The Duodenal Mucosal Bicarbonate Secretion

*Role of Melatonin in Neurohumoral Control and Cellular Signaling*

BY

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Dissertation presented at Uppsala University to be publicly examined in B42, BMC, Uppsala, Friday, October 3, 2003 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract
ISBN 91-554-5688-X

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 thesis concerns the influence of the central nervous system and the effects of the hormone melatonin on bicarbonate secretion in anesthetized rats in vivo. Effects of melatonin on intracellular calcium signaling by duodenal enterocyte in vitro were 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 α1-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 secretagogues orexin-A and bethanechol but not those to melatonin or vasoactive intestinal polypeptide.

In conclusion, the results strongly suggest that intestinal melatonin plays an important role in central nervous elicited stimulation of duodenal mucosal bicarbonate secretion. Sensitivity of this alkaline secretion to some peripheral stimulators markedly depends on the feeding status.

Keywords: alkaline secretion, central nervous system, duodenal enterocyte, duodenal ulcer, duodenum, enterochromaffin cell, human, intraarterial, intracellular calcium, intracerebroventricular, melatonin, rat, vagal nerve

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ISSN 0282-7476
ISBN 91-554-5688-X
urn:nbn:se:uu:diva-3521 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-3521)
List of Papers

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


II. Sjöblom, M., Flemström, G. Central nervous α1-adrenoceptor stimulation induces duodenal luminal release of melatonin. (manuscript)


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Abbreviations

ACh  acetylcholine
CA   carbonic anhydrase
$[Ca^{2+}]_i$  intracellular calcium concentration
CCK  cholecystokinin
CFTR cystic fibrosis transmembrane conductance regulator
CNS  central nervous system
EC cell enterochromaffin cell
ENS  enteric nervous system
HCl  hydrochloric acid
HCO$_3^-$  bicarbonate
HPLC high-performance liquid chromatography
ia  intraarterial
2-ibmt  2-iodo-N-butanoyl-5-methoxytryptamine
icv  intracerebroventricular
iv  intravenous
MAP  mean arterial blood pressure
PGs  prostaglandins
pH$_i$ intracellular pH
VIP  vasoactive intestinal polypeptide
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 thesis 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 (Guyton & Hall 2000). In 1910 Karl Schwarz published the first clinical observation that gastric acid was associated with gastric and/or duodenal ulcer disease (Schwarz 1910). He noted that acid caused mucosal damage and that this damage could be decreased by luminal neutralization. He formulated the famous dictum “Ohne sauren 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
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 (Marshall & Warren 1984, Kuipers et al. 1995). 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 (Granstam et al. 1990, Ainsworth et al. 1993) decreases duodenal bicarbonate secretion and that ethanol (Cooke 1976, Stern et al. 1984) 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 (Rhodes et al. 1966, Rune & Viskum 1969, Flemström 1994), but in the immediate vicinity of the surface epithelium the pH remains neutral (Flemström & Kivilaakso 1983). 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 (Flemström 1994, Flemström & Isenberg 2001). The mucus gel consists of ~5% mucins (glycoproteins) and >90% water (Allen et al. 1993). The glycoproteins are secreted by exocytosis by the surface epithelial cells and Brunner’s glands. Together with water, a gel continu-
ously 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 (Atuma et al. 2001). 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 (Flemström et al. 1999) and has been reported to delay back diffusion of hydrogen ions (Allen et al. 1993). 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 (Flemström 1994). 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 rodent enterocyte only stays alive for two to five days (Lipkin 1987). 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 (Lipkin 1987). During cell migration, from the crypt region to the villus tip, the duodencytes 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 (Lipkin 1987).

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 (Lundgren 1984, Jodal & Lundgren 1989). The blood flow provides the duodenal mucosa with $\text{HCO}_3^-/\text{CO}_2$ (Holm et al. 1990, Tsukamoto et al. 1992) 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 (Guyton & Hall 2000). A unique feature of the ENS is that it can manage its many functions without input from the brain or spinal cord (Gershon et al. 1994). 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 (1989) and Berthoud et al. (1990). 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 (Guyton & Hall 2000).

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 (Furness et al. 1999). 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\textsubscript{3}\textsuperscript{−} 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\textsubscript{3}\textsuperscript{−} secretion is the presence of acid in the duodenal lumen, and the acid-induced HCO\textsubscript{3}\textsuperscript{−} response is mediated by enteric nervous pathways, involving release of vasoactive intestinal polypeptide (VIP) and acetylcholine (Flemström 1994, Flemström & Isenberg 2001), as well as by E-type prostaglandins (PGs) released from mucosal cells (Takeuchi et al. 1997b).

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\textsubscript{3}\textsuperscript{−} transport by the duodenal mucosa in all species tested (Flemström et al. 1985, Wolosin et al. 1989, Flemström 1994, Glad et al. 2003). Other mediators stimulating duodenal bicarbonate transport include cholecystokinin (CCK), pancreatic polypeptide and neurotensin (Konturek et al. 1985), glucagons (Wenzl et al. 1987), pituitary adenylate cyclase-activating polypeptide (PACAP) (Takeuchi et al. 1997a, Takeuchi et al. 1998, Glad et al. 2003) and angiotensin II (Johansson et al. 2001).

The roles of PGs and nitric oxide (NO) in the HCO\textsubscript{3}\textsuperscript{−} 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 (Takeuchi et al. 2002). 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 (Sababi & Nylander 1994, Nylander et al. 2001).

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 (Takeuchi et al. 1993, Hällgren et al. 1995, Sababi et al. 1995, Sababi & Nylander 1996). Other studies, however, have shown that both luminal L-NAME (Holm et al. 1997, Holm et al. 1998a) and iv L-NAME (Bilski & Konturek 1994, Holm et al. 1997, Sugamoto et al. 2001) 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 (Takeuchi et al. 2002).

The bicarbonate secretion is inhibited by NSAIDs as well as by α₂-adrenoreceptor sympathetic stimuli. *H. pylori* infected patients with acute or chronic duodenal ulcer disease have impaired alkaline secretion (Isenberg et al. 1987), and eradication of the infection at least partly restores the secretion (Hogan et al. 1996). Further, Fändriks et al. showed that water extracts from *H. pylori* inhibit duodenal mucosal bicarbonate secretion in the rat (Fändriks et al. 1997).

**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 (Berthoud et al. 1990). 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 (Fändriks 1986, Nylander et al. 1987) and in rats (Jönson et al. 1986, Fändriks & Jönson 1990) 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 (Ballestros et al. 1991) and dogs (Konturek & Thor 1986). 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 HCO₃⁻ secretion (Lenz 1989). 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 (Flemström & Jedstedt 1989, Lenz 1989, Lenz et al. 1989, Flemström 1994), as well as by some benzodiazepines (Säfsten et al. 1991).
Furthermore, an up to a four-fold increase in secretion has been observed after intracerebroventricular (icv) infusion of the \( \alpha_1 \)-adrenoceptor agonist phenylephrine (Larson et al. 1996). 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 (Cheng & Leblond 1974). 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 \( \text{HCO}_3^- \) 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 enterotoxin).

The duodenal enterocytes possess different mechanisms for acid/base transport possibly reflecting the second messenger system activated. \( \text{HCO}_3^- \) and \( \text{CO}_2 \) reach the epithelium via the blood and \( \text{HCO}_3^- \) is imported at the basolateral membrane by \( \text{Na}^+(n)\)-\( \text{HCO}_3^- \) cotransport. \( \text{CO}_2 \) diffuses into the enterocytes and \( \text{HCO}_3^- \) is formed intracellularly by carbonic anhydrase conversion of \( \text{CO}_2 + \text{H}_2\text{O} \) to \( \text{HCO}_3^- + \text{H}^+ \). The enterocytes export \( \text{HCO}_3^- \) at the apical membrane by a \( \text{Cl}^-/\text{HCO}_3^- \) 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 \( \text{Cl}^- \) as well as \( \text{HCO}_3^- \) (Hogan et al. 1997, Seidler et al. 1997, Clarke & Harline 1998). An amiloride-sensitive \( \text{Na}^+/\text{H}^+ \) exchanger extrudes acid both at the apical and at the basolateral membrane. Fig. 1 shows a schematic illustration of the \( \text{HCO}_3^- \) 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 \( \text{HCO}_3^- \). In general, crypt cells are thought to have a secretory function, whereas the cells in the villi are mainly absorptive (Chang & Rao 1994). 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 \( \text{HCO}_3^- \) secretion in humans (Knutson et al. 1995), rabbits (Holm et al. 1990) and the guinea pig (Muallem et al. 1994). In rats, acetazolamide has been reported to decrease bicarbonate secretion (Flemström & Kivilaakso 1983), but other authors have found no effect (Takeuchi et al.
Furthermore, the CA isoenzyme II (CA II), associated with alkaline secretion, is located mainly in the villi and not in the duodenal crypts (Lönnertholm et al. 1989).

