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Functional aspects of the ECL cell in rodents

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List of papers

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

I. Guang-Lin Cui, Arne K Sandvik, Bjørn Munkvold, Helge L Waldum. Glycine-extended gastrin-17 stimulates acid secretion only via CCK-2 receptor-induced histamine release in the totally isolated, vascularly perfused rat stomach. *Acta Physiol Scand, accepted.*

II. Arne K. Sandvik, Guang-Lin Cui, Ingunn Bakke, Bjørn Munkvold, Helge L. Waldum. PACAP stimulates gastric acid secretion in the rat by inducing histamine release. *Am J Physiol, 2001; in press.*

III. Guang-Lin Cui, Gunnar Qvigstad, Sture Falkmer, Arne K Sandvik, Shiro Kawase, Helge L Waldum. Spontaneous ECLomas in cotton rats (*Sigmodon hispidus*): tumours occurring in hypoacidic/hypergastrinemic animals with normal parietal cells. *Carcinogenesis, 2000; 21:23-27*

IV. Guang-Lin Cui, Unni Syversen, Chun-Mei Zhao, Duan Chen, Helge L Waldum. Long-term omeprazole treatment suppresses body weight and bone mineralization in young male rats. *Scand J Gastroenterol, 2001; 36:1011-1015.*

V. Guang-Lin Cui, Arne K Sandvik, Bjørn Munkvold, Helge L Waldum. The effect of anaesthetic agents on gastrin and histamine-stimulated gastric acid secretion in the totally isolated vascularly perfused rat stomach. *Scand J Gastroenterol, submitted*

Abbreviations

CgA	chromogranin A
HDC	histidine decarboxylase
VMAT-2	vesicular monoamine transporter type 2
PTC	pancreastatin
IHC	immunohistochemistry
ABC	avidin-biotin peroxidase complex
ECLoma	ECL cell carcinoid
ECL cell	enterochromaffin-like cell
EC cell	enterochromaffin cell
Gly-G-17	glycine-extended gastrin-17
PACAP	pituitary adenylate cyclase-activating polypeptide
VIP	vasoactive intestinal peptide
CGRP	calcitonin gene-related peptide
H_2	histamine 2 receptor
H_3	histamine 3 receptor
PPI	proton pump inhibitor
H. pylori	helicobacter pylori
CCK-2	cholecystokinin-2 receptor
DXA	dual-energy X-ray absorptiometry
BMC	bone mineral content
BMD	bone mineral density
Reg	regeneration protein

Summary

Gastric acid plays an important role in digesting food (especially proteins), iron absorption, and destroying swallowed micro-organisms. H^+ is secreted by the oxyntic parietal cells. Its secretion is regulated by endocrine, neurocrine and paracrine mechanisms. Gastrin released from the antral G cell is the principal physiological stimulus of gastric acid secretion. The ECL cell is accepted as the source of histamine participating in the regulation of acid secretion and is functionally and trophically controlled by gastrin. Amidated gastrin is the main biologically active form of gastrin, and its main precursor Gly-G-17 was formerly thought to be without any biological activity. However, recent studies raised the possibility of both secretory and trophic effects of Gly-gastrin. No Gly-G-17 receptor has been cloned yet. In paper I, the effect of this peptide on gastric acid secretion was examined in the totally isolated vascularly perfused rat stomach. This study clearly demonstrates that the administration of Gly-G-17 in high doses was followed by an increase in histamine release and gastric acid output. Moreover, Gly-G-17-induced gastric acid secretion was completely inhibited by the H_2 receptor antagonist ranitidine. Thus, the natural interpretation of these data is that Gly-G-17 is a weak gastrin agonist, interacting with the CCK-2 receptor on the ECL cell, resulting in a subsequent release of histamine, which in turn stimulates the parietal cell. The stomach is also regulated by nerves, principally by the vagal nerves. The gastric neurons contain various neuropeptides, some of them, such as PACAP are known to influence gastric acid secretion. In paper II, PACAP was studied with respect to the effect on gastric acid secretion using totally isolated vascularly perfused rat stomachs, chronic fistula rats and isolated parietal cells. The results show that its stimulatory effect on gastric acid secretion is mainly due to an increase in histamine release from the ECL cell. PACAP is a powerful stimulator of histamine release from the ECL cell.

