Microdialysis to study the Gastrin-ECL cell Axis in the conscious rat

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Abstract

The ECL cells constitute the major endocrine cell population in the acid-producing part of the stomach. Gastrin from G cells in the antrum is the main stimulus of gastric acid secretion. Gastrin stimulates the ECL cells in the oxyntic mucosa to mobilize histamine, which in turn stimulates the parietal cells to produce hydrochloric acid. In the present study we assessed the usefulness and limitations of the technique of gastric submucosal microdialysis for the study of ECL-cell histamine mobilization in vivo. We found the microdialysis technique to be a reliable tool for the study of the ECL-cell histamine mobilization in conscious rats. Although there was an inflammatory response to the implantation of the microdialysis probe it did not seem to affect the ECL-cell histamine mobilization. In a previous microdialysis study, gastric submucosal microinfusion of endothelin and adrenaline induced massive release of histamine and caused severe local damage to the mucosa. The results of the present study indicate that ECL cells are the source of the histamine and that endothelin and adrenaline affect ECL cells in situ in an indirect manner. Also, the mobilized histamine does not contribute to the mucosal damage. Instead, the mucosal damage is caused by ischemia and acid back diffusion. We also examined whether the vagus and the sympathetic nervous system contributes to the food- and gastrin-evoked responses of ECL cells in situ. The results suggest that vagal activation does not stimulate histamine mobilization by a direct action on the ECL cells. Instead, the ECL cells operate under long-term tonic control by the vagus, and vagal denervation of the stomach leads to ECL-cell gastrin receptor desensitization. The sympathetic nervous system was of minor importance in the food-evoked histamine response. In order to study the neuronal and hormonal regulation of gastrin secretion from G cells in situ, we developed a method for antral submucosal microdialysis. From our results it seems that the vagus exerts both stimulatory and inhibitory effect on gastrin cells in the antrum, and that the antrum is dependent on an intact vagal innervation for a normal gastrin response to food intake. Also, local neurons seem to be crucial for the food-evoked gastrin mobilization, since blocking of local neurons abolished the gastrin response. In addition, when administered locally via the microdialysis probe, adrenaline, noradrenaline, isoprenaline, serotonin, carbachol and GRP were found to stimulate G-cell gastrin mobilization while somatostatin, galanin and bradykinin were found to inhibit omeprazole-induced gastrin mobilization.

Key words: ECL cells; G cells; histamine; gastrin; in vivo microdialysis; vagus; enteric nervous system

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Through nature´s inflexible grace I´m learning to live

John Myung

To my family

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ORIGINAL PAPERS

The thesis is based on the following papers, referred to by their roman numerals.

I **Ericsson P**, Norlén P, Bernsand M, Alm P, Höglund P, Håkanson R. ECL cell histamine mobilization studied by gastric submucosal microdialysis in awake rats: Methodological considerations. Pharmacol Toxicol 2003; 93: 57-65.

II Bernsand M, **Ericsson P**, Björkqvist M, Zhao C-M, Håkanson R, Norlén P. Submucosal microinfusion of endothelin and adrenaline mobilizes ECL-cell histamine in rat stomach, and causes mucosal damage: a microdialysis study. Br J Pharmacol 2003; 140: 707-717.

III Norlén P, **Ericsson P**, Kitano M, Ekelund M, Håkanson R. The vagus regulates histamine mobilization from rat stomach ECL cells by controlling their sensitivity to gastrin. J Physiology 2005; 564: 895-905.

IV **Ericsson P**, Håkanson R, Rehfeld JF, Norlén P. Antral submucosal microdialysis, a novel approach to study gastrin secretion *in vivo*. Manuscript

V **Ericsson P**, Håkanson R, Norlén P. Gastrin secretion in response to locally applied candidate messengers monitored by antral submucosal microdialysis in the conscious rat. Manuscript

INTRODUCTION

The stomach

The functional role of the stomach is to digest food and to serve as a reservoir for ingested food. Also, the low intraluminal pH creates a barrier to infectious pathogens (Waldum *et al.* 2002; Martinsen *et al.* 2005). The rat stomach can be divided into three different parts: rumen, corpus and antrum. The rumen (forestomach, not present in humans) is composed of non-glandular epithelium and has no secretory capacity. It functions as an expandable food reservoir. The corpus (oxyntic gland area) is the acid-producing part of the stomach. The oxyntic mucosa harbours different exocrine cells that secrete products, needed for the processing of food, into the lumen. Parietal cells secrete hydrochloric acid (HCl, see below) and chief cells secrete pepsinogen (an enzyme that upon activation digests proteins). In addition, mucous neck cells and surface mucous cells secrete mucus and bicarbonate, which forms a barrier that protects the gastric mucosa from irritants such as HCl and bile. The antrum secretes mucus into the lumen and controls the delivery of processed food into the duodenum. Besides its role in the process of digestion the stomach is also an endocrine organ, harbouring at least 6 different endocrine cell types. In the oxyntic mucosa of the rat, endocrine cells make up about 2% of the total number of epithelial cells (the corresponding figure in man being approximately 1%), making it one of the largest endocrine organs of the body. The endocrine cells of the oxyntic mucosa in the rat stomach comprise ECL cells (65-70%), A-like cells (20-25%), somatostatin (D) cells (5-10%) and D_1/P cells (1%) (Solcia *et al.* 1975; Capella *et al.* 1991; Sundler & Håkanson 1991; Nissinen & Panula 1993). These cells are of the closed type, which means that they are not in contact with the lumen. The endocrine cells in the antral mucosa harbours roughly equal numbers of gastrin (G) cells, somatostatin (D) cells and enterochromaffin (EC) cells. In contrast to the endocrine cells in the oxyntic mucosa, the endocrine cells in the antrum are of the open type: they reach the lumen of the gland with apical processes. Presumably, this allows them to respond to directly intraluminal stimuli.

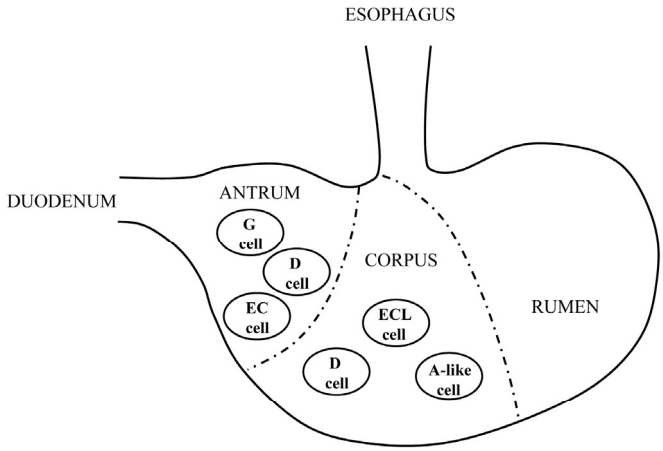


Fig. 1. Schematic illustration of the anatomy of the rat stomach and the distribution of gastric endocrine cells.

Innervation of the stomach

The enteric nervous system

The gastrointestinal tract has its own local nervous system, the enteric nervous system (ENS) that regulates the gastrointestinal movements and secretory activity. The enteric nervous system, constructed from 100 million neurones, is organized in two ganglionated plexa (nerve networks): the myenteric plexus and the submucosal plexus. The myenteric plexus is thought to be the main regulator of gastrointestinal movements while the submucosal plexus is responsible for controlling secretory functions and local blood flow. Neurons in the myenteric plexus synapse with neurons in the submucosal plexus, and vice versa, thereby enabling the two plexa to coordinate their functions. However, in the stomach wall the myenteric plexus predominate over the submucosal plexus and most of the nerve fibres found in the mucosa emanate from

myenteric ganglia. Thus, in the stomach the myenteric plexus is thought to control both motility and secretory activity.

The enteric nervous system consists of numerous different populations of neurons that contain different candidate neurotransmitters. In fact, often a single neuron contains more than one type of neurotransmitter. Candidate neurotransmitters in the enteric neurons include acetylcholine and noradrenaline as well as neuropeptides such as vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), gastrin releasing peptide (GRP), pituitary adenylate cyclase-activating peptide (PACAP), calcitonin gene-related peptide (CGRP), substance P, galanin, enkephalins and peptide YY (Schultzberger *et al.* 1980; Ekblad *et al.* 1985, 1991; Green & Dockray 1988; Furness *et al.* 1992; McConalogue & Furness 1994; Hannibal *et al.* 1998). In addition, the enteric nervous system contains also other neurochemicals, such as the amino acids aspartate, glycine, glutamate and γ-aminobutyric acid (GABA), and the amines serotonin and dopamine.

The autonomic nervous system

The stomach receives parasympathetic input via the vagus nerve and sympathetic input, via the splanchnic and mesenteric nerves. The postganglionic sympathetic fibers mainly innervate ganglions of the ENS and blood vessels. Although the sympathetic nervous system is thought to contribute to the regulation of gastric secretory activity, motility and local blood flow, the significance of the sympathetic contribution in the control of gastric functions are unclear. The vagus nerve is an important regulator of gastric secretory activity and motility, transmitting information between the central nervous system and the stomach via vagal efferents and afferents. The vagal innervation of the corpus is lateralized with little overlap, with a ventral and a dorsal vagal trunk innervating the ventral and dorsal side of the corpus, respectively (Legros & Griffith 1969; Håkanson & Liedberg 1971; Håkanson *et al.* 1984). However, in the antrum, the vagal innervation of the two sides seems to overlap (Berthoud *et al.* 2001). Instead of a direct innervation of effector cells, the vagus synapse on the enteric

nervous system mediating the vagal regulation. The traditional view, based on the fact that vagal fibres projecting to the ENS are far outnumbered by the multitude of enteric neurones, is that vagal fibres synapse on specific "mother cells" or "command neurones" in the myenteric ganglia (Langley 1922; Wood 1987). However, there are also reports suggesting that vagal fibres project to almost all myenteric neurones (Holst *et al.* 1997; Powley 2000; Hayakawa *et al.* 2006). The vagus nerve plays a central role in the regulation of acid secretion, which is illustrated by the fact that, in the rat, vagal excitation stimulates acid secretion (Brodie & Knapp 1966; Håkanson *et al.* 1980; Ekelund *et al.* 1982), whereas vagal denervation inhibits acid secretion (Håkanson *et al.* 1982a; Vallgren *et al.* 1983). Although vagal excitation has been shown to stimulate the release of several candidate messengers from enteric neurones and endocrine cells in the stomach, the relative importance of the various agents and the exact mechanisms mediating the vagal stimulation of acid secretion remain to be defined.

Gastric acid secretion

Gastric acid is secreted by the parietal cells in the oxyntic mucosal glands. Secretion of hydrochloric acid (HCl) depends on the proton pump, H^+/K^+-ATPase, that transports protons from the interior of the parietal cell to the glandular lumen in exchange for potassium ions (Ganser & Forte 1973; Lee *et al.* 1974; Sachs *et al.* 1976). The apical part of the parietal cells have an extensive network of channels called secretory canaliculi, and a large number of vesicles and tubules, the tubulovesicles (Helander & Hirschowitz 1972; Helander & Sundell 1984). Upon stimulation, the activity of H^+/K^+-ATPase is increased and the tubulovesicles, which contain the H^+/K^+-ATPase, fuse with the canalicular membrane to increase the secretory surface area (Forte *et al.* 1977; Wolosin & Forte 1981). Acid secretion in response to a meal can be divided into a cephalic phase and a gastric and intestinal phase. The cephalic phase, activated by the sight, smell and taste of food, appears to be mediated by cholinergic vagal neurones that, via the enteric nervous system, stimulate the parietal cells. In fact, the cephalic phase may account for 30-50% of the acid secretion during a meal (Richardson *et al.*

1977; Feldman *et al.* 1980). The gastric phase is started when food enters the stomach. Gastrin, the major mediator of the gastric phase (Feldman *et al.* 1978), is released in response to stomach distension (Lloyd *et al.* 1992; Mailliard & Wolfe 1989), increase in antral pH (Walsh *et al.* 1975; Schusdziarra *et al.* 1978), and the presence of certain food constituents such as amino acids and amines (Strunz *et al.* 1978; Lichtenberger *et al.* 1982a, b). When the gastric content is emptied into the duodenum (intestinal phase), various regulatory peptides are released –so called enterogastrones (Kosaka & Lim 1930), some stimulating and some inhibiting acid secretion (for review see Chew 1993).