**Figure 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.

### Melatonin

Melatonin is the major hormone of the pineal gland and was first isolated by Lerner and colleagues (Lerner et al. 1958). 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) (Pang et al. 1993, Vanecek 1998). 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 (Reiter 1991), scavenging of free radicals (Reiter et al. 1995, Tan et al. 2000), alleviation of jet lag (Brown 1994) and (in non-human mammals) seasonal reproductive behaviors (Reiter 1991).
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 (Raikhlin et al. 1975). 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 (Stefulj et al. 2001). The amount of melatonin in the gastrointestinal tract does not depend on the presence of light and is not reduced by pineal glandectomy (Bubenik & Brown 1997).

Distribution of peripheral melatonin

The major source of melatonin in the body is the gastrointestinal tract (Huether 1993, Bubenik 2001, Bubenik 2002). 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 (Huether 1993). A similar observation has been made for serotonin (5-HT), of which approximately 95% is found in the alimentary canal (Gershon 1999). 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 (Huether et al. 1992b), whereas melatonin released from the pineal gland is responsible for the higher concentrations during darkness (Reiter 1993). The pineal gland melatonin is released in a circadian fashion (Reiter 1991, Reiter 1993), while the melatonin produced in the gastrointestinal tract steadily enters the circulation (Bubenik et al. 1996, Bubenik et al. 1999). 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 (Huether et al. 1998). It has also been reported that, in both normal (Huether et al. 1992a, Huether et al. 1992b) and pineal glandectomized rats (Yaga et al. 1993), 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 (Yeleswaram et al. 1997).
Melatonin receptors

Melatonin acts principally via high-affinity receptors coupled to heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). Three receptor subtypes have been found (Witt-Enderby et al. 2003); two of them, the MT₁ and MT₂ melatonin receptors, have been identified in mammals in molecular cloning studies, and a third receptor, named MT₃, has been found though not yet cloned (Masana & Dubocovich 2001, Witt-Enderby et al. 2003). Melatonin receptors are distributed throughout the gastrointestinal tract (Bubenik et al. 1977, Bubenik 2001, Bubenik 2002).

The signaling properties of the MT₂ receptor are becoming clearer since the recent development of MT₂-selective ligands. Unfortunately, no high affinity MT₁ receptor ligands have yet been discovered. Ligands with high affinity for MT₁ receptors are required to further clarify the physiological and pathophysiological roles of the biological actions of melatonin. The two receptor subtypes MT₁ and MT₂ have in common that they inhibit cAMP formation and stimulate phosphatidylinositol hydrolysis (Witt-Enderby et al. 2003).

The melatonin receptors mentioned in this thesis are defined according to the nomenclature and classification of the Nomenclature Committee of the International Union of Pharmacology (Dubocovich et al. 1998). The denomination MT₁ corresponds to that of the recombinant receptor previously termed ML₁A or Mel₁A. MT₂ refers to the native functional receptors with pharmacological characteristics similar to those of the recombinant receptor MT₂ previously termed ML₁B or Mel₁B. MT₃ corresponds to the pharmacologically defined melatonin receptor subtype, with unknown gene sequence, previously referred to as ML₄.

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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 \textit{in vivo} and to study its effect on duodenal enterocyte intracellular calcium signaling \textit{in vitro}.

The specific aims were:

- to 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 \textit{in vivo}

- to elucidate the neurohumoral pathways mediating the increase in duodenal mucosal bicarbonate secretion elicited by phenylephrine administered icv

- to investigate duodenal mucosal bicarbonate secretion in pineal glandectomized rats \textit{in vivo}

- to study the release of melatonin from the duodenal mucosa

- to examine acid-stimulated duodenal mucosal bicarbonate secretion and the role of melatonin

- to compare basal and stimulated duodenal alkaline secretory rates in fed animals and in animals fasted for a short period (overnight)

- to develop a method for isolation of duodenal enterocytes suitable for studies of intracellular signaling

- to elucidate the effects of melatonin on intracellular calcium signaling in duodenal enterocytes
Materials & Methods

Animals
All experiments on animals were approved by the Uppsala Ethics Committee for Experiments with Animals and results from studies of 315 rats are presented in this thesis. Male outbred Sprague-Dawley rats (Study I [Paper I]; Møllegaard Breeding Center, Ejby, Denmark) weighing 190-260 g or F₁-hybrids of Lewis × Dark Agouti rats (Studies I, II, III, IV and V; Animal Department, Biomedical Center, Uppsala, Sweden) weighing 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, except in Study V [Paper V], where some rats were deprived of food for a short period (overnight).

Human biopsies
In Study IV [Paper IV], 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.

Four studies were performed in anesthetized rats in vivo (Studies I, II, III and V) and one study was performed in vitro (Study IV). In Study IV clusters of human and rat duodenal enterocytes were used. The methods are described separately below.
In vivo experiments

Anesthesia and general surgery

In most protocols 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 Ca2+ and 25 HCO3−) 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.

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 HCO3− 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. HCO3− secretion into the luminal perfusate was continuously titrated with 50 mM HCl at pH 7.4 under automatic control of a pH-stat system.
Rat proximal duodenum was cannulated in situ between two glass tubes connected to the same reservoir containing isotonic saline. The mucosal HCO₃⁻ secreted into the luminal perfusate was continuously titrated 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 brightness of the duodenal segment.

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.
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 Ca²⁺, 0.8 Mg²⁺, 132.8 Cl⁻, 25 HCO₃⁻, 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. 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 secretion) 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
In Study I, 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%.

In Study II [Paper II], melatonin was determined by high-performance liquid chromatography (HPLC) with electrochemical detection, running Chromelon™ 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 (HCO₃⁻) per centimeter of intestine per hour (µEq·cm⁻¹·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 HCO₃⁻ 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.
Experimental protocols for *in vivo* experiments

After anesthesia and operative procedures the animals were allowed to stabilize for 40-60 minutes. The basal rate of $\text{HCO}_3^-$ secretion was then titrated for 30-40 min. Test compounds were administered, and the secretion was measured continuously throughout the experiments. At the end of most experiments, the responsiveness of the preparation was tested by administering 20 $\mu$M prostaglandin E$_2$ (PGE$_2$) to the luminal perfusate. The prostaglandin was added as a small amount from an ethanol stock solution stored at $-20^\circ$C.

**Study I**

Peripheral melatonin mediates neural stimulation of duodenal mucosal bicarbonate secretion.

In this study the effects of melatonin on duodenal mucosal bicarbonate secretion were investigated. The central nervous influence on the alkaline secretion after stimulation with phenylephrine was also analysed. Rats were divided into 21 groups with respect to treatment, as shown below. Duodenal mucosal $\text{HCO}_3^-$ secretion, MAP and temperature were monitored continuously and recorded at 10-min intervals.

The drugs in the treatment groups were administered by continuous infusion, while those given as pretreatment were injected as a bolus dose. Series of control experiments were run as follows: Basal $\text{HCO}_3^-$ secretion was measured for 210 min in $F_1$-hybrids of Lewis x Dark Agouti rats and in such rats exposed to ia or iv infusion of saline alone. In Sprague-Dawley rats, basal $\text{HCO}_3^-$ secretion was measured during a period of 230 min. For comparison with effects of icv infusions in such animals, basal secretion was measured during a 30-min period and subsequently during a 170-min period with infusion of artificial cerebrospinal fluid alone. Similar measurements of basal $\text{HCO}_3^-$ secretion and of secretion during icv infusion were performed in the pituitary glandectomized and pineal glandectomized Sprague-Dawley rats.
Study II

Central nervous $\alpha_1$-adrenoceptor stimulation induces duodenal luminal release of melatonin.

