in $[Ca^{2+}]_i$ was followed by slow rhythmic oscillations in $[Ca^{2+}]_i$ of high amplitude which spread throughout the cluster of enterocytes. Oscillations (and spread of oscillations) were never observed in the absence of Ca^{2+} in the perfusate, suggesting that influx of Ca^{2+} contributes to the phenomenon. Presence of extracellular Ca^{2+} may also be important, however, in maintaining mucosal cell-to-cell communication. The melatonin-induced oscillations observed in clusters of rat as well as human duodenal enterocytes occurred with about the same frequency (~ one period in 5 min) (108). Thus, there was no decline but rather a time-dependent gain in amplitude, and oscillations spread within the cell cluster.

We used isolated clusters of enterocytes, a preparation that should be devoid of neural tissue (108). Pretreatment with the muscarinic antagonist atropine did not affect the basal $[Ca^{2+}]_i$ or the response to melatonin, further excluding the possibility that melatonin might act at muscarinic receptors at the enterocyte cell membrane. Cellular responses depend on the pattern and magnitude of $[Ca^{2+}]_i$ signaling (131) and as stated previously calcium is one of the major regulators of physiological

functions. We have preliminary data that support our theory that the increase in calcium activates enterocyte stimulus-secretion coupling. In clusters of duodenal enterocytes melatonin affects intracellular pH, suggesting activation of enterocyte acid/base transport (Sjöblom 2003, *unpublished observations*). The duodenal secretagogues dopamine, VIP and prostaglandin E2 increase cAMP production (132, 133), and intracellular cGMP is involved in mediating the HCO_3^- secretory responses to guanylin and heat stable enterotoxin (ST_a) (64). Interactions between these pathways and enterocyte $[Ca^{2+}]_i$ signaling would seem likely.

Ion transporters

Duodenal enterocytes export HCO_3^- by an apical Cl^-/ HCO_3^- exchanger as well as an anion conductive pathway, very probably the CFTR channel. Anion-channel dependent transport of HCO_3^- may be a property of crypt cells, where the CFTR channels are expressed at the greatest levels. The apical transporters in the villus cells, in contrast, constitute the electroneutral anion exchanger.

The duodenal transmucosal electrical potential difference was measured in some experiments (60). The PD was recorded between the duodenal mucosa and the posterior vena cava with a high-input impedance voltmeter via matched calomel half-cells. The results demonstrate that melatonin stimulates duodenal mucosal transport of HCO_3^- without a significant change in PD, indicating an electroneutral transport process.

Clinical relevance

Convincing evidence that melatonin stimulates HCO_3^- secretion in the rat has been provided in this work. Furthermore, centrally elicited stimulation induces duodenal luminal release of melatonin, most probably from the intestinal EC cells. In enterocytes, both of human and rat origin, melatonin increases intracellular calcium, suggesting that intestinal actions of the hormone may be similar in the two species.

Circadian rhythms in pain and discomfort are pathological features in gastroduodenal ulcer, and the incidence of gastroduodenal ulcer is reported to show peaks at certain periods of the year (134). Melatonin is the major hormone regulating circadian rhythms. Interestingly, there is a strong disturbance of melatonin secretion in both the exacerbation and in the remission stage of the disease in patients with duodenal ulcer (135). Studies in fasting animals have shown that the gastric secretions of HCO_3^- and mucus, both important in mucosal protection, exhibit day and night rhythms with peak times different from those of the mucosa-aggressive H^+ secretion (136). This phase shift in secretory rhythms may, in theory, result in circadian variations in mucosal vulnerability to acid injury.

Conclusions

The work presented in this study provides new and interesting knowledge about the central nervous as well as the peripheral regulation of the mucosa-protective bicar-

bonate secretion by the duodenal mucosa. The conclusions are based upon integrative animal experiments *in vivo* combined with *in vitro* experiments with tissues of human and rat origin. Fig. 11 summarizes the proposed role of melatonin in the regulation of HCO_3^- transport by the duodenal epithelium. The main findings are summarized as follows:

- Melatonin is a potent stimulant of duodenal mucosal bicarbonate secretion and seems to be involved in the acid stimulation of alkaline secretion.
- Endogenous melatonin is released from the duodenal mucosa after central nervous stimulation with the _1-adrenoceptor agonist phenylephrine and, furthermore, stimulates duodenal mucosal bicarbonate secretion.
- Intraarterial infusion close to the duodenum is more effective than intravenous infusion of duodenal secretagogues and also minimizes central nervous actions of infused drugs.
- Overnight fasting, a standard procedure in experimental studies of intestinal function, rapidly and profoundly downregulates the responses to the duodenal secretagogues orexin-A and bethanechol, but not to melatonin or VIP.
- A new method for isolating viable duodenal enterocytes was established. Clusters consisting of 10–50 cells of either human or rat origin are more viable than single cells and allow studies of both intracellular and intercellular signaling.
- Melatonin increases the intracellular calcium concentration in both human and rat duodenal enterocytes in clusters and appears to induce release of calcium from intracellular stores as well as influx of extracellular calcium. Further, duodenal enterocytes seem to function as a syncytium.

ACKNOWLEDGMENTS

The author expresses his sincere gratitude to Professor Gunnar Flemström, who supervised this work, and the coauthors for significant collaboration. This work was supported by grants from the Swedish Research Council (3515, 12205), the Wallenberg Foundation, the Anna Cederberg foundation, the Swedish Pharmaceutical Society, the Scandinavian Physiological Society, the Swedish Society of Medicine, the Medical Faculty of Uppsala University, Gästrike-Hälsinge Nation and the Swedish Society for Medical Research.

REFERENCES

1. Guyton AC, Hall JE. Textbook of Medical Physiology. Saunders W B Co, 2000.
2. Schwarz K. Ueber penetrierende Magen- und Jejunalgeschüre. Beiträge zur Klinischen Chirurgie, 67: 96–128, 1910.
3. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1: 1311–1315, 1984.
4. Kuipers EJ, Thijs JC, Festen HP. The prevalence of Helicobacter pylori in peptic ulcer disease. Aliment Pharmacol Ther, 9: 59–69, 1995.
5. Granstam SO, Jönson C, Fändriks L, Holm L, Flemström G. Effects of cigarette smoke and nicotine on duodenal bicarbonate secretion in the rabbit and the rat. J Clin Gastroenterol, 1: S19–S24, 1990.
6. Ainsworth MA, Hogan DL, Koss MA, Isenberg JI. Cigarette smoking inhibits acid-stimulated duodenal mucosal bicarbonate secretion. Ann Intern Med, 119: 882–886, 1993.