As mentioned above, aside from its stimulatory effect on gastric acid secretion, gastrin also has a trophic effect on the oxyntic mucosa, especially on the ECL cell. A definite connection has been found between hypergastrinemia and gastric carcinoids both in rats and humans. Moreover, some of the gastric adenocarcinomas in rodents with hypergastrinemia have been reclassified as ECLomas. However, spontaneous gastric ECL omas in laboratory animals are extremely rare. Japanese cotton rats (*Sigmodon hispidus*) have a very high incidence of gastric carcinomas occurring predominately in females, and which we previously showed, were associated with achlorhydria and hypergastrinemia. In paper III, the gastric carcinomas in cotton rats are described further. Particularly the oxyntic mucosa outside the tumour is shown to contain normal parietal cells indicating a normal ability to produce acid.

Long-term potent inhibitiors of acid secretion resulting in secondary hypergastrinemia will induce ECL cell hyperplasia and probably carcinoids. Accordingly, the induction of ECL-cell hyperplasia and carcinoids remains a topic of considerable concern, especially in young individuals. Furthermore, the stomach is important for the absorption of calcium. Achlorhydria has been described as causing impairement of calcium absorption. Thus, a mechanism related to gastric acid secretion has been postulated to be involved in osteopenia developing in patients after gastric resection. More specifically, a postulated peptide, gastrocalcin, has been hypothetised to exist in the ECL cell. We therefore examined the effect of long-term hypergastrinemia secondary to drug induced hypoacidity with respect to bone development in young male rats (paper IV). Long-term potent acid inhibition evoked a marked increase in plasma gastrin levels, leading to enlargement of oxyntic mucosa with ECL cell hyperplasia. However, body weight and bone mineral density were reduced in the

hypergastrinemic young male rats. These findings do not support the hypothesis that the ECL cell plays a role in bone metabolism.

Finally, anaesthetized animal models have been widely used to study gastric acid secretion. However, anaesthetic agents also affect acid output. Anaesthetic agents naturally reduce acid secretion by interaction with neural activity, but could also play a role by affecting the function of the different cells taking part in the regulatory chain of acid secretion as well as the parietal cell itself. In paper V, the totally isolated vascularly perfused rat stomach was used to study the effect of anaesthetic agents on the ECL cell and the parietal cell functions. The results indicate that anaesthetic agents can also affect gastric acid secretion through a direct inhibitory action on parietal cells and ECL cells.

Jeneral introduction

. The physiological role of the ECL cell in regulating gastric acid secretion

)ne of the main functions of the stomach is to produce hydrochloric acid (Beaumont 1833; 'avlov 1910), which plays an important role in protein digestion, iron absorption and articularly in destroying swallowed micro-organisms (Koelz 1992). The stomach is rich in ieuroendocrine cells (Capella *et al.* 1991; Sundler *et al.* 1991a; Solcia *et al.* 2000). At present it least six endocrine cells have been described in the stomach, these are G cells, D cells, ECL cells, A-like cells, D_1/ P cells, and EC cells. G cells are limited to the antral mucosa, while A-like and ECL cells are confined to the oxyntic mucosa. D and D_1/P cells are found in both the antral and oxyntic muocsa. These endocrine cells constitute approximately 2 % of he oxyntic mucosal cells in rodents, and 1.2 % in humans. Among these endocrine cells, the ECL cell is the most prevalent cell type in the oxyntic mucosa. They constitute about 65-70 % of the endocrine cell population in rodents. The ECL cell produces and stores histamine, nd is found in the oxyntic glands of all mammals studied so far. However, the localisation within the glands differs from one species to another. In rodents, they are mainly located in he basal third of the oxyntic mucosa. Furthermore, the stomach is innervated by different nerves (review, Sundler *et al.* 1991b; Ekblad *et al.* 2000), and the gastric wall contains intrinsic neurons producing different peptides influencing the stomach functions, including acid secretion. Gastric acid is produced by the parietal cell in the oxyntic mucosa (Koelz 1992), and the production of acid is regulated by neurons, hormones and paracrine substances. Gastrin released from the antral G cells, histamine from the oxyntic ECL cells and acetylcholine from postganglionic cholinergic neurons are the main stimuli of acid secretion (review, Makhlouf & Schubert 1990; review, Hersey & Sachs. 1995; Lloyd KCK, *et al.* 1992). Whereas gastrin has an indirect stimulatory effect on the parietal cell by stimulating release of histamine from ECL cells (Sandvik *et al.* 1986a; Waldum *et al.* 1991a),

acetylcholine has a direct effect on the parietal cell (Berglindh & Öbrink, 1976, Sandvik *et al.*1998).