Release of melatonin from the proximal duodenum after central nervous stimulation with phenylephrine was examined. Duodenal mucosal $\text{HCO}_3^-$ secretion, MAP and temperature were monitored continuously and recorded at 10-min intervals. Animals were divided into three groups with respect to treatments, as shown below.

All groups were examined according to the same study protocol. The experimental time was 160 min. The duodenal mucosal $\text{HCO}_3^-$ secretion was recorded every 10 min. The luminal perfusate was sampled at $t=0$, $t=60$ and $t=160$ for measurements of the amount of melatonin released into the perfusate, in an HPLC system. Phenylephrine was administered by continuous infusion, while the pretreatment drug luzindole was injected as a bolus dose.

\begin{table}[h]
\centering
\begin{tabular}{ll}
\textbf{Infusion} & \textbf{Treatment} & \textbf{Pretreatment} \\
\hline
ia & Melatonin & \\
ia & 2-Ibmt & \\
ia & 2-phenylmelatonin & \\
ia & Melatonin & Luzindole iv \\
ia & Melatonin & Prazosin iv \\
ia & Luzindole (low dose) & \\
ia & Luzindole (high dose) & \\
ia & 4-P-PDOT & \\
icv & Melatonin & \\
icv & Phenylephrine & Luzindole iv \\
icv & Phenylephrine & Pituitary glandectomy \\
icv & Phenylephrine & Pineal glandectomy \\
icv & Phenylephrine & Vagalectomy \\
icv & Phenylephrine & Sympathectomy \\
icv & Phenylephrine & Vagal- + sympathectomy \\
i & Melatonin & \\
i & 2-phenylmelatonin & \\
i & CRH & \\
i & MSH & \\
i & ACTH & \\
\end{tabular}
\end{table}

2-Ibmt = 2-iodo-N-butanoyl-5-methoxytryptamine. 4-P-PDOT = 4-phenyl-2-propionamidotetraline. CRH = corticotrophin-releasing hormone. MSH = $\gamma$-melanocyte-stimulating hormone. ACTH = adrenocorticotropic hormone.
Study III

Melatonin in the duodenal lumen is a potent stimulant of mucosal bicarbonate secretion.

Duodenal luminal acidification is the main physiological stimulus of alkaline secretion in the duodenum. The role of melatonin in the acid-induced HCO$_3^-$ response and the effects of melatonin perfused in the duodenal lumen were examined in Study III [Paper III]. Rats were divided into seven groups with respect to treatment, as shown below. Duodenal mucosal HCO$_3^-$ secretion and MAP were monitored continuously and recorded at 10-min intervals.

In the seven treatment groups, ia melatonin was given as a continuous infusion while the drugs used for pretreatment were injected as a bolus dose except for hexamethonium, which was given as a bolus dose followed by continuous infusion. The duodenal lumen was exposed to acid (pH 2.0) for 5 min and the pH of the luminal perfusate was then changed to 7.4.

<table>
<thead>
<tr>
<th>Infusion</th>
<th>Treatment</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>icv</td>
<td>Phenylephrine</td>
<td>Luzindole iv</td>
</tr>
<tr>
<td>icv</td>
<td>Phenylephrine</td>
<td>Luzindole iv</td>
</tr>
</tbody>
</table>

Study V

Short fasting dramatically decreases rat duodenal secretory responsiveness to orexin-A but not to VIP or melatonin.

In this study the actions of some intestinal secretagogues were compared between fed rats and rats fasted for a short period (overnight). The animals were divided into eight groups with respect to treatment, as shown below.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Treatment</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>luminal</td>
<td>Melatonin</td>
<td>Luzindole iv</td>
</tr>
<tr>
<td>luminal</td>
<td>Melatonin</td>
<td>Hexamethonium iv</td>
</tr>
<tr>
<td>luminal</td>
<td>Melatonin</td>
<td>Luzindole iv</td>
</tr>
<tr>
<td>luminal</td>
<td>Acid pH 2.0</td>
<td>Luzindole iv</td>
</tr>
<tr>
<td>ia</td>
<td>Melatonin</td>
<td>Domperidone iv</td>
</tr>
</tbody>
</table>
Duodenal mucosal HCO₃⁻ secretion MAP were monitored continuously and recorded at 10-min intervals. The ia infusion was given continuously.

<table>
<thead>
<tr>
<th>Infusion</th>
<th>Treatment</th>
<th>Feeding status</th>
</tr>
</thead>
<tbody>
<tr>
<td>ia</td>
<td>Orexin-A</td>
<td>Fed</td>
</tr>
<tr>
<td>ia</td>
<td>Orexin-A</td>
<td>Fasted</td>
</tr>
<tr>
<td>ia</td>
<td>Bethanechol</td>
<td>Fed</td>
</tr>
<tr>
<td>ia</td>
<td>Bethanechol</td>
<td>Fasted</td>
</tr>
<tr>
<td>ia</td>
<td>VIP</td>
<td>Fed</td>
</tr>
<tr>
<td>ia</td>
<td>VIP</td>
<td>Fasted</td>
</tr>
<tr>
<td>ia</td>
<td>Melatonin</td>
<td>Fed</td>
</tr>
<tr>
<td>ia</td>
<td>Melatonin</td>
<td>Fasted</td>
</tr>
</tbody>
</table>

In vitro experiments

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 Ca²⁺; 1.2 Mg²⁺; 121.8 Cl⁻; 1.2 SO₄²⁻; 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 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). HCO₃⁻ (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 ([Ca²⁺]), 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 Ca²⁺; 1.2 Mg²⁺; 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 (Shariatmadari et al. 2001). 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 [Ca²⁺] 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.

![Diagram of temperature-controlled perfusion chamber](image)

**Figure 4** Schematic illustration of the temperature-controlled perfusion chamber (from Shariatmadari et al. 2001).

### 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 achieved by use of SigmaPlot for Windows 4.01.

### Experimental protocol for *in vitro* experiments

**Study IV**

Melatonin-induced calcium signaling in clusters of human and rat duodenal enterocytes.

This study established a new method, using isolated clusters of inter-connected duodenal enterocytes from human biopsy specimens and scraped-off rat mucosa, for examining the effects of melatonin on intracellular calcium signaling. Changes in [Ca$^{2+}$], were measured by the dual-wavelength excitation ratio technique. The experiments were divided into 18 groups with respect to treatment, concentrations and species (R= rat, H= human), as shown below.

2-Ibm and 2-iodomelatonin are potent melatonin agonists. Luzindole and DH97 are MT$_2$ selective melatonin receptor antagonists. Control experi-
ments were performed by measuring $[Ca^{2+}]_i$ in human and rat preparations perfused with the electrolyte solution alone (no agonists or antagonists added).

<table>
<thead>
<tr>
<th>Species</th>
<th>Electrolyte perfusion solution mixed with</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Melatonin 0.1, 1.0, 5.0 &amp; 100 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 0.1, 1.0, 5.0, 10, 100, 500 &amp; 1000 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 1000, 1500 &amp; 2000 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 10, 100 &amp; 500 nM</td>
</tr>
<tr>
<td>H</td>
<td>Melatonin 10, 100 &amp; 500 nM</td>
</tr>
<tr>
<td>R</td>
<td>2-lbmt 10, 100, 500 &amp; 1000 nM</td>
</tr>
<tr>
<td>H</td>
<td>2-lbmt 10, 100, 500 &amp; 1000 nM</td>
</tr>
<tr>
<td>R</td>
<td>2-iodomelatonin 10, 100, 500 &amp; 1000 nM</td>
</tr>
<tr>
<td>H</td>
<td>2-iodomelatonin 10, 100, 500 &amp; 1000 nM</td>
</tr>
<tr>
<td>R</td>
<td>Luzindole 10, 100, 500, 1000, 1500 &amp; 2000 nM</td>
</tr>
<tr>
<td>H</td>
<td>Luzindole 10, 100, 500, 1000, 1500 &amp; 2000 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 10, 100 &amp; 500 nM in $Ca^{2+}$-free solution</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 10 &amp; 100 nM mixed with atropine 1.0 µM</td>
</tr>
<tr>
<td>H</td>
<td>Melatonin 500 nM mixed with luzindole 500 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 500 nM mixed with luzindole 500 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 500 nM mixed with DH 97 500 nM</td>
</tr>
<tr>
<td>R</td>
<td>Melatonin 10, 100 &amp; 500 nM followed by carbachol 100 µM and CCK-8 100 nM</td>
</tr>
<tr>
<td>H</td>
<td>Melatonin 10, 100 &amp; 500 nM followed by carbachol 100 µM and CCK-8 100 nM</td>
</tr>
</tbody>
</table>
Results

Study I Peripheral melatonin mediates neural stimulation of duodenal mucosal bicarbonate secretion.