7. Cooke AR. Gastric damage by drugs and the role of the mucosal barrier. Aust N Z J Med, 6: 26–32, 1976.
8. Stern AI, Hogan DL, Kahn LH, Isenberg JI. Protective effect of acetaminophen against aspirin- and ethanol-induced damage to the human gastric mucosa. Gastroenterology, 86: 728–733, 1984.
9. Rhodes J, Apsimon HT, Lawrie JH. pH of the contents of the duodenal bulb in relation to duodenal ulcer. Gut, 7: 502–508, 1966.
10. Rune SJ, Viskum K. Duodenal pH values in normal controls and in patients with duodenal ulcer. Gut, 10: 569–571, 1969.
11. Flemström G. Gastric and duodenal mucosal secretion of bicarbonate. Physiology of the gastrointestinal tract. (ed. LR Johnson et al.),. vol 2. Raven, New York, 1285–1309, 1994.
12. Flemström G, Kivilaakso E. Demonstration of a pH gradient at the luminal surface of rat duodenum in vivo and its dependence on mucosal alkaline secretion. Gastroenterology, 84: 787–794, 1983.
13. Flemström G, Isenberg JI. Gastroduodenal mucosal alkaline secretion and mucosal protection. News Physiol Sci, 16: 23–28, 2001.
14. Allen A, Flemström G, Garner A, Kivilaakso E. Gastroduodenal mucosal protection. Physiol Rev, 73: 823–857, 1993.
15. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol, 280: G922–G929, 2001.
16. Flemström G, Hällgren A, Nylander O, Engstrand L, Wilander E, Allen A. Adherent surface mucus gel restricts diffusion of macromolecules in rat duodenum in vivo. Am J Physiol Gastrointest Liver Physiol, 277: G375–G382, 1999.
17. Lipkin M. Proliferation and differentiation of normal and diseased gastrointestinal cells. Physiology of the gastrointestinal tract. (ed. LR Johnson et al.),. vol 2. Raven, New York, 255–284, 1987.
18. Lundgren O. Microcirculation of the gastrointestinal tract. Handbook of physiology. The cardiovascular system. Microcirculation, (ed. Renkin & Michel), vol. 4, 799–864, 1985.
19. Jodal M, Lundgren O. Neurohumoral control of gastrointestinal blood flow. Handbook in physiology: Section 6, The gastrointestinal system I, 1301–1334, 1989.
20. Holm L, Flemström G, Nylander O. Duodenal alkaline secretion in rabbits: influence of artificial ventilation. Acta Physiol Scand, 138: 471–478, 1990.
21. Tsukamoto Y, Goto H, Hase S, Arisawa T, Ohara A, Suzuki T, Hoshino H, Endo H, Hamajima E, Ohmiya N. Effect of duodenal mucosal blood flow on duodenal alkaline secretion in rats. Digestion, 51: 198–202, 1992.
22. Gershon MD, Kirchgessner AL, Wade PR. Functional anatomy of the enteric nervous system. Physiology of the gastrointestinal tract. (ed. LR Johnson et al.),. vol 1, Raven, New York, 381–422,1994.
23. Kirchgessner AL, Gershon MD. Identification of vagal efferent fibers and putative target neurons in the enteric nervous system of the rat. J Comp Neurol, 285: 38–53, 1989.
24. Furness JB, Kunze WA, Clerc N. Nutrient tasting and signaling mechanisms in the gut. II. The intestine as a sensory organ: neural, endocrine, and immune responses. Am J Physiol Gastrointest Liver Physiol, 277: G922–G928, 1999.
25. Takeuchi K, Yagi K, Kato S, Ukawa H. Roles of prostaglandin E-receptor subtypes in gastric and duodenal bicarbonate secretion in rats. Gastroenterology, 113: 1553–1559, 1997.
26. Flemström G, Kivilaakso E, Briden S, Nylander O, Jedstedt G. Gastroduodenal bicarbonate secretion in mucosal protection. Possible role of vasoactive intestinal peptide and opiates. Dig Dis Sci, 30: 63S–68S, 1985.
27. Wolosin JD, Thomas FJ, Hogan DL, Koss MA, O'Dorisio TM, Isenberg JI. The effect of vasoactive intestinal peptide, secretin, and glucagon on human duodenal bicarbonate secretion. Scand J Gastroenterol, 24: 151–157, 1989.
28. Glad H, Ainsworth MA, Svendsen P, Fahrenkrug J, Schaffalitzky De Muckadell OB. Effect of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide on pancreatic, hepatic and duodenal mucosal bicarbonate secretion in the pig. Digestion, 67: 56–66, 2003.
29. Konturek SJ, Bilski J, Tasler J, Laskiewicz J. Gut hormones in stimulation of gastroduodenal alkaline secretion in conscious dogs. Am J Physiol Gastrointest Liver Physiol, 248: G687–G691, 1985.
30. Wenzl E, Feil W, Starlinger M, Schiessel R. Alkaline secretion. A protective mechanism against acid injury in rabbit duodenum. Gastroenterology, 92: 709–715, 1987.