Gastrin, histamine and acetylcholine stimulate the parietal cell acid secretion via gastrin/CCK_2, H_2 and M_3 receptors, respectively. However, how the different stimuli act and interact with each other is a matter of controversy. Two hypotheses have been purposed: the transmission hypothesis and the permission hypothesis (Black & Shankley 1987). According to the transmission hypothesis, gastrin and acetylcholine stimulate release of histamine from the ECL cells which in turn acts on the parietal cells (Code 1956, 1965; Kahlson *et al.* 1973). The experimental findings that injection of gastrin and refeeding, as well as vagal stimulation lowered the histamine content and accelerated the formation of histamine in the gastric mucosa (Kahlson *et al.* 1964, 1967), that histamine H_2 receptor antagonists inhibited acid secretion stimulated by gastrin and cholinergic agents (Black *et al.* 1972; Carter *et al.* 1974; Ekblad *et al.* 1985; Ekelund *et al.* 1987), and that depletion of gastric histamine prevented acid secretion in response to gastrin (Andersson *et al.* 1996) all support the transmission hypothesis. However, after finding that both histamine H_2 receptor antagonists and anticholinergic agents inhibited gastrin as well as vagally stimulated acid secretion, Grossman & Konturek (1974) proposed the permission hypothesis. According to this hypothesis the parietal cell express receptors for gastrin, acetylcholine and histamine, and the secretagogues cooperate in stimulating the parietal cell. Supporting this view, gastrin, acetylcholine and histamine have been shown to potentiate each other in studies on isolated gastric glands and parietal cells (Berglindh 1977; Soll 1978, 1982).

The ECL cell

ECL cells occur in the stomach of all vertebrates and seem to be restricted to the basal half of the acid-producing part of the mucosa (Håkanson *et al.* 1986, 1994; Capella *et al.* 1971). The ECL cells display an irregular shape and contain numerous electron-lucent large secretory vesicles and a few small electron-dense membrane-enclosed granules as well as electron-lucent microvesicles (Håkanson *et al.* 1994; Capella *et al.* 1971; Böttcher *et al.* 1989). The granules (25-150 nm in diameter) arise in the Golgi and have an electron-dense core surrounded by an electron-lucent halo (Zhao *et al.* 1999, 2005). The granules are loaded with chromogranin A (CGA) and various proteolytic enzymes. In addition, they are thought to contain the precursor of the anticipated ECL-cell peptide hormone. During transport to the periphery of the cell, the granules take up preformed histamine from the cytosol, and turn into larger secretory vesicles (125-500 nm in diameter) (Chen *et al.* 1996; Zhao *et al.* 1999). Also, cleavage products of CGA (e.g. pancreastatin, see below) are generated during the transport. The secretory vesicles, containing secretory products (histamine, peptide hormone and CGA derived peptides), are then stored close to the plasma membrane. Upon stimulation, the secretory vesicles release their content by exocytosis.

Histamine

In the gastric mucosa, histamine is stored in both ECL cells and mast cells (Håkanson *et al.* 1983, 1986; Panula *et al.* 1985; Waldum & Sandvik 1989; Nissinen & Panula 1993). In some species (e.g. man and dog) the main storage site is mast cells, whereas in other species (e.g. rat and mouse) the main storage site is the ECL cells (Håkanson *et al.* 1986; Sundler & Håkanson 1991). In the rat stomach, the ECL cells are rich in the histamine-forming enzyme histidine decarboxylase (HDC) (Kubota *et al.* 1984; Håkanson *et al.* 1986; Andersson *et al.* 1992, 1996), whereas mast cells seem to lack HDC (Andersson *et al.* 1992, 1996). In the ECL cells, HDC occurs in the cytosol and catalyzes the decarboxylation of the amino acid L-histidine (Ganrot *et al.* 1962; Lovenberg *et al.* 1962). Once formed, histamine is taken up into the secretory vesicles

by a vesicular membrane amine transporter type 2 ($VMAT_2$) and stored until exocytosis (Weihe *et al.* 1994; Peter *et al.* 1995; Schuldiner *et al.* 1995; Dimaline & Struthers 1996; Zhao *et al.* 1997). HDC can be inhibited by the irreversible inhibitor α-FMH, resulting in rapid depletion (within 24 h) of ECL-cell histamine (Andersson *et al.* 1992, 1996; Norlén *et al.* 2001a).

Histamine is degraded by the two histamine-degrading enzymes diamine oxidase and histamine N-methyl transferase (Schayer 1966; Reilly & Schayer 1971; Lönnroth *et al.* 1989; Maslinski & Fogel 1991; Loiselle & Wollin 1993). The respective contribution of the two enzymes to the degradation of gastric histamine varies from species to species (and from tissue to tissue). In the rat stomach, diamine oxidase is thought to be most important (Schayer 1966).

Chromogranin A and Pancreastatin

Chromogranin A is widely distributed in the body, occurring in the central and peripheral nervous systems, as well as in many endocrine cells. Rat CGA is a 448 amino acid protein that can be processed by proteolytic enzymes (i.e. endoproteases) to yield CGA-derived peptides such as pancreastatin. Different CGA-derived peptides are found in the different endocrine cellpopulations in the rat stomach, indicating the presence of different endoproteases within their secretory vesicles (Norlén *et al.* 2001a). Despite much speculation, the physiological significance of the CGA-derived peptides remains unknown. Nonetheless, since the ECL cells is one of the richest (possibly the richest) source of CGA and pancreastatin, circulating pancreastatin can be used as a marker for high ECL-cell activity and proliferation (Håkanson *et al.* 1995; Kimura *et al.* 1997; Norlén *et al.* 1997).

Regulation of the ECL cell

Gastrin released from G cells is the main stimulator of the ECL cells. By acting on CCK_2 receptors on the ECL cells (Chiba *et al.* 1991; Asahara *et al.* 1994), gastrin

stimulates the release of histamine and CGA-derived peptides (Kahlson *et al.* 1964; Sandvik *et al.* 1987a; Chen *et al.* 1994). Also, stimulation with gastrin increases HDC activity (Kahlson *et al.* 1964; Håkanson *et al.* 1974) and expression of HDC mRNA and CGA mRNA (Dimaline & Sandvik 1991; Dimaline *et al.* 1993; Chen *et al.* 1994; Sandvik *et al.* 1994; Björkqvist *et al.* 1999). The ECL cells are also regulated by somatostatin released from nearby D cells. Somatostatin, acting on sst_2 receptors (Schindler & Huphrey 1999), is considered to be the main inhibitor of the ECL cells (Sandvik & Waldum 1988a; Kondo *et al.* 1993; Prinz *et al.* 1994; Lindström & Håkanson 2001). The ECL cells are thought to operate under nervous control. Several of the neurotransmitters and neuropeptides present in enteric neurons have been shown to stimulate (e.g. PACAP, VIP, adrenaline and noradrenaline), or inhibit (e.g. galanin) histamine mobilization from ECL cells both *in vitro* and *in situ* (Prinz *et al.* 1993, 1994; Lindström *et al.* 1997; Zeng *et al.* 1998; Håkansson *et al.* 2001; Norlén *et al.* 2001b; Bernsand *et al.* 2007). In addition, inflammatory mediators such as prostaglandin E_2 and IL-1β inhibits ECL-cell secretory activity (Prinz *et al.* 1997; Sandvik *et al.* 1988b; Lindström & Håkanson 1998, 2001; Norlén *et al.* 2001b; Björkqvist *et al.* 2005).

The D cell and Somatostatin

Somatostatin occurs in endocrine/paracrine cells in many tissues. Somatostatin (D) cells are present all along the digestive tract and evenly distributed in the rat oxyntic and antral mucosa (Alumets *et al.* 1977). In the rat oxyntic mucosa the D cells are of the closed type and found scattered all along the glands, whereas in the rat antral mucosa they are of the open type and found in the basal part of the glands. D cells have long slender cytoplasmic processes that end in club-like swellings, enabling the D cells to contact with nearby cells (enabling paracrine mode of signal transmission) (Alumets *et al.* 1979; Larsson *et al.* 1979). Somatostatin exerts an inhibitory effect on a number of target cells, including G cells and ECL cells (for a review see Gillies 1997).

The G cell

In the rat, the G cells are found in the basal part of the antral glands (Larsson *et al.* 1974). The G cells are flask shaped and reach the gland lumen through a narrow luminal surface covered with microvilli (Forssmann & Orci 1969). This thought to explain their ability to detect the presence of food in the stomach and changes in intragastric pH. The G cells contain numerous secretory membrane-enclosed granules located between the Golgi complex, where they are formed, and the basement membrane (Håkanson *et al.* 1982b; Sundler *et al.* 1991). The newly formed granules near the Golgi are small and electron-dense and contain the gastrin precursor progastrin (Rahier *et al.* 1987), as well as proteolytic enzymes and chromogranin A. As the granules are transported towards the plasma membrane, they mature into large electron-lucent secretory vesicles (Mortensen *et al.* 1979; Mortensen & McC 1980; Håkanson *et al.* 1982b). The mature secretory vesicles contain various forms of gastrin, which are generated from the processing of progastrin during transport of the granules (see below). The secretory vesicles are stored close to the plasma membrane until stimulation causes secretion (by exocytosis) of the stored products.

Gastrin

Like many other peptide hormones, gastrin requires extensive posttranslational processing to become biologically active (Merchant *et al.* 1994). The antral G cells synthesize several progastrin-derived fragments (e.g. gastrin-34 and gastrin-17) (Rehfeld *et al.* 1991). The gastrins share the same C-terminal tetrapeptide amide (Trp-Met-Asp-Phe-NH_2), which is required for full biological activity as this is the part that binds to gastrin/CCK_2 receptors (Tracy & Gregory 1964). The progastrin molecule of 83 amino acid residues, formed in the endoplasmatic reticulum, is transported to, and proceeds through the Golgi apparatus, where progastrin undergoes partial sulphation and phosphorylation (Brand *et al.* 1984; Dockray *et al.* 1987; Varro *et al.* 1994). After exiting from the Golgi, progastrin is packed in secretory granules that contain the enzymes necessary to complete the progastrin processing. The progastrin molecule is

cleaved and amidated to yield the various end products (for review see Rehfeld 2006). Amidated gastrin-17 (and gastrin-34) is the major form found in tissue and plasma (Rehfeld & Johnsen 1994). Only amidated gastrin, acting on gastrin/CCK_2 receptors, exerts acid stimulatory effects (Morley *et al.* 1965; McGuigan & Thomas 1972; Matsumoto *et al.* 1987; Hilsted *et al.* 1988).