The mechanisms of action of melatonin and melatonin receptor ligands on duodenal mucosal HCO$_3$\textsuperscript{-} secretion were investigated in the anesthetized rat in vivo. Melatonin, as well as the potent melatonin receptor agonist 2-iodo-N-butanoyl-5-methoxytryptamine, administered by continuous ia infusion, induced a marked increase in duodenal mucosal HCO$_3$\textsuperscript{-} secretion. An increase in alkaline secretion was also observed when melatonin was infused intravenously, but only at higher concentrations than those effective with ia infusion. The melatonin receptor antagonist (MT$_2$>MT$_1$) luzindole was used to block melatonin receptors. In a first step, we showed that luzindole had no effect on spontaneous HCO$_3$\textsuperscript{-} secretion. The subtype-selective antagonist was then used to characterize the response to exogenous melatonin. Pretreatment with luzindole given as a bolus dose 20 min prior to the start of ia melatonin infusion prevented the HCO$_3$\textsuperscript{-} response to melatonin. Neither melatonin nor luzindole significantly changed the MAP.

In a previous study it had been found that icv infusion of the $\alpha_1$-adrenoceptor agonist phenylephrine markedly stimulated duodenal alkaline secretion, but the neurohumoral pathways mediating this secretory response had not been elucidated. Intracerebroventricular infusion of phenylephrine increased the HCO$_3$\textsuperscript{-} secretion four-fold in Study I (Fig. 5a). A transient increase in MAP was observed during the first five minutes of the icv phenylephrine infusion. Luzindole abolished the duodenal secretory response to icv phenylephrine (Fig. 5b).

The inhibition of the icv phenylephrine-induced secretory response by the melatonin receptor antagonist luzindole prompted a study of the response in pineal glandectomized rats. Removal of the pineal gland inhibits central nervous (but not peripheral) production of melatonin. Pineal glandectomized rats showed the same rates of spontaneous duodenal HCO$_3$\textsuperscript{-} secretion as rats with an intact pineal gland and icv phenylephrine elicited the same increase in secretion (Fig. 5a).
It had also previously been reported that the pituitary peptide β-endorphin is a potent stimulant of the alkaline secretion. Thus, it was of interest to study the duodenal HCO₃⁻ secretion in pituitary glandectomized animals. The increase in secretion in response to icv phenylephrine infusion was not significantly different from that in non-pituitary glandectomized controls. Pituitary peptides thus seem unlikely to be mediators of the response to icv phenylephrine.

Bilateral section of the vagal trunks inhibits the rise in duodenal secretion induced by icv infusion of TRH and some other agents, but the same kind of vagotomy (at the sub-laryngeal level) did not affect the response to icv phenylephrine. In contrast, section both of the vagal trunks and of the sympathetic chains around the carotid arteries (at the sub-laryngeal level) significantly inhibited the duodenal secretory response to icv phenylephrine (Fig. 5b). Sympathectomy alone, like truncal vagotomy alone, was not sufficient to inhibit the response.

In conclusion, ia infusion of secretagogues close to the duodenum is significantly more effective than iv administration. Furthermore, close ia infusion should minimize any central nervous action of the compounds. Melatonin is a potent stimulant of duodenal mucosal bicarbonate secretion. Central nervous stimulation, with icv phenylephrine, does not act by releasing melatonin from the pineal gland, but very probably stimulates release of melatonin from the enterochromaffin cells in the intestinal mucosa. The brain-gut axis involved in mediation of the response includes the vagus nerves as well as the sympathetic chain. Further evidence that melatonin stimulates alkaline secretion by a peripheral action is that iv luzindole inhibits the response to ia and iv melatonin as well as to icv phenylephrine.

![Figure 5a](image)

*Bicarbonate secretion increased significantly after administration of phenylephrine icv. Neither pineal nor pituitary glandectomy inhibited the secretory response to icv phenylephrine.*
Figure 5b
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.

Study II  Central nervous $\alpha_1$-adrenoceptor stimulation induces duodenal luminal release of melatonin.

Results of Study I indicated that icv infusion of the $\alpha_1$-adrenoceptor agonist phenylephrine induced release of melatonin from the duodenal mucosa. The aim of the second study was therefore to confirm that central nervous stimulation does, indeed, release melatonin from the proximal duodenum.

Rats were anesthetized and the proximal segment of the duodenum with its blood supply intact was cannulated in situ and bicarbonate secretion was titrated by pH-stat. The release of melatonin into the duodenal luminal perfusate was determined by HPLC with electrochemical detection. Central nervous stimulation with phenylephrine induced a >10-fold increase in the release of melatonin (Fig. 6) and a three-fold increase in duodenal mucosal bicarbonate secretion. The melatonin receptor antagonist luzindole almost abolished the marked rise in alkaline secretion but did not inhibit the luminal release of melatonin. The results of this study thus strongly suggest that icv phenylephrine increases the bicarbonate secretion by the duodenal mucosa through intestinal release of melatonin, and several groups have identified EC cells as the source of intestinal melatonin.
Figure 6
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. $t = \text{experimental time.}$

Study III  Melatonin in the duodenal lumen is a potent stimulant of mucosal bicarbonate secretion.

The main physiological stimulant of duodenal mucosal bicarbonate secretion is the presence of acid (discharged from the stomach) in the duodenal lumen. Pathways that mediate this response have been reported to include VIP, acetylcholine and prostaglandins.

The aim of this study was to establish and elucidate the effects of lumenally administered melatonin and the role of melatonin in the acid-induced stimulation of the secretion.

In a first series of experiments the duodenal lumen was perfused with melatonin. The alkaline secretion started to increase 30 min after the start of the perfusion and peaked after another 60 min (Fig. 7). In previous experiments (Study I) melatonin was administered intraarterially or intravenously. The response to luminal melatonin was considerably stronger. In a second series of experiments the animals were pretreated with the predominantly MT$_2$ selective melatonin receptor antagonist luzindole. Luzindole injected iv 20 min prior to the start of the luminal melatonin
perfusion abolished the stimulatory response to this luminal melatonin (Fig. 7).

To elucidate the question whether melatonin acts directly on the duodenal enterocytes or indirectly via the enteric nervous system, animals were pretreated with hexamethonium. Hexamethonium is a selective nicotinic receptor antagonist, which blocks transmission in autonomic ganglia, and has previously been shown to cause total inhibition of the increase in HCO$_3^-$ secretion in response to luminal acid. Iv hexamethonium reduced the stimulation of duodenal alkaline secretion by luminal melatonin (Fig. 7). However, the HCO$_3^-$ secretory rate always remained significantly higher than that in untreated controls. Hexamethonium also caused a rapid and sustained decrease in MAP.

![Figure 7](image.png)

**Figure 7**
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.

Exposure of the duodenal mucosa to physiological concentrations of acid (HCl) has previously been found to markedly increase the alkaline secretion by the duodenal mucosa in all species tested. In *Study III* we therefore repeated established experiments by exposing the duodenal mucosa for 5 min to HCl at pH 2.0. The HCO$_3^-$ secretion increased and remained high throughout the experiments. The melatonin MT$_2$ selective antagonist luzindole was then used to study the involvement of melatonin in mediating the response to luminal acid. Luzindole was injected as a bolus in a dose, not affecting the HCO$_3^-$ secretion, 20 min prior to the challenge with luminal acid. The increase in HCO$_3^-$ secretion was significantly smaller than that in
animals exposed to luminal acid alone, but was still significantly higher than that in controls not exposed to acid.