The role of gastrin-histamine sequence in ECL cell-parietal cell axis

The existence of gastrin was postulated by Edkins (Edkins 1905). Gastrin was purified from antral mucosa by Gregory and Tracy (Gregory & Tracy, 1961). Its release is regulated by the luminal contents of the stomach, with proteins and their digestion products as the main physiological releasers (Strunz *et al.* 1987; Debas. 1987; Schubert *et al.* 1992; Ramos *et al.* 1992) whereas H^+ is the most important inhibitor of gastrin release (Debas 1987). Gastrin is a potent stimulus of gastric acid secretion (Blair *et al.* 1987; review, Sawada & Dickinson 1997). Already in 1920 histamine was found to stimulate acid secretion (Popielski 1920). However, the interactions between these two mediators remained controversal for a long time. With the introduction of the H_2 receptor antagonist the understanding of gastric physiology improved markedly (Black *et al.* 1972). The ECL cell was originally described by Håkanson et al. as early as in 1967 (Håkanson & Owman 1967). The ECL cell is the dominant endocrine cell in the oxyntic mucosa in all vertebrate species studied so far. However, its physiological function was also long disputed except in the rat where it was initially recognised as the major histamine producing cell of the stomach (Håkanson *et al.* 1986a). Gradually the physiological role of the ECL cell in other species has also been accepted (Waldum *et al.* 1991a). The totally isolated vascularly perfused rat stomach is a suitable model to assess the interaction between gastrin and histamine in the regulation of gastric acid secretion (Kleveland *et al.* 1986; 1987). In this model gastrin induces an immediate and concentration dependent histamine release from the ECL cell (Sandvik *et al.* 1987). With concommitant administration of the H_2-blocker ranitidine together with gastrin, the acid secretion in the isolated stomach model is reduced to baseline level. Therefore, the stimulation of gastrin in acid secretion is most likely via histamine release from the ECL cells only (Waldum *et al.* 1991a; Sandvik & Waldum 1991a; review, Waldum *et al.* 1993a).

This finding using isolated stomachs was supported by studies using isolated ECL cells (Brenna *et al.* 1991; Chuang *et al.* 1992; Prinz *et al.* 1993; 1994). Gastrin stimulation of histamine release from the ECL cell is mediated by the CCK-2 receptor located on the ECL cell (Sandvik & Waldum. 1991b; Ding & Håkanson 1996, Chen *et al.* 2000a). Not only histamine release but also the synthesis of histamine in the ECL cell is regulated by gastrin (Sandvik *et al.* 1994; Höcker et al. 1996). Administration of exogenous gastrin at a dose giving concentration in the physiological range can evoke a significant increase in histidine decarboxylase (HDC) activity (Ryberg *et al.* 1990; Chen *et al.* 1994; review, Håkanson *et al.* 1994a), as well as an increase in HDC mRNA abundance (Dimaline & Sandvik 1991; Dimaline & Baxendale 1998; Hollande *et al.* 1996; Hocker *et al.* 1996). HDC catalyses the formation of histamine from histidine (Kahlson *et al.* 1964). Endogenous hypergastrinemia after potent acid inhibition can induce a similar increase in HDC activity (Axelson *et al.* 1988; Ryberg *et al.* 1989; Brenna *et al.* 1991).

Furthermore, based on our results that gastrin-stimulated maximal gastric acid secretion is lower than histamine-stimulated maximal gastric acid secretion, histamine release from the ECL cell is considered as a limiting step in gastrin-stimulated maximal gastric acid secretion (Kleveland *et al.* 1987; Sandvik *et al.* 1987). These experimental data indicate that gastrin-stimulated maximal gastric acid secretion reflects both the ECL cell and parietal cell masses, while histamine-stimulated maximal gastric acid secretion reflects the parietal cell mass only (Waldum *et al.* 1998). Therefore, from a functional point of view, the stimulatory effect of gastrin on gastric acid secretion can be fully explained by an indirect action through histamine release from the ECL cell. Now it is generally accepted that the gastrin-histamine sequence is the main pathway for gastrin stimulation of gastric acid secretion (Waldum *et al.* 1991a).