Regulation of the G cell

Gastrin is secreted in response to a variety of food-related stimuli, such as elevated luminal pH (Walsh *et al.* 1975; Schusdziarra *et al.* 1978), intraluminal amino acids and amines (Strunz *et al.* 1978; Lichtenberger *et al.* 1982a, b, 1986; Dial *et al.* 1986), and distension of the stomach (Lloyd *et al.* 1992; Mailliard & Wolfe 1989; Schubert & Makhlouf 1993). Somatostatin-containing D cells have cytoplasmic processes that come in close contact with G cells, suggesting a functional linkage between the two cell types (Alumets *et al.* 1979; Larsson *et al.* 1979). Somatostatin inhibits gastrin release (Hayes *et al.* 1975; Saffouri *et al.* 1980) via somatostatin type-2 receptors (Zaki *et al.* 1996). Also, hormones reaching the antrum via the circulation contribute to the control of gastrin secretion (Chiba *et al.* 1980; Wolfe *et al.* 1983; Sandvik *et al.* 1987b). In addition, G-cell secretion is regulated by the enteric nervous system and the autonomic nervous system (via transmitters such as acetylcholine, gastrin-releasing peptide and galanin) (Dockray *et al.* 1979; Chiba *et al.* 1980; DuVal *et al.* 1981; Schubert *et al.* 1985; Madaus *et al.* 1988, 1990; Schepp *et al.* 1990a, b).

Microdialysis

Background

In many *in vivo* experimental models, the substances studied are monitored by sampling of blood. In order to enable sampling from extracellular environment, a "push-pull cannula" technique was developed (Fox & Hilton 1958; Gaddum 1961; Delgado 1962). In this method, two adjacent cannulas are used: one to introduce fluid

to obtain interstitial solutes, and one to pull the perfusate out (for analysis). The drawbacks are that the tissue may be disturbed by the streaming fluid, and that the inflow and the outflow must be perfectly balanced to avoid pressure build-up and tissue damage. In 1966, Bito et al. developed so-called "dialysis sacs" in order to assess the concentration of amino acids in extracellular tissue. The dialysis sacs, containing 6% dextran in saline, were implanted into the brain and subcutaneous tissue of the neck of dogs. After 10 weeks of equilibration with the surrounding extracellular fluid, the sacs were surgically removed and their contents analyzed. However, the dialysis sacs could only give information on the average concentration of extracellular solutes over a period of time. By combining the two techniques (push-pull cannula and dialysis sacs), Delgado et al. (1972) developed a "dialytrode" consisting of two adjacent cannulas connected by a small porous bag, which enabled continuous sampling without the risk of pressure build-up. In order to increase the membrane area, Ungerstedt and Pycock (1974) implanted hollow semipermeable fibers into the brain. The probes were perfused with a fluid. The "hollow fibre probe" has over the years developed into the "needle probes" used today. Although the microdialysis technique was originally developed, and has been most extensively used, for brain research, the range of applications has increased to cover a variety of biomedical sciences (for reviews see Benveniste 1989; Benveniste & Huttemeier 1990; Ungerstedt 1991; Justice 1993; Plock & Kloft 2005).

Principle of microdialysis

The general principle of the microdialysis probe is to mimic a capillary blood vessel. A capillary consist of a single layer of endothelial cells separated by thin intercellular clefts (approximately 6 nm wide). The clefts, which are slightly narrower than most plasma proteins, allow the passage of small molecules (but not proteins), between plasma and the surrounding tissue. The microdialysis probe consists of a semi-permeable membrane that is connected to an inflow and an outflow tube. A physiological perfusion fluid enters the probe through the inflow tubing at a constant flow rate, passes the membrane and is then transported through the outflow tubing and

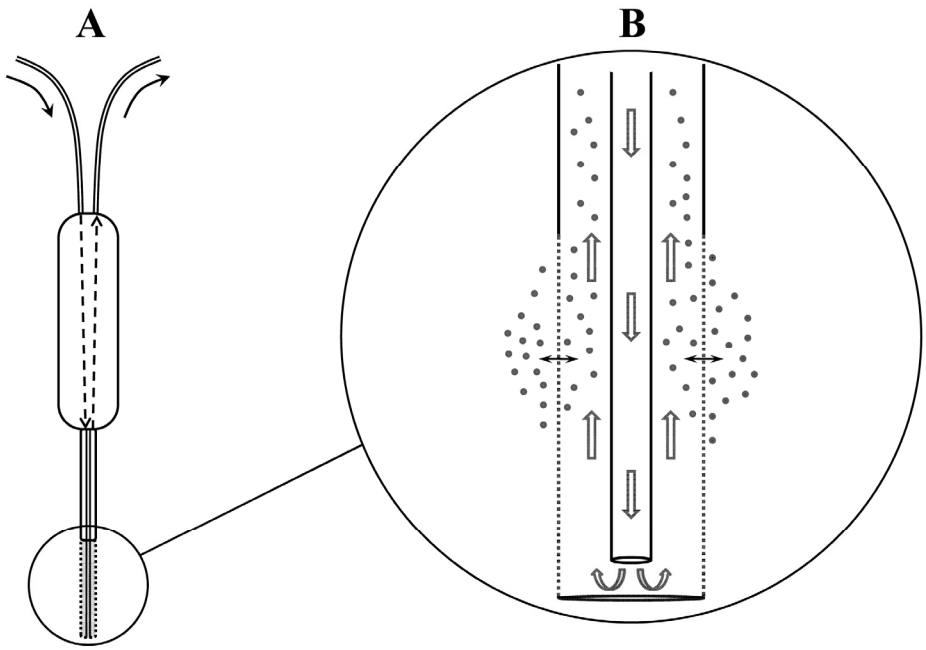


Fig. 2. A. Schematic illustration of a microdialysis needle probe. The perfusion fluid enters the probe through the inflow tubing, passes the membrane and is then transported through the outflow tubing and collected in a vial. B. The membrane of the microdialysis probe. The perfusion fluid is introduced via the inner concentric capillary. As the perfusion fluid flows along the membrane, the perfusate equilibrates with the fluid outside the membrane by diffusion in both directions. Any molecule can pass as long as they are smaller in size than the pores of the membrane.

collected in a vial. The membrane of the probe allows any molecule to pass freely, as long as they are smaller in size than the pores of the membrane, while preventing larger molecules (i.e. proteins and enzymes) from passing. The maximal size of the molecules that can pass the membrane is defined as the "cut-off". The perfusate is generally an aqueous solution with the same osmotic properties as the surrounding medium. As the perfusion fluid flows along the membrane, the perfusate equilibrates with the fluid outside the membrane by diffusion in both directions. Thus, the

microdialysis probe can be used both to collect a substance from, as well as administer it to, the surrounding fluid.

Recovery

The samples obtained through microdialysis do not contain the same concentrations of small molecules as the surrounding medium. Rather, the amount of a substance obtained during microdialysis depends on the "recovery". Relative recovery is defined as the ratio (%) between the concentration of a substance in the dialysate and the concentration of the same substance in the medium outside the membrane (absolute recovery is the amount of a substance obtained in the microdialysate per unit time). A number of factors influence the recovery of a substance. The relative recovery is inversely related to the perfusion flow rate, i.e. with increasing flow rate, molecules will have less time to diffuse over the membrane and the recovery will be lower. The recovery also depends on the properties of the dialysis membrane, such as membrane area and permeability. In addition, the relative recovery depends on the diffusion coefficient of the substance, which is a result of factors like molecular weight of the substance as well as the composition and temperature of the surrounding medium (for detailed discussions see Benveniste 1989; Benveniste & Huttemeier 1990). Moreover, diffusion is often impeded by surrounding structures such as cell membranes and connective tissue (Benveniste & Huttemeier 1990).

AIMS OF THE STUDY

- To assess the usefulness and limitations of the technique of gastric submucosal microdialysis for the study of ECL-cell histamine mobilization *in vivo*

- To investigate the histamine response to local administration of endothelin and adrenaline in the submucosa

- To examine whether the vagus and the sympathetic nervous system contributes to the food- and gastrin-evoked responses of ECL cells *in situ*

- To develop a method for the study of gastrin release by antral submucosal microdialysis

- To explore the importance of the vagus and of local neurons in the control of the G cells *in situ* in response to food and elevated intragastric pH

- To study the mobilization of gastrin from G cells *in situ* in response to locally applied candidate messenger compounds

MATERIAL AND METHODS

Animals

Male and female (as specified) Sprague-Dawley rats (250-300 g) were kept at a 12-h light and 12-h dark cycle in plastic cages (2-3 in each cage) with free access to standard rat food pellets (B & K Universal, Sollentuna, Sweden) and tap water. When the rats were to be fasted, they were housed individually in cages with wire mesh bottoms (free access to water) for 24 h before the experiments. In experiments involving refeeding the fasted rats were offered standard rat pellets and tap water for 3-4 h. Microdialysis experiments were performed on conscious animals, except those experiments that involved electrical vagal stimulation. During sampling of microdialysate they were kept in Bollman-type restraining cages. Starting 1 week prior to the experiments the rats were familiarized with the Bollman cages by daily training for 1-2 h. Blood samples (200 µl) for determination of serum gastrin were drawn from the tip of the tail. Each rat was killed by exsanguination from the abdominal aorta following an overdose of chloral hydrate intraperitoneally. The studies were approved by the local Animal Welfare Committee of Lund/Malmö.

Drugs

α-Fluoromethylhistidine (α-FMH) (paper II): α-FMH, an irreversible inhibitor of HDC (Andersson *et al.* 1990; 1992) was administered continuously via osmotic minipumps (24 h, 3 mg kg^{-1} h^{-1}). The osmotic minipumps (Alzet 2001 D, Alza, Palo Alto, CA, U.S.A.) were implanted subcutaneously in the neck under chloral hydrate anesthesia. Before implantation, the minipumps were activated in 0.9% saline at 37°C for 4 h.

Antiinflammtory agents (paper I): Predinsolone (Precortalone®) was obtained from Organon (Oss, Holland). It was given as a bolus dose (60 mg kg^{-1}, intramuscularly) 72 h before the experiment. Indomethacin (Sigma) was dissolved in 5% $NaHCO_3$ and given intraperitoneally (15 mg kg^{-1}) 2 h before the experiment.

Gastrin (papers I, II & III): Rats to be infused intravenously with gastrin were fitted with a polyethylene catheter in the right jugular vein. The catheter was passed under the skin to a point at the nape of the neck where it was fastened with sutures.

Gastrin receptor antagonist YF476 (paper III): The gastrin receptor antagonist YF476 was obtained from Dr A. Harris (Ferring, Copenhagen, Denmark) and emulsified in PEG 300. It was given as a bolus dose subcutaneously 24 h before start of the experiments (300 μmol kg^{-1}) (Kitano *et al.* 2000a).

Histamine (H_2) receptor antagonist Ranitidine (paper II): The histamine (H_2) receptor antagonist ranitidine hydrochloride (a gift from Astrazeneca, Mölndal, Sweden) was given as a bolus dose subcutaneously (40 mg kg^{-1}) 30 min before start of perfusion with endothelin.

Inhibitors of histamine-degrading enzymes (paper I): Metoprine, an inhibitor of histamine N-methyl transferase, was a gift from Professor T. Watanabe (Tohoku University, Japan). It was dissolved in 1% lactic acid in 0.9% saline and given as a bolus dose (10 mg kg^{-1}) subcutaneously 2 h before the experiment. Aminoguanidine (Sigma), an inhibitor of diamine oxidase, was dissolved in 0.9% saline and given as a bolus dose (10 mg kg^{-1}) subcutaneously 2 h before the experiment.

Insulin (paper III): Human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was injected subcutaneously at a dose known to induce vagal stimulation and acid secretion without affecting the serum gastrin concentration (0.6 IU kg^{-1}, Ekelund *et al.* 1982).