The results of this study thus demonstrate that luminal melatonin is a potent stimulant of duodenal mucosal bicarbonate secretion and, furthermore, strongly suggest that melatonin is an important mediator of acid-induced secretion. Melatonin seems to stimulate HCO$_3^-$ secretion by the duodenal mucosa by two routes, both by a direct effect on the duodenal enterocytes and by an indirect effect via activation of the enteric nervous system.

**Study IV  Melatonin-induced calcium signaling in clusters of human and rat duodenal enterocytes.**

A new method for isolating viable duodenal enterocytes was established. Clusters of interconnected enterocytes (10-50 cells) were obtained from human duodenal biopsy specimens and scraped-off rat duodenal mucosa. Effects on intracellular calcium signaling induced by melatonin, and melatonin receptor ligands, were studied in such clusters loaded with fura-2/AM. The excitation ratio was measured by the dual-wavelength technique by exposure to alternating 340 and 380 nm light. The isolation procedure provides tissue devoid of enteric neurons.

Melatonin, and the selective melatonin agonists 2-iodomelatonin and 2-ibmt, increased the intracellular calcium concentration in both human and rat duodenal enterocytes. Moreover, the median effective concentration (EC$_{50}$) of melatonin effects on rat duodenal enterocytes in clusters was as low as 17 ± 2.6 nM. The presence of the muscarinic receptor antagonist atropine in the perfusate did not affect the [Ca$^{2+}$]i response to melatonin. The findings demonstrate that melatonin acts directly at enterocyte membrane receptors and, in addition, that the responses occur in intestinal epithelium of both human and rat origin.

Three main types of signaling patterns were observed. In the major type of response, [Ca$^{2+}$]i spiked rapidly and then slowly returned to the baseline or near baseline value within 4-6 min (Fig. 8). A second type of signaling pattern was a rapid and profound increase in [Ca$^{2+}$], followed by a sustained plateau at about 75-100% of the maximal [Ca$^{2+}$], response. The third type of response was an initial transient increase in [Ca$^{2+}$], followed by slow, rhythmic oscillations of high amplitude. Oscillations continued throughout the perfusion period, even after cessation of the exposure to melatonin. The frequency of these [Ca$^{2+}$], oscillations was one period in about 5 to 6 minutes. Furthermore, there was a time delay between individual cells with respect to the start of each rise in [Ca$^{2+}$], suggesting a spread of this
signaling pattern within the cluster by cell-to-cell communication. Melatonin increased $[\text{Ca}^{2+}]_i$ also in the absence of $\text{Ca}^{2+}$ in the perfusate, but oscillations were never observed with $\text{Ca}^{2+}$-free perfusate.

Enterocytes in primary culture have previously been found to respond to carbachol and cholecystokinin octapeptide (CCK-8) with a transient increase in $[\text{Ca}^{2+}]_i$ (Chew et al. 1998). Similar responses to these intestinal secretagogues were observed in the present preparation of freshly isolated clusters of duodenal enterocytes.

Like other enterochromaffin cell products, melatonin may exert paracrine regulatory actions on epithelial function. It therefore seemed of interest to study effects of melatonin on the $[\text{Ca}^{2+}]_i$, response to other stimuli. Cell clusters were perfused with melatonin before exposure to carbachol and CCK-8. Marked enhancement of the enterocyte $[\text{Ca}^{2+}]_i$ response to the latter secretagogues was observed.

Luzindole, and the more selective MT$_2$ receptor antagonist N-Pentanoyl-2-benzyltryptamine (DH97), abolished the rise in enterocyte $[\text{Ca}^{2+}]_i$ in response to melatonin in both human and rat duodenal clusters, strongly suggesting that melatonin increases $[\text{Ca}^{2+}]_i$ via an action on enterocyte membrane MT$_2$ receptors. No effects on $[\text{Ca}^{2+}]_i$ were observed after perfusion with luzindole or DH97 alone. From our results we concluded that melatonin increases $[\text{Ca}^{2+}]_i$ in enterocytes from human and rat duodenal clusters. Melatonin appears to induce both release of $\text{Ca}^{2+}$ from intracellular stores and influx of extracellular $\text{Ca}^{2+}$. Further, duodenal enterocytes seem to function as a syncytium.

**Figure 8**
Isolated human duodenal enterocytes in clusters increased their intracellular calcium concentration after perfusion with melatonin.
Study V  Short fasting dramatically decreases rat duodenal secretory responsiveness to orexin-A but not to VIP or melatonin.

Ever since Pavlov published his classical work, at the end of the 19th century, experimental studies of gastrointestinal physiology and pathophysiology in intact animals have by tradition been conducted after the animals have been fasted overnight. Presence of food in itself has considerable effects on intestinal functions. We therefore compared the actions of known intestinal secretagogues such as melatonin, VIP and bethanechol as well as of the recently discovered peptide orexin-A in fed animals with those in animals fasted overnight.

All secretagogues in this study were administered close to the proximal duodenal segment by intraarterial infusion. The appetite-regulating peptide orexin-A caused marked and dose-dependent stimulation of the duodenal mucosal bicarbonate secretion in fed animals, but did not affect this secretion in animals deprived of food overnight (Fig. 9). Similarly, the short fasting caused a 100-fold increase in the amount of the muscarinic M3 agonist bethanechol required for stimulation of the secretion. In contrast, the secretory responses to both melatonin (Fig. 9) and VIP did not differ significantly between fed and fasted animals.

The conclusion drawn from this study is that some intestinal secretagogues are markedly dependent on previous intake of food. The mechanism underlying this phenomenon is not known. An attractive explanation for the changes in sensitivity to orexin-A and the muscarinic agonist bethanechol might be that intake of food *per se*, or intestinal intraluminal presence of food constituents, induces the expression of specific signal pathways or receptors in the intestinal mucosa. Since only very small amounts were required for stimulation of secretion, the results strongly suggest that the increases in secretion reflect actions of the duodenal mucosa *per se*, and that central nervous actions did not influence the responses.
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 $\text{HCO}_3^-$ secretion. No significant differences between the fed and fasted animals were observed.

**Figure 9**

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 $\text{HCO}_3^-$ secretion. No significant differences between the fed and fasted animals were observed.
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 HCO₃⁻ are higher in the duodenum than in the stomach and in other, more distal parts of the small intestine (Flemström 1994). The investigations described in this thesis 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 thesis 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 HCO₃⁻. 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 HCO₃⁻ secretion depends markedly on the feeding status of the animals.

The physiological relevance of bicarbonate secretion

Evidence for HCO₃⁻ secretion originating from the duodenal mucosa was first reported a century ago in a thesis from Pavlov’s laboratory in St. Petersburg (Ponomarew 1902). Since that time, both in vitro and in vivo studies have shown that the duodenal mucosa secretes HCO₃⁻ at high rates (Flemström 1994). 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 (Flemström & Kivilaakso 1983, Flemström et al. 1999, Flemström & Isenberg 2001). 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 HCO₃⁻ secretion probably accounts for ~40% of the neutralization of the gastric acid load to the duodenum; pancreatic and biliary HCO₃⁻ accounting for the remaining bulk neutralization (Ainsworth et al. 1991, Ainsworth et al. 1992).

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 (Vanecek 1998). 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 (Raikhlin & Kvetnoy 1976) and the total amount of melatonin in the alimentary tract is considerably higher (>400) than that in the CNS (Huether 1993). It should also be noted that EC cells are in close contact with fibers from the autonomic nervous system (Lundberg et al. 1978). The physiological role of the intestinal source of melatonin has not been fully established. Like the EC cell products guanylin (Joo et al. 1998) and serotonin (Gershon 1999), intestinal melatonin may have a role in the reaction between the mucosa and the luminal contents.

In Study III, luminal perfusion of melatonin induced high rates of duodenal mucosal HCO₃⁻ secretion and such an effect was also observed after iv or close ia infusion in Study I. 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 (Bubenik et al. 1999). 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 (Tan et al. 1999, Messner et al. 2001). 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 (Flemström et al. 1999). The high rates of mucosal HCO$_3^-$ secretion induced by melatonin in Study III would suggest that the mucus layer does not significantly inhibit the migration of melatonin from the luminal fluid to the epithelial surface.