31. Takeuchi K, Takehara K, Kato S, Yagi K. PACAPs stimulate duodenal bicarbonate secretion at PACAP receptors in the rat. Am J Physiol Gastrointest Liver Physiol, 272: G646–G653, 1997.
32. Takeuchi K, Yagi K, Sugamoto S, Furukawa O, Kawauchi S. Involvement of PACAP in acid-induced HCO3– response in rat duodenums. Pharmacol Res, 38: 475–480, 1998.
33. Johansson B, Holm M, Ewert S, Casselbrant A, Pettersson A, Fändriks L. Angiotensin II type 2 receptor-mediated duodenal mucosal alkaline secretion in the rat. Am J Physiol Gastrointest Liver Physiol, 280: G1254–G1260, 2001.
34. Takeuchi K, Kagawa S, Mimaki H, Aoi M, Kawauchi S. COX and NOS isoforms involved in acid-induced duodenal bicarbonate secretion in rats. Dig Dis Sci, 47: 2116–2124, 2002.
35. Sababi M, Nylander O. Elevation of intraluminal pressure and cyclooxygenase inhibitors increases duodenal alkaline secretion. Am J Physiol Gastrointest Liver Physiol, 266: G22–G30, 1994.
36. Nylander O, Hällgren A, Sababi M. COX inhibition excites enteric nerves that affect motility, alkaline secretion, and permeability in rat duodenum. Am J Physiol Gastrointest Liver Physiol, 281: G1169–G1178, 2001.
37. Takeuchi K, Ohuchi T, Miyake H, Niki S, Okabe S. Effects of nitric oxide synthase inhibitors on duodenal alkaline secretion in anesthetized rats. Eur J Pharmacol, 231: 135–138, 1993.
38. Hällgren A, Flemström G, Sababi M, Nylander O. Effects of nitric oxide inhibition on duodenal function in rat: involvement of neural mechanisms. Am J Physiol, 269: G246–G254, 1995.
39. Sababi M, Nilsson E, Holm L. Mucus and alkali secretion in the rat duodenum: effects of indomethacin, N omega-nitro-L-arginine, and luminal acid. Gastroenterology, 109: 1526–1534, 1995.
40. Sababi M, Nylander O. Comparative study of the effects of nitric oxide synthase and cyclo-oxygenase inhibition on duodenal functions in rats anaesthetized with inactin, urethane or alpha-chloralose. Acta Physiol Scand, 158: 45–52, 1996.
41. Holm M, Johansson B, von Bothmer C, Jönson C, Pettersson A, Fändriks L. Acid-induced increase in duodenal mucosal alkaline secretion in the rat involves the L-arginine/NO pathway. Acta Physiol Scand, 161: 527–532, 1997.
42. Holm M, Johansson B, Pettersson A, Fändriks L. Acid-induced duodenal mucosal nitric oxide output parallels bicarbonate secretion in the anaesthetized pig. Acta Physiol Scand, 162: 461–468, 1998.
43. Bilski J, Konturek SJ. Role of nitric oxide in gastroduodenal alkaline secretion. J Physiol Pharmacol, 45: 541–553, 1994.
44. Sugamoto S, Kawauch S, Furukawa O, Mimaki TH, Takeuchi K. Role of endogenous nitric oxide and prostaglandin in duodenal bicarbonate response induced by mucosal acidification in rats. Dig Dis Sci, 46: 1208–1216, 2001.
45. Isenberg JI, Selling JA, Hogan DL, Koss MA. Impaired proximal duodenal mucosal bicarbonate secretion in patients with duodenal ulcer. N Engl J Med, 316: 374–379, 1987.
46. Hogan DL, Rapier RC, Dreilinger A, Koss MA, Basuk PM, Weinstein WM, Nyberg LM, Isenberg JI. Duodenal bicarbonate secretion: eradication of Helicobacter pylori and duodenal structure and function in humans. Gastroenterology, 110: 705–716, 1996.
47. Fändriks L, von Bothmer C, Johansson B, Holm M, Bolin I, Pettersson A. Water extract of Helicobacter pylori inhibits duodenal mucosal alkaline secretion in anesthetized rats. Gastroenterology, 113: 1570–1575, 1997.
48. Berthoud HR, Jedrzejewska A, Powley TL. Simultaneous labeling of vagal innervation of the gut and afferent projections from the visceral forebrain with dil injected into the dorsal vagal complex in the rat. J Comp Neurol, 301: 65–79, 1990.
49. Fändriks L. Sympatho-adrenergic inhibition of vagally induced gastric motility and gastroduodenal HCO-3 secretion in the cat. Acta Physiol Scand, 128: 555–562, 1986.
50. Nylander O, Flemström G, Delbro D, Fändriks L. Vagal influence on gastroduodenal HCO3– secretion in the cat in vivo. Am J Physiol Gastrointest Liver Physiol, 252: G522–G528, 1987.
51. Jönson C, Nylander O, Flemström G, Fändriks L. Vagal stimulation of duodenal HCO3(-)-secretion in anaesthetized rats. Acta Physiol Scand, 128: 65–70, 1986.
52. Fändriks L, Jönson C. Vagal and sympathetic control of gastric and duodenal bicarbonate secretion. J Intern Med Suppl, 732: 103–107, 1990.
53. Ballesteros MA, Wolosin JD, Hogan DL, Koss MA, Isenberg JI. Cholinergic regulation of human proximal duodenal mucosal bicarbonate secretion. Am J Physiol Gastrointest Liver Physiol, 261: G327–G331, 1991.