Omeprazole (papers II, IV & V): The proton pump inhibitor omeprazole (a gift from AstraZeneca, Mölndal, Sweden) was dissolved in 0.25% methyl cellulose. It was given to the rats (400μmol kg^{-1}) once daily (for 4 or 7 days) in the morning by oral gavage (Larsson *et al.* 1986).

Surgery

Anaesthesia: Surgery was performed under chloral hydrate anesthesia (300 mg kg^{-1} intraperitoneally). No antibiotics were used. Buprenorphine (Temgesic®, Schering-Plough, NJ, USA) was given subcutaneously (0.05 mg kg^{-1}) at the time of surgery to alleviate postoperative pain. No mortality was associated with the surgery. Experiments involving electrical stimulation of the vagus were performed on rats anaesthetized with fluanisone/fentanyl/midazolam (15/0.5/7.5 mg kg^{-1}, intraperitoneally).

Unilateral vagal denervation (paper III & IV): Unilateral vagotomy was performed by opening the abdominal cavity by a midline incision and by exposing the anterior vagus nerve along the oesophagus below the diaphragm before cutting it as close as possible to the stomach. The posterior vagus nerve was left intact. The rats were fitted with microdialysis probes at the same time.

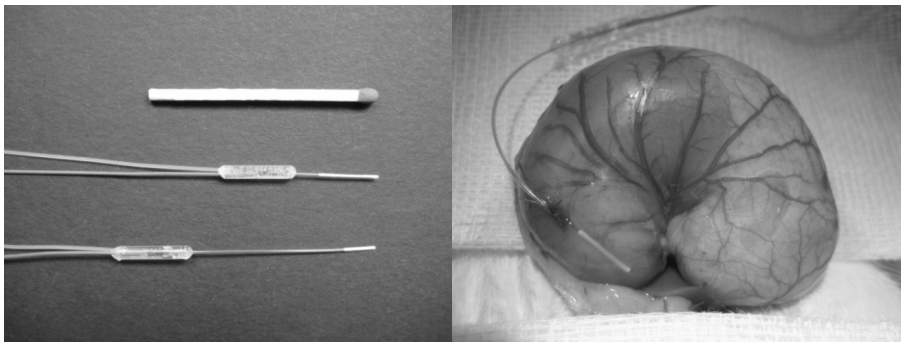
Bilateral vagal denervation (paper IV): Total abdominal vagotomy was achieved by cutting both vagal trunks immediately below the diaphragm. A pyloroplasty was performed at the same time to prevent gastric dilation (Håkanson & Liedberg 1971). The effectiveness of the operation was verified by demonstrating hypergastrinemia (Håkanson *et al.* 1982a; Vallgren *et al.* 1983) in the fasted state 3 days after surgery. The rats were fitted with microdialysis probes at the same time.

Upper abdominal sympathectomy (paper III): The celiac and superior mesenteric ganglia were extirpated (Holmes *et al.* 1967). The ganglia were excised by removal of tissue between the ventral surface of the aorta and the pancreas (between the celiac and superior mesenteric arteries). The aorta was freed from surrounding fat and connective tissue, which was also excised. Excised tissue was fixed in 4% formaldehyde and examined for the presence of ganglionic cells by routine histology (haematoxtlin-eosin staining) to verify success of surgery. Sham operation consisted of laparotomy (midline abdominal incision). The rats were left to recover for 2 weeks before implantation of microdialysis probes.

Pylorus ligation (paper III & IV): The abdomen was opened by a midline incision and a loose fitting loop (noose) of silk thread (4-0) was placed around the pylorus. The ends of the thread were passed through a plastic catheter that was passed through the abdominal opening and tunnelled under the skin to a point at the nape of the neck where it exited and was fastened by sutures. In this way, the two ends of the thread could be easily accessed at the time of the experiment (3 days later). At the same time each rat was fitted with a microdialysis probe on the dorsal side of the stomach. By pulling the ends of the thread the loop tightens around the pylorus (no anaesthesia), ligating the pylorus without disturbing the rat. The ligation was maintained until the animals were killed 4 h later (the tightness of the ligation was verified at necropsy). The gastric juice was collected and its volume determined.

Microdialysis

Implantation of the microdialysis probe: Flexible microdialysis probes (in the antrum: MAB3.35.4, AgnTho's AB, Stockholm, length 4 mm, outer diameter 0.57 mm, 35 kDa cut-off; in the fundus: MAB3.8.10, AgnTho's AB, Stockholm, length 10 mm, outer diameter 0.57 mm, 35 kDa cut-off) were used. The abdomen of the anaesthetized rat was opened by a midline incision. The serosa and the muscle layers of the dorsal or the ventral aspect (at times both) of the antrum or fundus were tangentially puncured by a needle (22 G) and a tunnel (5-8 mm long in the antrum, 12-15 mm long in the fundus) was made in the submucosal layer. In the antrum, the orientation of the tunnel was from the border between fundus and antrum towards the pylorus, ending 1-2 mm proximally to the sphincter. In the fundus, the orientation of the tunnel was from the major to the minor curvature. The microdialysis probe was gently inserted into the tunnel and kept in place with sutures at the tunnel entrance. The inlet and outlet tubes were passed through the abdominal opening and tunneled under the skin to a point at the nape of the neck where they were affixed with sutures.



A **B**

Fig. 3. A. The microdialysis probe models used in the present study, MAB 3.8.10 and MAB 3.35.4 (bottom). B. Microdialysis probe (MAB 3.35.4) implanted on the dorsal side of the antrum.

Fig 4. Sampling of microdialysate during refeeding. The rats are kept in Bollman-type restraining cages during the experiment.

Sampling of microdialysate: If not otherwise stated, microdialysate was sampled 3 days after implantation of the microdialysis probe (Paper I). All rats were conscious during the experiments except those that were subjected to electrical vagal stimulation. The inlet tube was connected to a microinfusion pump (Model 361, Sage Instrument, ATI Orion, Boston, USA) and the outlet was allowed to drain into 300 µl polyethylene vials. Perfusion of the microdialysis probes with 0.9% saline (1.2 µl min^{-1}) started at 7 a.m. When sampling from antrum, a 45 min equilibration period was used, and when sampling from fundus, a 1-2 h equilibration period was used before collection of microdialysate commenced. This equilibration period was found to be appropriate after experiments in which microdialysate was sampled every 30 min for 3 h after start of perfusion. At times, blood was drawn during the experiment for serum gastrin determination. Each rat and each probe was used once only (if not otherwise stated). The position of the probe in the submucosa was verified at autopsy. The microdialysate samples and the serum samples were stored at –20°C until measurement of gastrin or histamine.

Recovery of histamine in vivo (Paper I): To be able to calculate the *in vivo* recovery (relative recovery, see *Introduction*) of histamine, the actual concentration of histamine in the submucosa must be determined. This was achieved by the use of 1) the zero flow method (Jacobson *et al.* 1985; Parsons & Justice 1992) and 2) the no net-flux method (Lönnroth *et al.* 1987; Justice 1993) (see below). Once the submucosal histamine concentration had been determined, the *in vivo* recovery could be calculated.

Calculation of submucosal histamine concentration (paper I): *The zero flow method:* The microdialysis probes were perfused with saline at increasing rates (10, 15, 20, 34, 74 and 150 µl/h). 15 µl samples of microdialysate were collected at each perfusion rate. Each time the perfusion rate was increased sampling was preceded by an equilibration period corresponding to 10-15 µl. For calculation of the submucosal concentration of histamine a population approach of the method of Jacobson *et al.* (1985) was used. The microdialysate histamine concentration (C_{dial}) is a function of the perfusion rate (F), the submucosal histamine concentration (C_{hist}), and the product of

the mass transfer coefficient and the active area of the probe (K), according to the formula: $C_{dial} = C_{hist} (1 – \exp(-K/F))$

(see paper I for details)

The no-net-flux method: The microdialysis probes were perfused with increasing concentrations of histamine (0, 75, 150 and 300 nM histamine). Each time the perfusion solution was changed, sampling was preceded by an equilibration period. After equilibration for 15 min, microdialysate was collected for 30 min. This procedure was repeated for each concentration of histamine. Subtracting the influx histamine concentration from the efflux histamine concentration yields the Δ histamine concentration. The microdialysate histamine concentration that resulted in a Δ histamine concentration=0 (influx concentration equal to efflux concentration) was calculated for each rat (see paper I for details).

Recovery of gastrin in vitro (paper IV): Flexible microdialysis probes (MAB3.35.4, AgnTho's AB, Stockholm, length 4 mm, outer diameter 0.57 mm, 35 kDa cut-off) were used. Microdialysis probes were placed in vials containing serum (37°C): 1) from omeprazole treated rats with a gastrin concentration of 480 pmol l^{-1}, or 2) serum from normal fasted rats, but with synthetic rat gastrin-17 added up to a final concentration of 35 nmol l^{-1}. The probes were perfused with 0.9% saline (1.2 µl min^{-1}). The gastrin concentration in the perfusate was determined and the recovery of gastrin (%) was calculated.

Electrical vagal stimulation (papers III & IV): These experiments involved anaesthetized male rats (see Anaesthesia). The rats (fasted for 24 h) were kept on a warm surface (37°C) during the experiment. The abdomen was opened and the anterior vagus gently exposed. The anterior vagus was stimulated electrically by means of a pair of platinum electrodes (diameter 0.25 mm, distance between electrodes 2 mm). A Grass stimulator (S48 stimulator, Astro-Med. Inc., W Warwick, RI, USA) was used to generate 1 ms impulses of 5 V at 1 Hz, 5 Hz or 20 Hz. After 2 h of basal sampling of microdialysate, sampling started 3 min (the time taken for the perfusion medium to travel from the microdialysis membrane to the outlet of the probe) after start of

electrical stimulation. Samples were collected every 20 min for 1 h. Blood samples were collected at -30 min and 15 min.

Histological and chemical analyses of gastric wall and oxyntic mucosa

Collection of tissue samples from the gastric wall (papers I, II & IV): The stomachs were rinsed with ice-cold saline. For light microscopy, specimens of the gastric wall (2 x 4 mm or 5 x 5 mm) were collected from the area surrounding the microdialysis membrane. For electron microscopy, minute oxyntic mucosal specimens (0.5 mm cubes) were collected close to the probes.

Histochemistry (papers I, II & IV): Sections of the gastric wall (antral or fundic) showing the position of the microdialysis probe in the submucosal compartment and degree of inflammatory response were stained with haematoxylin and eosin/erythrosine (for details, see paper I & IV). Alternatively, carbodiimide-fixed oxyntic specimens were used for histamine immunostaining (Panula *et al.* 1988). The tissue samples were fixed in 4% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide hydrochloride overnight at 4°C, and rinsed with 20% sucrose in 0.1 mol l^{-1} phosphate buffer (pH 7.4). The specimens were frozen at −80°C, and sections were cut in a cryostat at 10 μm thickness and were thawed onto gelatin-coated slides. The sections were incubated with the histamine antiserum (final dilution 1:1000) overnight at 4°C (Håkanson *et al.* 1986). After washing in buffer, the sections were incubated at room temperature for 1 h with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (diluted 1:40). After rinsing, the sections were mounted in buffer: glycerol (1:4), and examined in a fluorescence microscope.