In Study I, we demonstrated for the first time that melatonin and melatonin receptor agonists increase the duodenal HCO$_3^-$ secretion in rats. 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$; Dubocovich 1988).

We showed in Study III that luminal melatonin is a potent stimulator of the HCO$_3^-$ secretion by the duodenal mucosa. When rats were pretreated with iv luzindole, the effects of luminal melatonin were efficiently abolished. However, it was also found in Study III that the ganglion-blocking agent hexamethonium (a nicotinic receptor antagonist) reduced the magnitude of the stimulatory effect of luminal melatonin on HCO$_3^-$ secretion. 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 in Study IV that melatonin increased the intracellular Ca$^{2+}$ in isolated duodenal enterocytes. 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.

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 (Overstreet et al. 1998) or used in humans for treatment of sleep disturbances or depression (Wetterberg 1999).

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 current experiments 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 (Merle et al. 2000a, Merle et al. 2000b). 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 (Flemström & Kivilaakso 1983) and ~pH 3 in humans (Feitelberg et al. 1992), neural reflexes and mucosal production of prostaglandins are stimulated.

The results obtained in Study III show that the melatonin antagonist luzindole decreases the HCO$_3^-$ response to acid. 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 (Flemström & Kivilaakso 1983, Paimela et al. 1990, Sababi et al. 1995). 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 (Holm et al. 1998b).

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 (Chang & Rao 1994).

On the basis of recent studies of intracellular pH ($pH_i$) in apical villus cells \textit{in situ}, it has been suggested as an additional mucosal protective mechanism that an acidic $pH_i$ facilitates basolateral uptake of base ($HCO_3^-$), increasing intracellular neutralization (Kaunitz & Akiba 2001). 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 (Lönnerholm \textit{et al.} 1989). 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 $\alpha_1$-adrenoceptor agonist phenylephrine has previously been shown to increase the duodenal secretory rate in rats (Larson \textit{et al.} 1996). 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 (Lenz \textit{et al.} 1989), CRH (Lenz 1989) and bombesin (Flemström & Jedstedt 1989), and of some benzodiazepines (Säfsten \textit{et al.} 1991). Bilateral ligation of the vagal trunks at the sub-laryngeal level inhibits the stimulation of duodenal (Lenz \textit{et al.} 1989) and pancreatic (Messmer \textit{et al.} 1993) 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.

It was found in Study I that icv phenylephrine stimulated the duodenal bicarbonate secretion. 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. Differences between effects of truncal vagotomy alone and of extended peri-carotid nervectomy have been observed previously in studies of duodenal distension-secretory interactions (Sababi & Nylander 1994). These differences may reflect intercommunications between the vagal and sympathetic neural pathways at the cervical level (Weijnen \textit{et al.} 2000) and
the anatomical mixing of pathways (Yang et al. 1999). Phenylephrine possibly mediates duodenal bicarbonate secretion by a different central mechanism than the other aforementioned neuropeptides and drugs.

The results of Studies I and II demonstrate that the melatonin receptor antagonist luzindole is a potent inhibitor of the duodenal secretory response to icv phenylephrine. Central nervous melatonin had no effect on the secretion. It was also established in Study I that the basal HCO₃⁻ secretion in both pineal glandectomized and pituitary glandectomized animals was the same as that in untreated controls. 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 HCO₃⁻ secretion in intact animals.

The release of melatonin from the duodenal mucosa into the luminal perfusate after icv administration of phenylephrine was investigated in Study II. Compared to control animals, phenylephrine induced an approximately 10-fold intraluminal increase in the melatonin level. Pretreatment with luzindole almost abolished the marked increase in bicarbonate secretion induced by icv phenylephrine, but did not inhibit the luminal release of melatonin. In Study I 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. 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 (Pavlov 1898). The presence of food itself has considerable effects on intestinal functions (Furness et al. 1999). We therefore examined the question whether the fasting procedure *per se* influenced the duodenal alkaline secretory response to some secretagogues.

It was established in Study V that feeding induced or very markedly potentiated the response of the duodenal HCO₃⁻ secreting epithelium to some stimuli but not to others. The most pronounced difference was noted after administration of orexin-A. Orexins (A and B) were originally discovered in the CNS as peptides that increased the appetite for food in
animals (Sakurai et al. 1998). Both orexins are also found in neurons and in neuroendocrine cells of the intestine (Kirchgessner & Liu 1999, Kirchgessner 2002), and orexin immunoreactivity is co-localized with VIP and choline acetyltransferase (Näslund et al. 2002). Both OX1 and OX2 receptors are thus expressed throughout the intestine in different cell types (Näslund et al. 2002). OX1 receptors are expressed mainly in neurons, while OX2 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 (Kirchgessner 2002) and to reduce (Satoh et al. 2001) 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.

In Study V, orexin-A caused a robust increase in the HCO₃⁻ secretory rate in fed animals, but did not affect that in animals fasted overnight. Similarly, fasting reduced the secretory sensitivity to the muscarinic agonist bethanechol by a dosefactor of ~100. In contrast, the HCO₃⁻ secretory responses to melatonin 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 ([Ca²⁺]ᵢ) at a constant resting level (around 100 nM) (Berridge et al. 2000). 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 [Ca²⁺]ᵢ signaling is probably of utmost importance in control of various aspects of enterocyte function, but very few studies of [Ca²⁺]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) (Lipkin 1987). 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 (Dawson & Schwenk 1989). The results in Study IV 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\(^{2+}\)] signaling. 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 (Weinlich et al. 1998). The findings in Study IV show further, for the first time, that melatonin has a direct action on duodenal epithelium. Melatonin increases [Ca\(^{2+}\)], in duodenal enterocytes from both rats and humans. Low concentrations of melatonin, with EC\(_{50}\) 17.0 ± 2.6 nM, and of agonists 2-iodomelatonin and 2-ibmt, increased enterocyte [Ca\(^{2+}\)]. 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\(^{2+}\)], spiked rapidly and then slowly returned to baseline or almost baseline values. In a smaller number of cells, [Ca\(^{2+}\)], tended to remain at a plateau level. The magnitude of the initial rise in [Ca\(^{2+}\)], 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 (Chew et al. 1998). Interestingly, as shown in Study I, 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 (Sjöblom et al. 2001). 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\(^{2+}\)], in mediating melatonin-induced stimulation of the secretion.

Perfusion with calcium-free solutions abolished the plateau phase but not the initial increase in [Ca\(^{2+}\)], 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\(^{2+}\)], 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 M\(_3\) receptors) induced biphasic [Ca\(^{2+}\)], responses (Chew et al. 1998), similar to those observed with melatonin. The sustained phase of the rise in [Ca\(^{2+}\)], was, as found here with melatonin, attributable to extracellular Ca\(^{2+}\).

Another interesting type of [Ca\(^{2+}\)], response to melatonin was observed in the responding preparations. The initial transient increase in [Ca\(^{2+}\)], was followed by slow rhythmic oscillations in [Ca\(^{2+}\)], 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 Study IV of clusters of rat as well as human duodenal enterocytes occurred with about the same frequency (~ one period in 5 min). 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 in Study IV, a preparation that should be devoid of neural tissue. Pretreatment with the muscarinic antagonist atropine did not affect the basal [Ca\(^{2+}\)], 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 (Barrett & Keely 2000) 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 E\(_2\) increase cAMP production (Säfsten & Flemström 1993, Reimer et al. 1994), and intracellular cGMP is involved in mediating the HCO\(_3^-\) secretory responses to guanylin and heat stable enterotoxin (ST\(_a\)) (Seidler et al. 1997). Interactions between these pathways and enterocyte [Ca\(^{2+}\)]\(_i\) signaling would seem likely.

Fig. 10 summarizes the proposed role of melatonin in the regulation of HCO\(_3^-\) transport by the duodenal epithelium.

**Figure 10**
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.
Ion transporters

Duodenal enterocytes export $\text{HCO}_3^-$ by an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger as well as an anion conductive pathway, very probably the CFTR channel. Anion-channel dependent transport of $\text{HCO}_3^-$ 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 in Study I. 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 $\text{HCO}_3^-$ without a significant change in PD, indicating an electroneutral transport process.