54. Konturek SJ, Thor P. Relation between duodenal alkaline secretion and motility in fasted and sham-fed dogs. Am J Physiol Gastrointest Liver Physiol, 251: G591–G596, 1986.
55. Lenz HJ. Regulation of duodenal bicarbonate secretion during stress by corticotropin-releasing factor and beta-endorphin. Proc Natl Acad Sci USA, 86: 1417–1420, 1989.
56. Flemström G, Jedstedt G. Stimulation of duodenal mucosal bicarbonate secretion in the rat by brain peptides. Gastroenterology, 97: 412–420, 1989.
57. Lenz HJ, Vale WW, Rivier JE. TRH-induced vagal stimulation of duodenal HCO3– mediated by VIP and muscarinic pathways. Am J Physiol Gastrointest Liver Physiol, 257: G677–G682, 1989.
58. Säfsten B, Jedstedt G, Flemström G. Effects of diazepam and Ro 15–1788 on duodenal bicarbonate secretion in the rat. Gastroenterology, 101: 1031–1038, 1991.
59. Larson GM, Jedstedt G, Nylander O, Flemström G. Intracerebral adrenoceptor agonists influence rat duodenal mucosal bicarbonate secretion. Am J Physiol Gastrointest Liver Physiol, 271: G831–G840, 1996.
60. Sjöblom M, Jedstedt G, Flemström G. Peripheral melatonin mediates neural stimulation of duodenal mucosal bicarbonate secretion. J Clin Invest, 108: 625–633, 2001.
61. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. Am J Anat, 141: 537–561, 1974.
62. Clarke LL, Harline MC. Dual role of CFTR in cAMP-stimulated HCO3– secretion across murine duodenum. Am J Physiol Gastrointest Liver Physiol, 274: G718–G726, 1998.
63. Hogan DL, Crombie DL, Isenberg JI, Svendsen P, Schaffalitzky de Muckadell OB, Ainsworth MA. CFTR mediates cAMP- and Ca2+-activated duodenal epithelial HCO3– secretion. Am J Physiol Gastrointest Liver Physiol, 272: G872–G878, 1997.
64. Seidler U, Blumenstein I, Kretz A, Viellard-Baron D, Rossmann H, Colledge WH, Evans M, Ratcliff R, Gregor M. A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca(2+)-dependent HCO3- secretion. J Physiol, 505: 411–423, 1997.
65. Chang EB, Rao MC. Intestinal water and electrolyte transport. Mechanisms of physiological and adaptive responses. In: Johnson LR, Jacobson ED, Christensen J, Alpers D, Walsh JH, eds. Physiology of the gastrointestinal tract. 3 ed. New York: Raven, 1994:2027–2081.
66. Knutson TW, Koss MA, Hogan DL, Isenberg JI, Knutson L. Acetazolamide inhibits basal and stimulated HCO3– secretion in the human proximal duodenum. Gastroenterology, 108: 102–107, 1995.
67. Muallem R, Reimer R, Odes HS, Schwenk M, Beil W, Sewing KF. Role of carbonic anhydrase in basal and stimulated bicarbonate secretion by the guinea pig duodenum. Dig Dis Sci, 39: 1078–1084, 1994.
68. Takeuchi K, Ohtsuki H, Okabe S. Mechanisms of protective activity of 16,16-dimethyl PGE2 and acetazolamide on gastric and duodenal lesions in rats. Dig Dis Sci, 31: 406–411, 1986.
69. Takeuchi K, Tanaka H, Furukawa O, Okabe S. Gastroduodenal HCO3-secretion in anesthetized rats: effects of 16,16-dimethyl PGE2, topical acid and acetazolamide. Jpn J Pharmacol, 41: 87–99, 1986.
70. Lönnerholm G, Knutson L, Wistrand PJ, Flemström G. Carbonic anhydrase in the normal rat stomach and duodenum and after treatment with omeprazole and ranitidine. Acta Physiol Scand, 136: 253–262, 1989.
71. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal factor that lightens melanocytes. J Am Chem Soc, 80: 2587, 1958.
72. Pang SF, Dubocovich ML, Brown GM. Melatonin receptors in peripheral tissues: a new area of melatonin research. Biol Signals, 2: 177–180, 1993.
73. Vanecek J. Cellular mechanisms of melatonin action. Physiol Rev, 78: 687–721, 1998.
74. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev, 12: 151–180, 1991.
75. Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, Ortiz GG, Acuna-Castroviejo D. A review of the evidence supporting melatonin's role as an antioxidant. J Pineal Res, 18: 1–11, 1995.
76. Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR. Significance of melatonin in antioxidative defense system: reactions and products. Biol Signals Recept, 9: 137–159, 2000.
77. Brown GM. Light, melatonin and the sleep-wake cycle. J Psychiatry Neurosci, 19: 345–353, 1994.