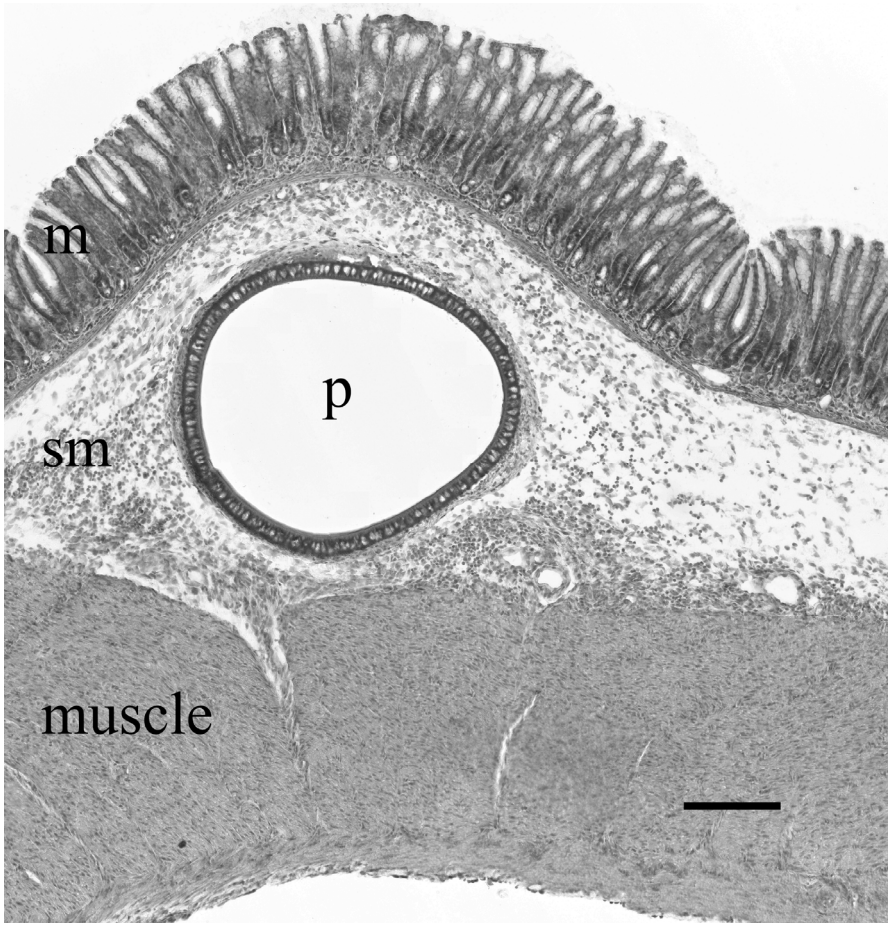


Fig. 5. Transverse section (12µm thick) of the antral wall (stained with haematoxylin and erythrosin), showing mucosa (m), microdialysis probe (p) in the submucosa (sm), and muscularis externa (muscle). Bar = 200 µm. The probe resembles a large blood vessel.

Electron microscopy (paper II): The oxyntic mucosal specimens were immersed in 2% glutaraldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4) at room temperature overnight. The specimens were postfixed for 1 h in 2% osmium tetroxide before being dehydrated in graded ethanol solutions, and embedded in epoxy resin. Sections were cut at 60 nm on an ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined in a JEM-100 CX electron microscope. The ECL cells were recognized by their characteristic ultrastructure (Zhao et al. 1999)

Determination of histamine (papers I, II & III): Histamine was measured in 10 μl microdialysate (diluted 1:10) and in 10 μl culture medium, using a commercially available radioimmunoassay kit (Immunotech, Marseille, France). The histamine concentration was expressed as nmol l^{-1} or pmol $well^{-1}$. All agents used in the infusion experiments were tested with respect to their interference with the radioimmunoassay of histamine. Only gastrin was found to interfere, precluding the use of doses higher than 0.1 mmol l^{-1} for local administration. For determination of histamine concentration in tissue, oxyntic mucosa was homogenized in ice-cold sodium phosphate buffer, pH 7.4, to a concentration of 100 mg ml^{-1}. The homogenate was diluted 1:10 in 3% trichloroacetic acid, and placed in boiling water for 5 min. The precipitated material was spun down, and the clear supernatant was diluted 1:50 with redistilled water. Histamine was determined spectrophotofluorometrically, and expressed as μg g^{-1} (Håkanson & Rönnberg 1974; Rönnberg & Håkanson 1984).

Determination of HDC activity (paper II): Oxyntic mucosa was homogenized in ice-cold sodium phosphate buffer, pH 7.4, to a concentration of 100 mg ml^{-1}. Aliquots (80 μl) of the oxyntic mucosal homogenates were incubated with L-[l-^{14}C]histidine (specific activity 50 mCi $mmol^{-1}$), 0.5 mmol l^{-1} L-histidine and 0.01 mmol l^{-1} pyridoxal-5-phosphate, in a total volume of 160 μl at 37°C for 1 h, as described previously (Larsson *et al.* 1986). HDC activity was expressed as pmol $^{14}CO_2$ mg^{-1} h^{-1}.

Determination of gastrin in microdialysate (papers IV & V): Gastrin in the microdialysate was measured by a radioimmunoassay using antiserum no. 92132/5 with rat gastrin-17 as standard and monoiodinated ^{125}I-gastrin-17 as tracer (Stadil & Rehfeld 1972; Rehfeld 1998). The gastrin concentrations in the microdialysate were measured in 15 µl of microdialysate and expressed as picomole equivalents of rat gastrin-17 per liter. All agents used in the infusion experiments were tested with respect to their interference with the radioimmunoassay of histamine. ANF, CCK-8s, endothelin, leu-enkephalin, met-enkephalin, PACAP-27, secretin and substance P were found to interfere in the RIA, and they were therefore excluded from the study (for a detailed description of the radioimmunoassay, see Paper IV).

Determination of gastrin in serum (papers II-V): The concentration of gastrin in serum was measured by a radioimmunoassay using antiserum no. 2604 (Rehfeld *et al.* 1972) with rat gastrin-17 as standard and monoiodinated ^{125}I-gastrin-17 as tracer (Stadil & Rehfeld 1972). The serum gastrin concentration was measured in 50 µl of serum and expressed as picomole equivalents of rat gastrin-17 per liter.

RESULTS AND DISCUSSION

In 1964 Kahlson and coworkers demonstrated that gastrin infusion and refeeding following a fasting period resulted in a transient reduction in the histamine content in the rat oxyntic mucosa. In order to study histamine mobilization from the ECL cells, different *in vitro* experimental models have been developed, including: isolated oxyntic mucosa (Rangachari 1975), the totally isolated vascularly perfused rat stomach (Sandvik *et al.* 1987a) and isolated ECL cells (Soll *et al.* 1981; Prinz *et al.* 1993; Lindström *et al*. 1997). Although *in vitro* models allow more direct studies of the ECL cells, they are "unphysiological" in that they do not take into account the role of neuronal and hormonal circuits. Demonstration of ECL cell histamine mobilization has proven difficult *in vivo* however. Once released, histamine is being continuously degraded by extracellular enzymes. Also, circulating basophiles release histamine upon damage, which complicates determination of plasma histamine from blood samples.

Recently a protocol was developed in our group to make use of the microdialysis technique to study histamine mobilization from gastric ECL cells in conscious rats (Kitano *et al.* 2000b). Since then, this technique has been used in a number of studies to characterize regulation of ECL-cell secretory activity *in vivo* (Norlén *et al.* 2000a, b, 2001b; Konagaya *et al.* 2001; Håkanson *et al.* 2001; Bernsand *et al.* 2007; Kitano *et al.* 2005; Fykse *et al.* 2006). Also, the microdialysis technique has been used for the study of the regulation of Ghrelin cells (Dornonville de la Cour *et al.* 2007) and D cells (Bernsand *et al.* 2007) *in vivo*.

The aim of Paper I *was to determine the actual submucosal histamine concentration in the acid-producing part of the rat stomach and to assess the usefulness and limitations of gastric submucosal microdialysis for the study of ECL-cell histamine mobilization in conscious rats.*

Calculation of submucosal histamine concentration: The histamine concentration in the microdialysate is proportional, rather than identical, to the "true" submucosal concentration. Therefore, an attempt was made to determine the "true" submucosal histamine concentration in fasted and freely fed rats. With the no net-flux method (Lönnroth *et al.* 1987; Justice 1993), the submucosal histamine concentration was estimated by perfusing the microdialysis probes with varying concentrations of histamine (see *Material and Methods*), while the zero flow method (Jacobson *et al.* 1985; Parsons & Justice 1992) was used to estimate the submucosal histamine concentration by perfusing microdialysis probes at increasing flow rates (see *Material and Methods*). By using these two methods, the submucosal histamine concentration was found to be 75 - 90 nM in fasted rats, and 90-100 nM in freely fed rats. The surprisingly small difference in submucosal histamine concentration between fasted and freely fed rats may be explained by the fact that rats have nocturnal habits: They eat mainly during the night. Food intake results in increased serum gastrin levels that stimulate histamine mobilization from the ECL cells. When the rats stop eating in the morning, gastrin levels slowly begin to decrease towards fasting levels. The *in vivo* recovery experiments were performed during daytime, and freely fed rats were thus in a transition between the fed and the fasted state. Once the submucosal histamine concentration had been determined, the *in vivo* recovery (relative recovery, the ratio between the concentration of histamine in the microdialysate and in the submucosa) could be calculated. The *in vivo* recovery was found to be 49% at the flow rate used (74 µl h^{-1}). In a previous study the corresponding *in vitro* recovery of histamine was found to be in the range of 50-60% (Kitano *et al.* 2000b). Diffusion of most hydrophilic substances is impeded by impermeable cell membranes (Nicholson *et al.* 1979). Thus, the diffusion coefficient for histamine in the submucosa, comprising interstitial-, intracellular- and vascular compartments, is expected to be lower than that of an aqueous solution (Benveniste & Huttemeier 1990). That considered, the differences found between *in vivo* and *in vitro* recovery of histamine were surprisingly small.

Importance of the composition of the perfusion medium: Exocytosis (release of histamine) in ECL cells depends upon Ca^{2+} entry, and so, we wanted to investigate to what extent ECL-cell histamine mobilization depends on the perfusion medium and, especially, on the Ca^{2+} concentration of the medium. Perfusion with various physiological salt solutions, 0.9 % saline or Krebs-Ringer variants (0, 1.2 and 3.4 mM Ca^{2+}) failed to affect either basal or gastrin-induced histamine mobilization, indicating that the Ca^{2+} concentration in the submucosa is sufficient to allow the ECL cells to secrete histamine, and that the perfusion medium has little impact on the mucosal Ca^{2+} concentration.

Importance of histamine-degrading enzymes: The distance histamine must diffuse from the ECL cells to reach the microdialysis probe was found to be approximately 200 μm. Once released from the ECL cells, histamine is rapidly degraded. Two enzymes, diamine oxidase and N-methyl transferase, are thought to be of greatest importance. Pretreatment with metoprine, an inhibitor of histamine N-methyl transferase, did not significantly affect either basal or gastrin-stimulated histamine mobilization. However, pretreatment with aminoguanidine (inhibitor of diamine oxidase) raised both basal and gastrin-stimulated histamine mobilization (~50 %), supporting the view that in rats, diamine oxidase may be more important than N-methyl transferase for the degradation of ECL-cell histamine (*see also* Schayer 1966). Moreover, the fact that quite significant degradation of histamine occurs when histamine is collected locally in the submucosa, illustrates the advantage of local microdialysis compared to measurements of histamine in the gastric venous outflow or in peripheral blood.