Clinical relevance

Convincing evidence that melatonin stimulates $\text{HCO}_3^-$ secretion in the rat has been provided in this thesis. 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 (Gibinski 1987). 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 (Malinovskaya et al. 2001). Studies in fasting animals have shown that the gastric secretions of $\text{HCO}_3^-$ and mucus, both important in mucosal protection, exhibit day and night rhythms with peak times different from those of the mucosa-aggressive $\text{H}^+$ secretion (Larsen et al. 1991). 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 thesis provides new and interesting knowledge about the central nervous as well as the peripheral regulation of the mucosa-protective bicarbonate 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. 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 $\alpha_1$-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.
Future perspectives

Previous studies have provided information about the regulation of duodenal mucosal bicarbonate secretion in several species. The findings in the present studies extend the understanding of the neurohumoral control of this mucosa-protective secretion, but also raise a number of challenging questions.

It would be interesting to determine whether melatonin also affects physiological processes other than secretion. Effects on intestinal motility and paracellular permeability may be of particular interest in the small intestine. It would also be of great interest to examine the effects elicited by icv phenylephrine in these respects.

In line with the work in Studies I & II, it would be interesting to elucidate the actual release of melatonin. We speculate that melatonin is released from the enterochromaffin cells, but this has not been confirmed by properly designed experiments. It would be very interesting to study the enterochromaffin cells per se, the innervation of these cells and their release of melatonin and other products under physiological and pathophysiological conditions; and also to determine whether HCl increases the release of melatonin.

It would be exciting to verify the presence of melatonin receptor subtype(s) in the duodenum. Since no selective MT₁ melatonin receptor agonist or antagonist has yet been discovered, there are no pharmacological means of elucidating the receptor subtypes present in the duodenum. Immunohistochemical staining of the different melatonin receptor subtypes with antibodies seems an appropriate alternative. Furthermore, it seems most interesting to investigate the number of receptors for melatonin and other secretagogues such as orexin, bethanechol and VIP in fed and fasted animals.

Studies in fasting animals have demonstrated that the gastric secretions of HCO₃⁻ and mucus, both important in mucosal protection, show day and night rhythms with peak times different from that of the mucosa-aggressive H⁺ secretion (Larsen et al. 1991). It would be interesting to find out if there is a similar phase shift in the duodenal mucosal bicarbonate secretory rate. Since melatonin has been shown to stimulate the alkaline secretion during the daytime (Studies I, II, III and V), it would be of interest to study the
secretory rate in darkness when the release of melatonin from the pineal gland is high.

In Study IV three different types of calcium responses were observed. Since changes in intracellular calcium are involved in numerous cellular processes, it is of utmost interest to couple such changes to physiological processes. We have preliminary data, from studies of rat duodenal enterocytes, showing that melatonin also affects the intracellular pH. This indicates that the calcium increase induced by melatonin most probably is involved in regulation of ion transport, but a considerable amount of work is required for characterization of the stimulus-secretion coupling. It would also be interesting to find those acid/base transporters activated by melatonin and to determine whether different types of calcium responses initiate different types of transporters.
Tolvfingertarmens sekretion av bikarbonatjoner ($\text{HCO}_3^-$) är en viktig fysiologisk försvarsmechanism mot det sura sekret som töms från magsäcken. Magsaften innehåller ofta, förutom saltsyra, slemhinneskadande proteinnedbrytande enzymer, etanol och läkemedel. Dessa kan liksom vid hämning av tarmhinnans försvarsmekanismer orsaka skador eller sår. Bikarbonatsekretionen i tolvfingertarmen är betydligt högre än i andra delar av tunntarmen.

Tidigare observationer har visat att bikarbonatsekretionen är kraftigt sänkt hos patienter med sår i tolvfingertarmen. Infektion med bakterien Helicobacter pylori förefaller, trots att infektionen är lokaliserad till magsäcken och inte till tolvfingertarmen, vara en viktig orsak till den nedsatta bikarbonatsekretionen. Mekanismen för hur en infektion i magäckens påverkar tolvfingertarmens sekretion är dock okänd. Andra möjliga orsaker till den nedsatta bikarbonatsekretionen kan vara defekter i den neurohumorala kontrollen av sekretionen, dvs dess reglering med nerver och hormoner, och/eller defekter i cellmembranernas bikarbonattransport. Vid skador eller sår i tolvfingertarmen är en kombination av ovan nämnda defekter sannolik.

Melatonin är ett hormon som reglerar en rad olika biologiska rytmer. Melatonin frisätts från tallkottkörteln i hjärnan när det är mörkt. Produktion av melatonin har även, oberoende av tid på dygnet, påvisats i specialiserade endokrina (enterokromaffina) celler i tarmhinnan. Smärtor från magtarmkanalen som visar en dygnsvariation kan vara melatoninberoende.

Studie I

Peripheral melatonin mediates neural stimulation of duodenal mucosal bicarbonate secretion.
Markus Sjöblom, Gunilla Jedstedt & Gunnar Flemström


I den första studien i avhandlingsarbetet var målsättningen dels att studera den centralnervösa regleringen av tolvfingertarmens bikarbonatsekretion, dels att studera om hormonet melatonin påverkar sekretionen. En tidigare studie hade visat att centralnervös stimulering, genom intracerebroventrikulär (icv) infusion, av α₁-adrenoceptoragonisten fenylefrin ökade tolvfingertarmens bikarbonatsekretion kraftigt. Hur stimuleringen medieras hade dock inte kunnat utredas.


Melatonin ökade tolvfingertarmens bikarbonatsekretion både efter intravenös (iv) och ia infusion. Om djuren förbehandlades med melatoninreceptor antagonisten luzindole iv förhindrades ökningen av alkalisk sekretion.


Icv infusion av hypofyshormonerna CRH, MSH och ACTH påverkade inte tolvfingertarmens bikarbonatssekretion. Hypofyshormoner och melatonin från tallkottkörtel konstaterades inte delaktiga i den centralnervösa medieringen av icv fenylefrin. Om man kapade de vagala och de sympatiska nervbanorna i halsregionen förhindrades helt bikarbonatsekretionsökningen efter icv fenylefrin stimulering. Om man endast kapade av dessa nervbanor så stimulerade icv fenylefrin bikarbonatsekretionen.
Sammanfattningsvis kunde vi i Studie I, som första forskningsgrupp, visa att melatonin stimulerar bikarbonatsekretionen i tolvfingertarmen. Vidare kunde vi visa att icv fenylefrin stimulerar bikarbonatsekretionen genom att aktivera vagala och sympatiska nerver. Dessa nerver stimulerar sannolikt enterokromaffina celler i tarmslonhinnan att frisätta melatonin. Effekten av icv fenylefrin togs bort helt med iv luzindole.

Studie II

Central nervous α1-adrenoceptor stimulation induces duodenal luminal release of melatonin.
Markus Sjöblom & Gunnar Flemström
Manuscript.

En möjlig förklaring till resultaten i Studie I var att ökningen av tolvfingertarmens bikarbonatsekretion efter icv infusion med fenylefrin beror på frisättning av melatonin från tarmslonhinnan. Målsättningen med Studie II var att undersöka om centralnervös stimulering med fenylefrin de facto ger en frisättning av melatonin från tolvfingertarmen.

Sövning och operation utfördes i enlighet med Studie I med enda skillnaden att prover togs kontinuerligt från den luminala recirkulerande perfusionslösningen. Från dessa prov analyserades mängden melatonin med hjälp av en HPLC.


Sammantaget visar resultaten i den här studien att icv infusion av fenylefrin stimulerar tolvfingertarmens bikarbonatsekretion genom frisättning av melatonin från tarmslonhinnan. Ett rimligt antagande är att det frisatta melatoninet kommer från tarmslonhinnas enterokromaffina celler då flera studier har visat att tarmslonhinnans melatonin produceras i dessa celler.
Studie III

Melatonin in the duodenal lumen is a potent stimulant of mucosal bicarbonate secretion.
Markus Sjöblom & Gunnar Flemström

Ett surt pH i tarmlumen är det fysiologiskt viktigaste stimulit till en ökad bikarbonatsekretion från tolvfingertarmens slemhinna. Den syrainducerade signalen medieras genom frisättning av VIP och acetylkolin från det enteriska nervsystemet samt av prostaglandiner från tarmcellerna. I Studie III var målsättningen därför att undersöka om melatonin är involverad i medieringen av den syrainducerade bikarbonatsekretionen samt att undersöka om luminalt melatonin har någon effekt på bikarbonatsekretionen.