Neural regulation

The nervous system is implicated in [illegible]tion of gastric acid secretion (review, Walsh 1998). On one hand it integrates [illegible]formation arising in the stomach, on the other hand, it generates stimuli to the [illegible]ly mediated by the vagal nerves. The vagal efferent fibers are preganglionic. [illegible] innervate stomach endocrine or exocrine cells (Ekblad *et al.* 2000). The [illegible]agal preganglionic neurons is the intrinsic neurons that are located in the [illegible]glion cells. The intrinsic neurons contain acetylcholine and different peptides [illegible]. 1985; 1991; 2000; Hashiguchi *et al.* 1993), as GRP, VIP, galanin, and PAC[illegible]. At present at least fourteen different peptides can be demonstrated in intrinsic nerve fibers (review, Chen *et al.* 1999; Ekblad et al. 2000). They innervate the G, D, ECL and parietal cells. The effect of vagal nerves on gastric acid secretion is complex. Acetylcholine, one of the main gastric acid secretagogues, was also once thought to stimulate gastric acid secretion partly via histamine release from the ECL cell (Prinz *et al.* 1993). But, we and others have recently found that carbachol, a cholinergic agent, does not stimulate histamine release from ECL cells in the isolated vascularly perfused rat stomach or isolated ECL cells (Sandvik *et al.* 1998; Lindström *et al.* 1997). Therefore acetylcholine mainly has a direct effect on acid secretion acting on a M3 receptor on the parietal cell. In vivo, galanin and PYY have been shown to inhibit histamine release from isolated ECL cells (Sandor *et al.* 1996; Zeng *et al.* 1997; 1998a). VIP induces somatostatin release from D cells, but stimulates histamine release from ECL cells probably via a PACAP receptor (Sandor *et al.* 1996; Lindström *et al.* 1997; Lindström & Håkanson 2001). PACAP is a potent stimulator of histamine release from ECL cells. Several PACAP receptors have been identified in different types of cells (review, Läuff *et al.* 1999). The stimulatory effect of PACAP on gastric secretion is mediated by the PACAP-1 receptor on ECL cells (Zeng *et al.* 1998b; 1999; Pisegna *et al.* 2000). However, Li et al. recently reported that PACAP inhibited

gastric acid secretion when studied in the totally isolated rat stomach (Li *et al.* 2000). The intrinsic nerve fibers contain a number of neuropeptides, and the same fiber may contain and release several messengers, one having excitory and the other an inhibitory effect. Therefore, it is not strange that there have been many disputes concerning the effects of the intrinsic neurons on acid secretion.

Besides its regulatory effect on the oxyntic ECL cell, neuropeptides also exert an effect on antral G and D cells (Koop *et al.* 1987; Sandvik *et al.* 1989; Scubert & Makhlouf 1997b; review, Schubert *et al.* 1992). The vagal nerves stimulate gastrin release via GRP. The interaction between CGRP, somatostatin and gastrin seems to be very complex, but CGRP may play an important role in acid mediated paracrine regulation of gastrin release (Manela *et al.* 1995; Ren *et al.* 1992; 1998). Also galanin is thought to exert its inhibitory effect on the G cells (Scheep *et al.* 1990). Moreover, these results have been confirmed by in vivo studies. Hence, it seems that the vagal nerves can participate in the regulation of gastric acid secretion at different levels by controlling the release of different neuropeptides.