Local inflammatory response: Inflammatory mediators, such as prostaglandin E_2 and IL-1β, released during an inflammatory response are powerful inhibitors of synthesis and release of histamine from ECL cells (Prinz *et al.* 1997; Lindström & Håkanson 1998, 2001; Norlén *et al.* 2001b; Björkqvist *et al.* 2005). Introduction of the

microdialysis probe into the gastric submucosa is associated with some tissue damage and a reactive inflammatory response which could affect the ECL cells. In Paper I, variations in histological parameters reflecting the inflammatory response after implantation of the microdialysis probe were measured over a 10 day period. Histological examination revealed minor local bleedings in 1/3 of the rats. In addition, low grade edema, fibrosis, and a low to moderate number of inflammatory cells were seen around the probe in most rats. The edema persisted throughout the study. Invasion of inflammatory cells peaked during the first 3-4 days after implantation of the probe and declined subsequently. Fibrosis was slow and remained modest for the first couple of days, increasing thereafter. However, pretreatment with antiinflammatory agents (prednisolone and indomethacin) failed to affect the concentration of histamine in the microdialysate, indicating that although there is a moderate inflammatory reaction, it does not impair the mobilization of ECL-cell histamine.

When to perform microdialysis after surgery? All things considered, day 3 or 4 after implantation of the probe was considered suitable for performing gastric submucosal microdialysis studies since at this point in time the edema was stable and the fibrosis was still inconspicuous. Also, often the microdialysis experiments are performed on rats fasted for 24 h. The fasting included, less than 3 days would not give rats sufficient with time to recover from the surgery.

To summarize, gastric submucosal microdialysis is a reliable method to study ECL-cell histamine mobilization, offering the advantage that "real-time" studies can be performed in conscious animals. The potential problems of the technique are: 1) the inflammatory response to the implantation of the microdialysis probe may affect the results. However, the results suggest that the impact of the inflammatory response is minor and does not affect the results of the experiments. 2) Histamine may be degraded before reaching the interior of the probe, resulting in misleadingly low

values. Indeed, blockade of histamine-degrading enzymes doubled the recovery of histamine. The fact that quite significant degradation of histamine occurs when histamine is collected in the submucosa indicates that local microdialysis should be advantageous, compared to measurements of histamine in the gastric venous outflow or in peripheral blood.

Paper II *examines endothelin- and adrenaline-evoked histamine mobilization. Specifically, the aim was to identify the source of gastric histamine mobilized by endothelin and adrenaline, to explain why the histamine response to endothelin and adrenaline is short-lasting, to establish whether the effects of endothelin and adrenaline on the mucosal histamine stores are direct or indirect, and to investigate whether the spectacular local release of histamine contributes to the mucosal injury or vice versa.*

Endothelin and adrenaline mobilize ECL-cell histamine and cause mucosal damage

In a previous microdialysis study, gastric submucosal microinfusion of endothelin and adrenaline induced a large but short-lasting (1-2 h) release of histamine; the peak response was much greater than that induced by any other stimulus (e.g. gastrin, VIP and PACAP) (Norlén *et al.* 2001b). Also, the two agents were found to cause severe local damage to the mucosa (along the probe). There may be different explanations for this mucosal damage: Both agents are powerful vasoconstrictors and known to generate local tissue damage in the gastric mucosa (Whittle & Esplugues 1988; Tepperman & Whittle 1991; Whittle 1993). Alternatively, their ability to mobilize large amounts of gastric histamine (Norlén *et al.* 2001b; Kitano *et al.* 2005; Bernsand *et al.* 2008) could contribute to the mucosal damage by stimulating acid secretion locally.

Mechanisms behind the gastric mucosal injury: The gastric mucosal damage seen after local infusion of adrenaline and endothelin was found to be independent of the concurrent massive histamine release: Local administration of high concentrations of histamine alone did not induce any mucosal damage, and local administration of histamine H_1- and H_2-receptor antagonists failed to prevent the endothelin-induced mucosal damage. It thus seems more likely that the mucosal damage was related to the vasoconstrictory effects of adrenaline and endothelin. Subjecting rats to gastric ischemia (clamping of gastric arteries) will induce severe mucosal damage. Also, the effect of the ischemia can be prevented by acid blockade (Kitano *et al.* 1997, 2005). This was confirmed in the present study, where inhibition of acid secretion (by systemic administration of the H_2-receptor antagonist ranitidine or the proton pump inhibitor omeprazole) prevented the endothelin-induced mucosal injury, without preventing the mobilization of histamine. The results indicate that adrenaline and endothelin cause ischemia that undermines the resistance of the mucosal barrier to the tissue-damaging effect of acid back diffusion.

Is histamine mobilized from ECL cells or mast cells? Histamine is present in both ECL cells and mast cells in the rat oxyntic mucosa although the ECL cells are far more numerous than the mast cells which harbors about 20% of the histamine in the rat stomach (Håkanson *et al.* 1986, Andersson *et al.* 1992). Local administration of adrenaline and endothelin was associated with a reduced histamine concentration in the mucosa close to the probe. Histological examination revealed a reduced intensity of histamine immunostaining in ECL cells with a reduced number of histamine-storing secretory vesicles in the ECL cells close to the probe, while the mast cells seemed unaffected. α-FMH has been shown to deplete ECL cells of histamine (by irreversible inhibition of HDC), while leaving mast cells unperturbed (Andersson *et al.* 1992). In the present study, systemic pretreatment with α-FMH almost abolished the histamine response to local administration of endothelin, indicating that the ECL cells are the source of histamine. Moreover, in rats with high ECL cell HDC activity (induced by omeprazole) endothelin and adrenaline mobilized much larger amounts of histamine than in control rats. Taken together, the results indicate that the ECL cells are the

prime source of mobilized histamine. In accordance with our findings, Kitano et al. (2005) reported that there was no difference between wild-type rats and mast cell-deficient rats in the histamine response to ischemia (evoked by clamping of the celiac artery). Also, pretreatment with α-FMH virtually abolished the histamine response to ischemia (Kitano *et al.* 2005).

Why is the histamine response to adrenaline and endothelin short-lasting? The pattern of histamine mobilization differed greatly between gastrin on the one hand and adrenaline and endothelin on the other. Local administration of gastrin produced a dose-dependent sustained increase in the microdialysate histamine concentration without affecting the local histamine concentration in the tissue. Also, the local HDC activity was increased by gastrin. The continuous histamine response to gastrin probably reflects the fact that histamine is being continuously replaced due to accelerated formation.

In the short term, endothelin mobilized more histamine that did gastrin. Local administration of endothelin and adrenaline produced a massive short-lasting (1-2 h) mobilization of histamine with a peak response up to 5 and 10 times greater than the integrated response and the peak response to gastrin, respectively, without stimulating the HDC activity. Also, a near-maximally effective dose of endothelin released about 70% of the histamine store, an effect that is unlikely to be explained by exocytosis. In fact, in a later study it was shown that ischemia (evoked by local infusion of vasoconstrictors or clamping of the celiac artery) induced massive histamine release but failed to mobilize pancreastatin, indicating a non-exocytotic release of histamine (Bernsand *et al.* 2008). In the present study, the ECL cells were shown to need several days to recover from a single dose of endothelin or adrenaline before they were able to respond to a second challenge. However, the fact that the ECL cells did recover indicates that the two vasoconstrictors did not damage them irrevocably. The results favor the view that mobilization of ECL-cell histamine by endothelin and adrenaline is short-lasting, because the store of releasable histamine is rapidly exhausted and because histamine resynthesis is slow.

Is the effect of adrenaline and endothelin direct or indirect? Endothelin was inactive, and adrenaline only moderately active (compared to gastrin) in releasing histamine from ECL cells in primary culture (see also Lindström *et al.* 1997). This indicates that endothelin and adrenaline affect ECL cells *in situ* in an indirect manner. It is not inconceivable that vasoconstriction (and possibly ischemia) is the causative factor.

Paper III *was designed to investigate the nervous regulation of rat stomach ECL cells. The aim was to examine whether the vagus and the sympathetic nervous system contribute to the food- and gastrin-evoked responses of ECL cells in situ. This was tested by 1) acute vagal excitation, and 2) vagal or sympathetic denervation, using gastric submucosal microdialysis.*

Food intake is thought to stimulate gastric acid secretion not only via activation of the gastrin-ECL cell-parietal cell axis but also via vagal excitation (of the ECL cells or the parietal cells or both). In the rat, the importance of the vagus is illustrated by the fact that vagal excitation induced by pylorus ligation or insulin hypoglycaemia stimulates acid secretion (Brodie & Knapp 1966; Håkanson *et al.* 1980; Ekelund *et al.* 1982), and that vagal denervation inhibits both basal and stimulated acid secretion (Håkanson *et al.* 1982a; Vallgren *et al.* 1983). In addition, unilateral vagotomy results in ECL-cell hypoplasia on the denervated side of the stomach, suggesting a tonic influence of the vagus on the ECL-cell population (Håkanson *et al.* 1984, 1992; Axelson *et al.* 1988).Vagal excitation is thought to stimulate the parietal cell through cholinergic mechanisms since acid secretion can be inhibited by muscarinic receptor blockers (e.g. atropine and pirenzepine) (Ekelund *et al.* 1987; Riedel *et al.* 1988), although cholinergic agents (e.g. carbachol and bethanecol) are poor secretagogues in the chronic gastric fistula rat (Ding & Håkanson 1996; Nishida *et al.* 1996). The ECL cells do not seem to operate under cholinergic control (Lindström *et al.* 1997; Lindström & Håkanson 2001; Norlén *et al.* 2001b), but respond with secretory activation to a

number of neuropeptides (e.g. PACAP and VIP) and to adrenaline and noradrenaline (Lawton *et al.* 1995; Lindström *et al.* 1997; Zeng *et al.* 1998, 1999; Lindström & Håkanson 2001; Norlén *et al.* 2001b; Paper II).

Histamine mobilization in response to vagal stimulation: Vagal activation was induced by one-sided electrical vagal stimulation (anaesthetized rats), acute pylorus ligation or insulin hypoglycaemia. Electrical vagal stimulation has previously been shown to stimulate gastrin secretion in rats (Schubert *et al.* 1982; Alino *et al.* 1983). In the present study, both gastrin and histamine were released in response to electrical vagal stimulation (5 & 20 Hz). At 5 Hz, the microdialysate histamine concentration peaked within 10-20 min (2-fold increase) and then declined to prestimulation levels. The histamine response was preceded by a 4-fold increase in the serum gastrin concentration. Such an increase in serum gastrin most likely stimulates ECL-cell histamine mobilization (Konagaya *et al.* 2001), and it is therefore not surprising that one-sided electrical vagal stimulation resulted in histamine mobilization on both sides of the stomach (albeit more effectively on the stimulated side). Pretreatment with gastrin receptor antagonist (YF476) virtually abolished the histamine response, indicating that electrical vagal stimulation mobilizes ECL-cell histamine via release of gastrin rather than via a direct action on the ECL cells. Acute pylorus ligation mobilized only small amounts of ECL-cell histamine and gastrin (not statistically significant), which is in agreement with an earlier report demonstrating the lack of effect of pylorus ligation on the ECL-cell histidine decarboxylase activity (Zhao *et al.* 1996). However, in Paper IV (see below), using rats with microdialysis probes implanted in the antral submucosa, the gastrin concentration in both serum and microdialysate was significantly increased following pylorus ligation. In Paper III, insulin hypoglycaemia also failed to mobilize ECL-cell histamine and gastrin.

Histamine mobilization following unilateral vagal denervation: We monitored food- and gastrin-evoked ECL-cell histamine mobilization after unilateral vagotomy, taking advantage of the fact that each vagal trunk innervates one side of the stomach only and that denervation of one side does not impair the functional capacity of the

other (Håkanson *et al.* 1984, 1992). By placing microdialysis probes on both sides of the stomach, histamine mobilization on the intact side of the stomach can be compared with that on the vagotomized side. In intact rats (fasted for 24h) the histamine response to refeeding was equally large on the two sides of the stomach. Intake of food resulted in a 4-fold increase (peak within 20-40 min) in the microdialysate histamine concentration, followed by a gradual decline to a level 2-fold over basal (4h), while the serum gastrin concentration was increased 2- to 3-fold. Unilateral vagotomy reduced the histamine response to food intake by 56% on the denervated side as compared with the intact side of the stomach without affecting circulating gastrin levels. Since circulating gastrin should affect ECL cells on either side of the stomach similarly and simultaneously, the histamine response to refeeding observed following unilateral vagotomy is most likely not explained by perturbations in the serum gastrin concentration. In fact, when food-evoked gastrin mobilization was monitored in a similar experiment using microdialysis probes in the antral submucosa (see Paper IV), unilateral vagotomy was found to increase the secretory activity of the G cells.