Sövning och operation utfördes i enlighet med Studie I.


Närvaro av syra (pH 2.0) i tolvfingertarmslumen i 5 min inducerade en kraftig ökning av bikarbonatsekretionen. Om djuren förbehandlades med luzindole iv minskade sekretionssvaret på luminal syra signifikant, men bikarbonatsekretionen var fortfarande högre än i kontrollgruppen som inte exponerats för syra.

Sammanfattningsvis visar resultaten i Studie III att luminalt melatonin kraftigt stimulerar tolvfingertarmens bikarbonatsekretion. Dessutom är melatonin en mediator av den syrainducerade sekretionen. Effekterna av luminalt melatonin aktiverar dels tolvfingertarmens sekretoriska celler direkt, dels dessa celler via det enteriska nervsystemet.
Studie IV

Melatonin-induced calcium signaling in clusters of human and rat duodenal enterocytes.
Markus Sjöblom, Bengt Säfsten & Gunnar Flemström


Melatonin och de selektiva melatoninreceptoragonisterna 2-ibmt och 2-iodomelatonin inducerade ökningar i [Ca^{2+}], både i celler från människa och rätta. EC_{50} värdet för melatonin var 17 ± 3 nM. Närvaro av den muskarineriga receptorantagonisten atropin påverkade inte svaren på melatonin vilket tyder på att melatonin har en direktverkande effekt på tarmceller. Tre typer av kalciumsvar observerades. Huvudtypen var en snabb ökning i [Ca^{2+}], som därefter återgick till normalvärde efter 4-6 min. En annan typ av signalering var en snabb ökning i [Ca^{2+}], som låg kvar på 75-100% av den maximala kalciumkonsentration. Den tredje typen av svar karakteriserades av en initial ökning av den [Ca^{2+}], följt av rytmiska oscillationer som fortsatte även efter det att melatonin tagits bort från perfusatet. Melatoninreceptorantagonisterna luzindole eller DH97 förhindrade kalciumöknning efter stimulering med melatonin. Om cellpreparationen perfunderades med melatonin före tillsats av de kända sekretagogerna CCK-8 och karbakol kunde ett potentierat kalciumsvar observeras. Sammanfattningsvis lyckades vi utveckla en ny metod att isolera viabla cellförband från tolvfingertarmen om ca 10-100 celler. Upp till sex timmar efter isoleringen var >85% av cellerna från både rätta och människa viabla.
Fördelen med cellförband, jämfört med enskilda celler, är att man kan studera både intracellulär och intercellulär (cell-till-cell) signalering. Melatonin inducerade [Ca\(^{2+}\)]-ökningar. Dessutom potentierte melatonin svaren efter stimulering med CCK-8 och karbakol. Cellförbanden förefaller attilda ett funktionellt syncytium. Fyndet att melatonin inducerar ökning av [Ca\(^{2+}\)], och modulerar svarsnivån på andra substanser är en viktig upptäckt och kan ge nya terapeutiska möjligheter.

Studie V

Short fasting dramatically decreases rat duodenal secretory responsiveness to orexin-A but not to VIP or melatonin. Gunnar Flemström, Markus Sjöblom, Gunilla Jedstedt & Karl EO Åkerman Am J Physiol Gastrointest Liver Physiol. (July 17), 10.1152/ajpgi.00193.2003

Ända sedan Pavlovs klassiska studier i slutet av 1800-talet har flertalet experimentella heldjursförsök i gastrointestinalfysiologi och patofysiologi utförts efter det att djuren (liksom patienter) fastats över natten. Närvaro av mat i magtarmkanalen påverkar tarmens olika funktioner. I detta arbete jämfördes fyra sekretagogers effekter på tolfvingertarmens bikarbonatsekretion i fastade djur med effekter i djur med kontinuerlig tillgång på mat. De olika sekretagogerna var VIP, melatonin, betanekol och orexin-A, en peptid som blivit känd för sin centralnervösa reglering av aptit.

Sövning och operation utfördes i enlighet med Studie I. Orexin-A och betanekol inducerade en kraftig och dosberoende ökning av tolfvingertarmens bikarbonatsekretion i djur som haft fri tillgång på föda. Däremot saknade orexin-A effekt på sekretionen i djur som varit fastade. Mängden betanekol som behövdes för att stimulera de fastade djuren var 100 gånger högre än i djur som haft tillgång på mat. Det var ingen skillnad mellan fastade och icke-fastade djur i sekretionssvaret efter stimulering med melatonin och VIP.

Acknowledgments

The work in this thesis has been performed at the Department of Neuroscience, and the former Department of Physiology, BMC at Uppsala University. I wish to express my sincere gratitude to all persons, who in different ways have smoothed the progress and supported me during my graduate studies. Particularly, I would like to thank:

Gunnar Flemström, my supervisor, for introducing me into the exciting scientific field of duodenal bicarbonate transport, for your invaluable scientific wisdom and knowledge. Thank you for encouraging me to participate in numerous international conferences. Your confidence in me has been very inspiring.

Olof Nylander, my enthusiastic co-supervisor, for significant help & advices, never-ending flow of ideas, for sharing your teaching skills and broad knowledge in physiology. I appreciate our small talk and friendship.

Karl Åkerman, co-supervisor, for your support and competent guidance on cell experiments.

Gunilla Jedstedt for all the skillful and friendly help during these years, for teaching me the surgical procedures and for nice conversations during our many hours in the cell lab.

Bengt Säfsten, co-author, for collaboration and providing duodenal biopsies.

Jon Isenberg & Birgitta Mårtensson for advice, friendship and good times during your stay in Uppsala.

Jyrki Kukkonen, Göran Sperber, Urban Höglund, Jann Hau & Hans-Erik Carlsson for creating a productive working atmosphere and for valuable discussions.

Lena Karlsson & Ann-Sofie Göransson for excellent administrative assistance.

Birgitta Klang & Gunno Nilsson for all kind, and most appreciated, help concerning student lab. Without you teaching would not be that fun.

Annika Jägare for your encouragement and for good times in the lab.

Erik Ekström, Stig Norberg & Emma Andersson for help and all sorts of problem solving.

Sussie, Susanne M, Eva & Göte at the Animal Department for help with anesthesia and taking very good care of my animals.
Seniors at the Integrative research group: Örjan, Peter, Lena, Mats S, Erik, Angelica, Britta & Mats W for good times and stimulating discussions. Margareta & Pierre at Photo for help with posters, photos and figures. Maud Marsden for excellent linguistic revision of the thesis.

A special thanks to all former and present PhD-colleagues for a nice blend of science and social life. Mia for being there for me from day one and still... Your help and encouragement is most appreciated. Johanna for your great spirit, valuable help and “cat-sitting”. You have both been great companions at congress travels. Thanks Tomas for nice discussions, Cissi for great travel company, Gabriella for chit-chat and wine tasting. Magnus, Liselotte, Klas, Mahinda, Fredrik, Russell, Joel, Lina, Louise & Micke for good times.

My best friends, Mia&Thomas for sharing life’s “ups and downs” and for bringing TLE to my life. Anna&Olof for first-class dinners and warm friendship. Lotta&Stefan for friendship, good times and great hospitality. Johanna, Mattias&Sara, Sanna&Magnus, Laura&Peter, Petter&Christine, Michael&Helena, Bella&Marcus and Perilla&Martin for being friends who are always there.

My family-in-law for their support.
My relatives and friends from Blekinge: Kurt & Eivor, Christin & Hans-Olof, Linda, Robert, Conny & Tina and Kim. Mormor for making me feel special, for “pluppavälling” and for following me on my path trough life, and morfar (I miss you).
My sister Åsa, and Per, for friendship, interesting Yatzy-evenings and good times.
My parents, Agneta & Kennet, for bringing me up to who I am, for support, love, encouragement and for always being there when needed.
Tuss & Norpan.
Viktoria, my wife, the most important person in my life for giving me love, support and the best motivation one can imagine to finish this thesis, for coaching me when wheels were spinning too slowly.

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Financial support for these studies was provided 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.

Uppsala, August 2003
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