78. Raikhlin NT, Kvetnoy IM, Tolkachev VN. Melatonin may be synthesised in enterochromaffin cells. Nature, 255: 344–345, 1975.
79. Stefulj J, Hörtner M, Ghosh M, Schauenstein K, Rinner I, Wölfler A, Semmler J, Liebmann PM. Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J Pineal Res, 30: 243–247, 2001.
80. Bubenik GA, Brown GM. Pinealectomy reduces melatonin levels in the serum but not in the gastrointestinal tract of rats. Biol Signals, 6: 40–44, 1997.
81. Huether G. The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. Experientia, 49: 665–670, 1993.
82. Bubenik GA. Localization, physiological significance and possible clinical implication of gastrointestinal melatonin. Biol Signals Recept, 10: 350–366, 2001.
83. Bubenik GA. Gastrointestinal melatonin: localization, function, and clinical relevance. Dig Dis Sci, 47: 2336–2348, 2002.
84. Gershon MD. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. Aliment Pharmacol Ther, 13 Suppl 2: 15–30, 1999.
85. Huether G, Poeggeler B, Reimer A, George A. Effect of tryptophan administration on circulating melatonin levels in chicks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. Life Sci, 51: 945–953, 1992.
86. Reiter RJ. The melatonin rhythm: both a clock and a calendar. Experientia, 49: 654–664, 1993.
87. Bubenik GA, Pang SF, Hacker RR, Smith PS. Melatonin concentrations in serum and tissues of porcine gastrointestinal tract and their relationship to the intake and passage of food. J Pineal Res, 21: 251–256, 1996.
88. Bubenik GA, Hacker RR, Brown GM, Bartos L. Melatonin concentrations in the luminal fluid, mucosa, and muscularis of the bovine and porcine gastrointestinal tract. J Pineal Res, 26: 56–63, 1999.
89. Huether G, Messner M, Rodenbeck A, Hardeland R. Effect of continuous melatonin infusions on steady-state plasma melatonin levels in rats under near physiological conditions. J Pineal Res, 24: 146–151, 1998.
90. Huether G, Hajak G, Reimer A, Poeggeler B, Blömer M, Rodenbeck A, Rüther E. The metabolic fate of infused L-tryptophan in men: possible clinical implications of the accumulation of circulating tryptophan and tryptophan metabolites. Psychopharmacology, 109: 422–342, 1992.
91. Yaga K, Reiter RJ, Richardson BA. Tryptophan loading increases daytime serum melatonin levels in intact and pinealectomized rats. Life Sci, 52: 1231–1238, 1993.
92. Yeleswaram K, McLaughlin LG, Knipe JO, Schabdach D. Pharmacokinetics and oral bioavailability of exogenous melatonin in preclinical animal models and clinical implications. J Pineal Res, 22: 45–51, 1997.
93. Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA. Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci, 72: 2183–2198, 2003.
94. Masana MI, Dubocovich ML. Melatonin receptor signaling: finding the path through the dark. Sci STKE, 2001: PE39, 2001.
95. Bubenik GA, Brown GM, Grota LJ. Immunohistological localization of melatonin in the rat digestive system. Experientia, 33: 662–663, 1977.
96. Dubocovich ML, Cardinali DP, Guardiola-Lemaitre B, Hagan RM, Krause DN, Sugden D, Vanhoutte PM, Yocca FD. Melatonin receptors. The IUPHAR compendium of receptor characterisation and classification. London: IUPHAR Media, 1998:187–193.
97. Hoffman RA, Reiter RJ. Rapid pinealectomy in hamsters and other small rodents. Anat Rec, 153: 19–21, 1965.
98. Shariatmadari R, Lund PE, Krijukova E, Sperber GO, Kukkonen JP, Åkerman KEO. Reconstitution of neurotransmission by determining communication between differentiated PC12 pheochromocytoma and HEL 92.1.7 erythroleukemia cells. Pflügers Arch, 442: 312–320, 2001.
99. Ponomarew SJ. Die Physiologie des Brunnerschen Teiles des Zwölffingerdarms, Thesis in St. Petersburg, Russia. J Botkinshospital: 1–8, 1902.
100. Ainsworth MA, Ladegaard L, Svendsen P, Cantor P, Olsen O, Schaffalitzky de Muckadell OB. Pancreatic, hepatic, and duodenal mucosal bicarbonate secretion during infusion of secretin and cholecystokinin. Evidence of the importance of hepatic bicarbonate in the neutralization of acid in the duodenum of anaesthetized pigs. Scand J Gastroenterol, 26: 1035–1041, 1991.
101. Ainsworth MA, Svendsen P, Ladegaard L, Cantor P, Olsen O, Schaffalitzky de Muckadell OB.