Unilateral vagotomy was shown to cause a shift to the right of the gastrin dose-histamine response curve and the serum gastrin concentration-histamine response curve. In fact, the histamine response to near-maximally effective doses of gastrin was unaffected by unilateral vagotomy but reduced by 60% at serum gastrin concentrations within the physiological range. The desensitization of the gastrin receptor after vagotomy was reflected by a 7-fold increase in the gastrin EC50 value. Previously, in vagally intact rats, it was noted that upon sustained exposure to high concentrations of gastrin the gastrin receptor of the ECL cells changes from a high affinity to a low affinity state (Konagaya *et al.* 2001). The present study suggests that also the vagus is capable of controlling the sensitivity of the gastrin receptor. Since vagal denervation was found to shift the serum gastrin concentration-histamine response curve to the right, it is tempting to speculate that vagal stimulation may shift the concentration-response curve to the left. This would explain the greater histamine response on the stimulated than on the non-stimulated side during one-sided electrical vagal stimulation.

Histamine mobilization following sympathetic denervation: Previously, it has been shown that sympathetic nerve stimulation may activate the ECL cells directly as well as indirectly: the sympathetic transmitters noradrenaline and adrenaline are capable of acting directly on β-adrenergic receptors on the ECL cells (Lindström *et al.* 1997; Lindström & Håkanson, 2001), but there is also evidence suggesting that they may activate ECL cells in situ in an indirect manner (conceivably via vasoconstriction-evoked ischaemia) (Norlén *et al.* 2001b; Paper II). In Paper III, surgical sympathectomy had no effect on either basal, gastrin- or food-evoked histamine mobilization, suggesting that under normal circumstances the sympathetic nervous system plays a minor role in the food-evoked ECL cell histamine response.

In conclusion, in Paper III, we obtained no evidence that acute vagal activation induced histamine mobilization by a direct action on the ECL cells. Nonetheless, ablation of the vagal input to the stomach impaired the histamine response of the ECL cells to both food and gastrin. Unilateral vagotomy resulted in a rightward shift of the serum gastrin concentration-histamine response curve on the denervated side of the stomach. This observation is in line with the view that the vagus controls the ECL cell activity and that the suppressed responsiveness of the ECL cells to gastrin after vagotomy reflects gastrin receptor desensitization. From the results in Paper III, it seems that the sympathetic nervous system plays a minor role (if any) in the food-evoked histamine response.

In Paper IV and V *the microdialysis technique was used to study the regulation of gastrin secretion from antral G cells.*

G cells secrete gastrin in response to a variety of food-related stimuli. This pathway is thought to involve both neuronal and paracrine/endocrine pathways (see *Introduction*). Gastrin secretion from antral G cells has been the subject of numerous studies in the

past using either *in vivo* or *in vitro* techniques. Although *in vivo* models allow studies of intact animals, it is usually difficult to decide whether the G-cell response is a direct effect of the experimental intervention or a consequence of confounding systemic effects. Also, gastrin is usually monitored in the peripheral circulation, a procedure that makes it difficult to detect rapid fluctuations. While *in vitro* methods, such as isolated stomachs (Chiba *et al.* 1980; DuVal *et al.* 1981; Sandvik *et al.* 1987a, b), antral sheets (Hayes & Williams 1975) or isolated G cells (Lichtenberger *et al.* 1982a; Dial *et al.* 1986), enable more direct studies of the G cell, the nervous and hormonal circuits, that may affect the ability of the G cells to respond to stimuli, are either non-existent or not operative. The microdialysis technique combines the advantages of *in vivo* and *in vitro* methods, in that specific cell populations can be studied in whole animals under physiologically relevant experimental conditions. Hence, the possibility to use microdialysis to monitor gastrin secretion from G cells would offer new opportunities to study how these cells are regulated.

The aim of Paper IV *was to develop a method for the study of gastrin release by antral submucosal microdialysis, to compare measurement of gastrin in blood and microdialysate following a series of treatments known to influence gastrin secretion, and to explore the importance of the vagus and of local neurons in the control of the G cells in relation to other means of stimulating gastrin secretion (food, elevated antral pH).*

In Paper IV we developed a protocol for antral submucosal microdialysis to monitor the concentration of gastrin in, and to introduce substances locally into, the antral submucosa (reverse microdialysis) of conscious rats. Implantation of the probe is known to cause tissue damage and an inflammatory response in the stomach wall (Paper I). Indeed, histological analysis of probe-carrying antral wall (3 days after implantation) revealed inflammatory cells around the probe and mild oedema in the

submucosa. The probe was invariably found in the submucosa and the distance between the membrane of the probe and the basal part of the antral mucosa, where most of the G cells are located, was found to be 255±20 µm.

In vitro recovery: To be able to translate the gastrin concentration in the microdialysate into the "true" submucosal concentration, the recovery of gastrin in the microdialysate had to be determined. By placing microdialysis probes in rat serum (37°C) containing different concentrations of gastrin (480 pmol l^{-1} and 35 nmol l^{-1}), the *in vitro* recovery of gastrin was found to be approximately 5%. The low recovery is thought to reflect the relatively large molecular weight of gastrin.

The *in vivo* recovery was not determined due to technical difficulties (the microdialysate gastrin concentrations seen under basal conditions were close to the lower detection limit of the gastrin assay). The recovery of gastrin is expected to be somewhat smaller *in vivo* than *in vitro* (see Paper I). Nonetheless, since the *in vitro* recovery was independent of the gastrin concentrations of the medium, we expect changes observed in microdialysate gastrin to parallel changes in the submucosa.

Gastrin in serum and microdialysate: In order to compare gastrin concentrations in serum and microdialysate, simultaneous sampling of serum and microdialysate was performed from rats with different basal gastrin levels (fasted and hypergastrinemic rats). The measurements revealed a close correlation between the serum gastrin concentration and the antral submucosal gastrin concentration. Also, in hypergastrinemic omeprazole-treated rats with microdialysis probes implanted in both antrum and corpus, the gastrin concentration in the microdialysate was found to be 14 times higher in antral probes than in probes in the corpus. Considering the recovery, the calculated gastrin concentration in the extracellular compartment of the corpus of omeprazole-treated rats was similar to that observed in serum from these rats. There have been suggestions that secreted gastrin reaches the oxyntic mucosa via a microcirculatory short-cut (i.e. a portal system) (Taylor & Torrance, 1975). The findings of Paper IV are in line with the view that gastrin reaches the corpus via the systemic circulation rather than via a direct route from antrum to corpus: The

concentration would presumably have been considerably higher in the corpus than in the circulation if circulating gastrin was distributed via the corpus.

Gastrin secretion in response to food: In male rats, intake of food resulted in a 3-fold increase in circulating gastrin concentrations during the first h, followed by a decline to a level still 2-fold over basal (after 3h). In male rats equipped with bilateral probes, the corresponding microdialysate gastrin concentration peaked during the first hour with an almost 20-fold increase over basal, after which it declined to a level about 2- to 3-fold above basal. The gastrin response was similar on either side of the antrum. It can be noted that the peak gastrin response was much more prominent in microdialysate than in the circulation. Presumably, rapid local changes are blunted in the circulation due to distribution of gastrin in the bloodstream and other body compartments. In female rats the increase in microdialysate gastrin after food intake was less prominent (peak increase 6-fold over basal) than in male rats. Conceivably, this reflects the long term difference in food consumption between male and female rats (Lichtenberger *et al.* 1976); the difference may manifest itself in females in a smaller number of G cells and in a smaller gastrin response to food.

Vagal and local neuronal control of gastrin secretion

The role of the vagus in the regulation of G-cell gastrin secretion is not fully understood. Gastrin secretion has been reported to be either stimulated or inhibited by vagal excitation (for reviews see Grossman 1978; Soll & Walsh 1979). The G cells are probably not directly innervated by vagal fibers. Instead, the vagus is thought to act via the enteric nervous system (Berthoud *et al.* 1990; Holst *et al.* 1997).

Gastrin mobilization in response to vagal stimulation: Vagal activation was induced by one-sided electrical vagal stimulation (anaesthetized rats) or by acute pylorus ligation (conscious rats). Electrical vagal stimulation is known to stimulate gastrin release (Uvnäs *et al.* 1975; Nishi *et al.* 1985; Madaus *et al.* 1990; Paper III). In Paper IV, electrical vagal activation for 1 h raised the microdialysate gastrin concentration

on the stimulated side, as well as circulating gastrin concentrations at all frequencies tested (1, 5 and 20 Hz). The increase was 3- to 10-fold in the microdialysate and 2- to 4-fold in serum. Berthoud et al. (2001) reported that electrical stimulation of the ventral vagal trunk in anaesthetized rats activated not only the enteric neurons on the ventral side of the antrum but also one third of the neurons on the dorsal side, indicating an overlap in the vagal innervation of the antrum. Indeed, in Paper IV using rats with probes on both sides of the antrum, gastrin was released from both sides of the antrum in response to selective electrical stimulation (5 Hz) of the anterior vagus.
Pylorus ligation is said to induce vagal excitation (Brodie 1966; Brodie & Knapp 1966). In Paper IV, pylorus ligation resulted in a prompt but transient increase in the microdialysate gastrin concentration. It has previously been reported that pylorus ligation does not affect the serum gastrin concentration (Alumets *et al.* 1982; Zhao *et al.* 1996). It is possible that the discrepancy may be explained by differences in the experimental setup: In our experiments, pylorus ligation was applied in conscious rats, whereas previous studies used rats that were anaesthetized during ligation (Alumets *et al.* 1982; Zhao *et al.* 1996). However, in Paper III, the increase in gastrin serum concentration following acute pylorus ligation was not statistically significant.

Gastrin mobilization following vagal denervation: Bilateral vagal denervation suppresses acid secretion and causes secondary hypergastrinemia (Håkanson *et al.* 1982a; Vallgren *et al.* 1983). Unilateral vagal denervation, on the other hand, suppresses acid secretion on the denervated but not on the intact side (Håkanson & Liedberg 1971) and does not affect circulating gastrin levels. This was confirmed in Paper IV as bilateral vagotomy resulted in a 5- to 10-fold increase in the serum gastrin concentration (rats fasted for 24h), which unilateral vagotomy failed to do. We monitored food-evoked gastrin mobilization after unilateral vagotomy in rats with probes implanted on both sides of the antrum. In intact rats the microdialysate gastrin response to food (see above) was similar on either side. However, in unilaterally vagotomized rats, food-evoked gastrin secretion on the denervated side differed greatly from that on the innervated side. While food intake resulted in an immediate increase in microdialysate gastrin concentration (10-fold increase within 20-40 min)

followed by a gradual decline to a level 4-fold above basal on the intact side of the antrum, the microdialysate gastrin concentration increased and stayed elevated (10-fold over basal) on the vagotomized side throughout the experiment. It thus seems that the vagus is capable not only of stimulating gastrin secretion (as e.g. shown by the gastrin response to electrical vagal stimulation and pylorus ligation), but also of lowering gastrin secretion, as shown by the relative increase in the integrated gastrin response on the vagotomized side of the antrum. This paradoxical effect of unilateral vagotomy can be interpreted to mean that a vagally dependent inhibition of the G cells is eliminated by the vagotomy (desinhibition).

The proton pump inhibitor omeprazole is a powerful inhibitor of acid secretion (Larsson *et al.* 1986). Blockade of acid secretion, whatever the cause, leads to hypergastrinemia (Håkanson *et al.* 1982a; Ryberg *et al.* 1988). In Paper IV, the concentration of gastrin in the circulation as well as in the antral microdialysate was increased in both omeprazole-treated rats and bilaterally vagotomized rats. The combination of bilateral vagal denervation and omeprazole treatment was not more effective than omeprazole treatment alone when it comes to raising the microdialysate (or serum) gastrin concentration. These results are in line with the view that lack of acid in the lumen represents an important stimulus of gastrin secretion, and that an intact vagal innervation is not crucial for the ability of the G cells to respond to a prolonged period of achlorhydria.

Local neuronal control of gastrin secretion: Tetrodotoxin (TTX) is a potent neurotoxin that blocks action potentials in nerves (Kao 1966). With the aim of investigating the influence of local neurons (enteric neurons) on the G cells, TTX was administered via the microdialysis probe in order to inactivate local neurons (Boehnke & Rasmusson 2001; van Duuren *et al.* 2007). In fasted male rat equipped with bilateral probes, unilateral infusion of TTX reduced the gastrin response to food dose-dependently on the TTX-treated side. At the highest concentration of TTX (100 µmol l^{-1}) the food-evoked gastrin response was virtually abolished on the TTX-treated side, while a normal (15-fold) increase was seen on the control side of the antrum. The amount of food ingested was not affected by TTX. Also, gastrin was released in

response to local infusion of GRP during concomitant administration of TTX, indicating that the G cells ability to release gastrin was not impaired by TTX (Ericsson, unpublished observation). G cells are known to respond, directly or indirectly, to food-related stimuli (see *Introduction*). Thus, the failure of food to release gastrin after local administration of TTX suggests that the gastrin response is mediated by local neurons or at least dependent on the permissive action of such neurons. Interestingly, in omeprazole-treated hypergastrinemic rats, local infusion of TTX (100 μmol l^{-1}) promptly lowered the microdialysate gastrin concentration by 65%. Hence, while the gastrin response to elevated antral pH (following omeprazole treatment) was unaffected by vagotomy (see above) it was clearly dependent upon the enteric nervous system.

To summarize, the microdialysis technique was found to be useful for the study of local gastrin secretion in conscious rats and to offer advantages over measurements of gastrin in serum in that 1) rapid changes in gastrin concentrations were more prominent in antral microdialysate than in serum, and 2) the technique allows each side of the antrum to serve as a convenient control for the other when studying effects of local stimulation or inhibition. From the results it seems that the vagus exerts both stimulatory and inhibitory effect on gastrin cells in the antrum, and that the antrum is dependent on an intact vagal innervation for a normal gastrin response to food intake. Also, local neurons seem to be of paramount importance in the process of food-evoked gastrin mobilization, since local administration of TTX abolished the gastrin response. In addition, the gastrin response to lack of acid seems to depend on local, but not vagal, neurons.

The aim of Paper V *was to study the effects of locally applied candidate neurotransmitters and regulatory peptides on gastrin secretion in intact, conscious rats.*

Stimulatory agents

Stimulatory effects were assessed in fasted (24h) but otherwise untreated rats.

Catecholamines: Both adrenaline and noradrenaline mobilized gastrin quite effectively: at a near-maximally effective concentration, adrenaline and noradrenaline produced a peak 35 and 20 times over the basal level, respectively. It has been shown that gastrin release can be stimulated by β-adrenergic agonists (Hayes *et al.* 1978; Buchan 1991). In Paper V, local infusion of the β-adrenergic agonist isoprenaline greatly increased microdialysate levels: a 20-fold increase was seen at the highest concentration tested. In Paper V, local infusion of isoprenaline induced a sustained increase in the microdialysate gastrin concentration, whereas the gastrin response to adrenaline and noradrenaline was transient. Both adrenaline and noradrenaline are powerful α-adrenergic agonists and vasoconstrictors. Hence, it cannot be excluded that the gastrin-mobilizing effect of these agents is, at least in part, due to vasoconstriction-evoked ischemia rather than to a direct, receptor-mediated effect on the G cells (for reports on the effect of ischemia on ECL-cell histamine mobilization see Paper II, Kitano *et al.* 2005 and Bernsand *et al.* 2008).

Serotonin: Serotonin has previously been suggested to have weak stimulatory effects on gastrin secretion *in vitro* (Koop & Arnold 1984; Hierro *et al.* 1980). In the present study, local infusion of serotonin was found to raise the concentration of microdialysate gastrin 2- to 3-fold. This is of interest since serotonin is present in large quantities in neighboring EC cells. Thus, we suggest that the G cells may be controlled by EC cells.

Carbachol: Acetylcholine and cholinergic agonists (i.e. carbachol and metacholine) are known to stimulate gastrin secretion both *in vivo* and *in vitro*. Cholinergic agonists stimulate gastrin release from isolated G cells (Sugano *et al.* 1987; Weigert *et al.* 1994), and gastrin release stimulated by distension of the stomach (Schubert & Makhlouf, 1993), intraluminal peptone (Schubert & Makhlouf, 1992), and electrical vagal excitation (Allescher *et al.* 1987), is at least in part dependent on cholinergic (muscarinic) mechanisms. Interestingly, acetylcholine inhibits somatostatin secretion

in the stomach: cholinergic stimulation of gastrin secretion may therefore be secondary to inhibition of somatostatin secretion (Saffouri *et al.* 1980; DuVal *et al.* 1981). In the present study, local infusion of carbachol stimulated gastrin mobilization concentration-dependently: a 4-fold increase was seen at a near-maximally effective carbachol concentration.

Gastrin releasing peptide (GRP): GRP and its amphibian homologue bombesin have been shown to stimulate gastrin release in both isolated stomachs (DuVal *et al.* 1981) and isolated G cells (Sugano *et al.* 1987; Schepp *et al.* 1990a; Weigert *et al.* 1998). On the other hand, GRP and bombesin have also been shown to stimulate somatostatin release from isolated stomach (Sandvik *et al.* 1989) and from isolated D cells (Schaffer *et al.* 1997). Thus, based on these studies, GRP may both stimulate (directly) and inhibit (indirectly via somatostatin, see below) gastrin release. In the present study, local infusion of GRP increased microdialysate gastrin levels concentration-dependently: At a near-maximally effective concentration the elevation in microdialysate gastrin levels was almost 10-fold.

In addition, GABA has been shown to stimulate gastrin release and inhibit somatostatin release *in vitro*. The effects of GABA are thought to reflect $GABA_A$-receptor-mediated activation of the cholinergic pathway (Harty & Franklin, 1983, 1986; Weigert *et al.* 1998). In the present study, however, neither inhibitory nor stimulatory effects were noted.

Inhibitory agents

Inhibitory effects were assessed in freely fed rats treated with omeprazole for 4 days (400 μmol/kg/day)

Somatostatin: Somatostatin-containing D cells have cytoplasmic processes that come in close contact with G cells, suggesting a functional linkage between the two cell types (Alumets *et al.* 1979; Larsson *et al.* 1979). Somatostatin inhibits gastrin release (Hayes *et al.* 1975; Saffouri *et al.* 1980) via somatostatin type-2 receptors

(Zaki *et al.* 1996). As expected, local infusion of somatostatin was found to inhibit omeprazole-evoked gastrin mobilization concentration-dependently.

Galanin: Galanin has been shown to inhibit gastrin release *in vitro* (isolated stomachs and isolated G cells) (Madaus *et al.* 1988; Schepp *et al.* 1990b). The galanin-induced inhibition was not mediated by either somatostatin release or neural pathways (Madaus *et al.* 1988), which indicates that galanin may have a direct inhibitory effect on the G cell (Schepp *et al.* 1990b). In the present study, local infusion of galanin inhibited omeprazole-evoked gastrin mobilization concentration-dependently. The inhibitory effect of galanin was slightly less powerful (approximately 80%) than that of somatostatin.

Bradykinin: Local infusion of bradykinin inhibited omeprazole-evoked gastrin mobilization concentration-dependently. In fact, bradykinin was equally powerful in inhibiting omeprazole-evoked gastrin release as somatostatin. To our knowledge, there are no previous reports demonstrating an inhibitory effect of bradykinin on gastrin release. One may speculate that e.g. inflammation in the antral mucosa (e.g. as a result of *Helicobacter pylori* infection), could have inhibitory effects on G cells due to increased production of bradykinin.

CGRP has also been shown to inhibit gastrin secretion by stimulating somatostatin release in the rat stomach (Ren *et al.* 1992, 1998; Manela *et al.* 1995; Kawashima *et al.* 2002). However, local administration of CGRP failed to affect the gastrin microdialysate concentration.

To summarize, local administration of adrenaline, noradrenaline, isoprenaline, serotonin, carbachol and GRP stimulated gastrin mobilization, whereas somatostatin, galanin and bradykinin had inhibitory effects. The results of the present study are in line with the view that the G cells operate under neurocrine and paracrine control. In addition, the fact that bradykinin inhibited gastrin release suggests that inflammation in the antral mucosa could have inhibitory effects on the G cell.

SVENSK SAMMANFATTNING

Magsäcken innehåller flera olika hormonproducerande celltyper. Av dessa spelar G-celler och ECL-celler en avgörande roll för reglering av magsäckens syraproduktion. Vid födointag stimuleras G-cellerna i antrum (magsäckens nedre del) att frisätta gastrin som via blodet når ECL-cellerna i den syraproducerande delen av magsäcken. Gastrin stimulerar ECL-celler att frisätta histamin, vilket i sin tur stimulerar så kallade parietalceller att utsöndra saltsyra. Vi har studerat frisättning av signalsubstanser från G-celler och ECL-celler med hjälp av mikrodialys. Denna metod bygger på att ett tubformat genomsläppligt dialysmembran placeras under slemhinnan i magsäcken. Genom att låta en vätska långsamt passera under membranet kan man samla upp substanser från den omgivande vävnaden. På omvänt sätt kan man via dialysmembranet även tillföra substanser (t.ex. signalsubstanser) lokalt i vävnaden och på så sätt studera deras effekt på de närliggande cellerna. Mikrodialysmetoden utvärderades dels för studier av ECL-cellernas histaminutsöndring, och dels för studier av G-cellernas gastrinutsöndring. Metoden visade sig vara pålitlig. Lokal infusion av kärlsammandragande ämnen i magsäckens submukosa har visats orsaka histaminfrisättning och sår i magsäcksslemhinnan. Vi fann att histaminet kommer från ECL-cellerna men att det inte orsakar skadorna. Skadorna tros bero på att magsäckens skydd mot magsyran försvagas då blodflödet minskar i slemhinnan. Nervös kontroll av G- och ECL-celler studerades noggrant. Vagusnerven, som förmedlar signaler mellan hjärnan och magtarmkanalen, visade sig både kunna stimulera och inhibera G-cellernas gastrinutsöndring, och ökade ECL-cellernas känslighet för gastrin. Vi såg även att G-cellerna är beroende av det lokala nervsystemet för att kunna frisätta gastrin vid födointag. Vidare kunde vi genom att lokalt tillföra olika signalsubstanser som normalt finns i magsäcken visa att flera av dessa påverkar gastrinutsöndringen.

Sammanfattningsvis har mikrodialys visat sig vara en värdefull metod för att i detalj kartlägga regleringen av G-celler och ECL celler.

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