Relative importance of pancreatic, hepatic, and mucosal bicarbonate in duodenal neutralization of acid in anaesthetized pigs. Scand J Gastroenterol, 27: 343–349, 1992.
102. Raikhlin NT, Kvetnoy IM. Melatonin and enterochromaffine cells. Acta Histochem, 55: 19–24, 1976.
103. Lundberg JM, Dahlström A, Bylock A, Ahlman H, Pettersson G, Larsson I, Hansson HA, Kewenter J. Ultrastructural evidence for an innervation of epithelial enterochromaffine cells in the guinea pig duodenum. Acta Physiol Scand, 104: 3–12, 1978.
104. Joo NS, London RM, Kim HD, Forte LR, Clarke LL. Regulation of intestinal Cl- and HCO3- secretion by uroguanylin. Am J Physiol Gastrointest Liver Physiol, 274: G633–G644, 1998.
105. Sjöblom M, Flemström G. Melatonin in the duodenal lumen is a potent stimulant of mucosal bicarbonate secretion. J Pineal Res, 34: 288–293, 2003.
106. Tan D, Manchester LC, Reiter RJ, Qi W, Hanes MA, Farley NJ. High physiological levels of melatonin in the bile of mammals. Life Sci, 65: 2523–2529, 1999.
107. Messner M, Huether G, Lorf T, Ramadori G, Schworer H. Presence of melatonin in the human hepatobiliary-gastrointestinal tract. Life Sci, 69: 543–551, 2001.
108. Sjöblom M, Säfsten B, Flemström G. Melatonin-induced calcium signaling in clusters of human and rat duodenal enterocytes. Am J Physiol Gastrointest Liver Physiol, 284: G1034–G1044, 2003.
109. Overstreet DH, Pucilowski O, Retton MC, Delagrange P, Guardiola-Lemaitre B. Effects of melatonin receptor ligands on swim test immobility. Neuroreport, 9: 249–253, 1998.
110. Wetterberg L. Melatonin and clinical application. Reprod Nutr Dev, 39: 367–382, 1999.
111. Merle A, Delagrange P, Renard P, Lesieur D, Cuber JC, Roche M, Pellissier S. Effect of melatonin on motility pattern of small intestine in rats and its inhibition by melatonin receptor antagonist S 22153. J Pineal Res, 29: 116–124, 2000.
112. Merle A, Faucheron JL, Delagrange P, Renard P, Roche M, Pellissier S. Nycthemeral variations of cholecystokinin action on intestinal motility in rats: effects of melatonin and S 20928, a melatonin receptor antagonist. Neuropeptides, 34: 385–391, 2000.
113. Feitelberg SP, Hogan DL, Koss MA, Isenberg JI. pH threshold for human duodenal bicarbonate secretion and diffusion of CO2. Gastroenterology, 102: 1252–1258, 1992.
114. Holm M, Johansson B, Pettersson A, Fändriks L. Carbon dioxide mediates duodenal mucosal alkaline secretion in response to luminal acidity in the anesthetized rat. Gastroenterology, 115: 680–685, 1998.
115. Kaunitz JD, Akiba Y. Integrated duodenal protective response to acid. Life Sci, 69: 3073–3081, 2001.
116. Messmer B, Zimmerman FG, Lenz HJ. Regulation of exocrine pancreatic secretion by cerebral TRH and CGRP: role of VIP, muscarinic, and adrenergic pathways. Am J Physiol Gastrointest Liver Physiol, 264: G237–G242, 1993.
117. Weijnen JA, Surink S, Verstralen MJ, Moerkerken A, De Bree GJ, Bleys RL. Main trajectories of nerves that traverse and surround the tympanic cavity in the rat. J Anat, 197: 247–262, 2000.
118. Yang M, Zhao X, Miselis RR. The origin of catecholaminergic nerve fibers in the subdiaphragmatic vagus nerve of rat. J Auton Nerv Syst, 76: 108–117, 1999.
119. Sjöblom M, Flemström G. Central nervous alpha1-adrenoceptor stimulation induces duodenal luminal release of melatonin. J Pineal Res, 36: 103–108, 2004.
120. Pavlov JP. Die Arbeit der Verdaungsdrüsen. JF Bergman Verlag, 1898.
121. Flemström G, Sjöblom M, Jedstedt G, Åkerman KE. Short fasting dramatically decreases rat duodenal secretory responsiveness to orexin A but not to VIP or melatonin. Am J Physiol Gastrointest Liver Physiol, 285: G1091–G1096, 2003.
122. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richarson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell, 92: 573–585, 1998.
123. Kirchgessner AL. Orexins in the brain-gut axis. Endocr Rev, 23: 1–15, 2002.
124. Kirchgessner AL, Liu M. Orexin synthesis and response in the gut. Neuron, 24: 941–951, 1999.
125. Näslund E, Ehrström M, Ma J, Hellström PM, Kirchgessner AL. Localization and effects of orexin on fasting motility in the rat duodenum. Am J Physiol Gastrointest Liver Physiol, 282:

G470–G479, 2002.
126. Satoh Y, Uchida M, Fujita A, Nishio H, Takeuchi T, Hata F. Possible role of orexin A in nonadrenergic, noncholinergic inhibitory response of muscle of the mouse small intestine. Eur J Pharmacol, 428: 337–342, 2001.
127. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol, 1: 11–21, 2000.
128. Dawson JR, Schwenk M. Isolated epithelial cells. Progr Pharmacol Clin Pharmacol, 7: 21–41, 1989.
129. Weinlich M, Heydasch U, Mooren F, Starlinger M. Simultaneous detection of cell volume and intracellular pH in isolated rat duodenal cells by confocal microscopy and BCECF. Res Exp Med (Berl), 198: 73–82, 1998.
130. Chew CS, Säfsten B, Flemström G. Calcium signaling in cultured human and rat duodenal enterocytes. Am J Physiol Gastrointest Liver Physiol, 275: G296–G304, 1998.
131. Barrett KE, Keely SJ. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. Annu Rev Physiol, 62: 535–572, 2000.
132. Reimer R, Odes HS, Muallem R, Schwenk M, Beil W, Sewing KF. Cyclic adenosine monophosphate is the second messenger of prostaglandin E2- and vasoactive intestinal polypeptide-stimulated active bicarbonate secretion by guinea-pig duodenum. Scand J Gastroenterol, 29: 153–159, 1994.
133. Säfsten B, Flemström G. Dopamine and vasoactive intestinal peptide stimulate cyclic adenosine-3',5'-monophosphate formation in isolated rat villus and crypt duodenocytes. Acta Physiol Scand, 149: 67–75, 1993.
134. Gibinski K. A review of seasonal periodicity in peptic ulcer disease. Chronobiol Int, 4: 91–99, 1987.
135. Malinovskaya N, Komarov FI, Rapoport SI, Voznesenskaya LA, Wetterberg L. Melatonin production in patients with duodenal ulcer. Neuroendocrinol Lett, 22: 109–117, 2001.
136. Larsen KR, Moore JG, Dayton MT. Circadian rhythms of acid and bicarbonate efflux in fasting rat stomach. Am J Physiol Gastrointest Liver Physiol, 260: G610–G614, 1991.

About the author

Markus Sjöblom received the Israel Hwasser award from the Uppsala Medical Association for the best dissertation in basic medicine in the academic year 2003/2004. He is at present carrying out his postdoctoral training at the Division of Physiology, Department of Neuroscience, Uppsala University.

Corresponding author: Markus Sjöblom, Ph.D.
Uppsala University
Division of Physiology
Department of Neuroscience
P.O. Box 572
751 23 Uppsala, Sweden
e-mail: markus.sjoblom@fysiologi.uu.se