Paracrine regulation

Gastric acid secretion, besides being regulated by the hormonal and neural route, is also regulated by paracrine factors (Schubert *et al.* 1987a; Sandvik & Waldum 1988; Schubert & Makhlouf 1996). Somatostatin, a principal paracrine inhibitory factor of gastric acid secretion, can exert its inhibitory effect on the gastric acid secretion (review, Makhlouf & Schubert 1990; review, Sandvik & Waldum 1991; Aurangk *et al.* 1997; Wyatt *et al.* 1996). Both antral and oxyntic somatostatin may inhibit acid secretion by paracrine mechanism. The reciprocal paracrine pathway between D and G cells is well known (Larsson *et al.* 1979; Holst *et al.* 1992), and the ECL cell is also in close contact with the oxyntic D cell (Solcia *et*

al. 1981; review, Arnold *et al.* 1992; Vuyyuru *et al.* 1997). Luminal acid is an important regulator of antral gastrin and somatostatin release, while the D cell in the oxyntic mucosa is of the closed type being influenced by intramural cholinergic and non-cholinergic neurons (Ekblad *et al.* 2000). There is now good evidence that acid releases somatostatin from the antral D cells (Shulkes & Read 1991). Futhermore, it has been shown that gastric acidity regulates gene expression of gastrin and somatostatin (Brand & Stone 1987; Wu *et al.* 1990; Sandvik *et al.* 1993). The antral somatostatin acts on the antral G cells, while the oxyntic somatostatin affects both the ECL cell and the parietal cell. Thus, somatostatin inhibits acid secretion via the action on different cells of the G-ECL-parietal cell axis. In connection with the above findings, an inverse relationship between antral gastrin and somatostatin cells has been demonstrated (Arnold *et al.* 1982; Lamberts *et al.* 1991; Chen *et al.* 1992). The G/D cell ratio is increased in conditions with hypoacidity and decreased in hyperchlorhydria (Creutzfeldt *et al.* 1986). Furthermore, histamine via H_3 receptors can act on D cells to stimulate release of somatostatin, which in turn inhibits histamine release (Prinz *et al.* 1993; Modlin *et al.* 1996).

2. The ECL cell during hypergastrinemia secondary to acid inhibition

Apart from a stimulatory action on gastric acid secretion, gastrin has also a trophic effect on the oxyntic mucosa (review. Håkanson et al. 1986b; review, Koh & Chen 2000) particularly on the ECL cell (Håkanson *et al.* 1994b), which is stimulated to replicate (Tielemans *et al.* 1990a; 1990b; Ryberg *et al.* 1990). Moreover, ample evidence supports the view that the trophic as well as secretory effect of gastrin on the ECL cell are mediated by the CCK-2 receptor (Sandvik & Waldum 1991; Eissele *et al.* 1992; Simon *et al.* 1995; Ding *et al.* 1997; Chen *et al.* 2000a). In rats, infusion of gastrin leads to ECL cell proliferation (Ryberg *et al.* 1990a). The ECL cell hyperplasia is most likely explained by an increased self replication (Tielemans *et al.* 1990a). Moreover, a concentration dependent relationship has been well established between gastrin and ECL density in rats (Brenna & Waldum 1992). The trophic effect of gastrin on the ECL cell can also be mimicked by endogenous hypergastrinemia induced by long-term potent acid inhibitors such as H_2 receptor blockers and PPIs (Sundler *et al.* 1986; Tielemans *et al.* 1989; Wallmark *et al.* 1990; Ryberg *et al.* 1989b; 1990b; Lee *et al.* 1992; Brenna *et al.* 1992b). Whether hypoacidity and hypergastrinemia are induced by a H_2 blocker or a PPI, there is a similar concentration dependent relationship between serum gastrin and the ECL cell density. Thus, there seems to be little doubt that gastrin rather than achlorhydria or drugs per se is reponsible for the ECL cell growth stimulation (Larsson *et al.* 1986; Sundler et al. 1986b; Ryberg et al. 1990a; review, Carlsson et al. 1990; review, Håkanson *et al.* 1986c; review, Håkanson & Sundler 1990; review, Creutzfeldt 1994). It has become apparent that rat ECL cells, in response to hypergastrinemia whether endogenous or exogenous, show hypertrophy within days, hyperplasia within weeks (Larsson *et al.* 1988a; Eissele *et al.* 1991; Chen *et al.* 1994; review, Chen *et al.* 1999) and carcinoids after months through a sequence of diffuse-linear-micronodular hyperplasia to ECL carcinoids (Havu *et al.* 1986; 1990; Larsson *et al.* 1988b; Hirth *et al.* 1988; Håkanson *et al.* 1994b). More recently,

by using the elutriation centrifugation method, our group has shown that gastrin only exerts a specific proliferative effect on the ECL cell but not on the parietal cell (Bakke *et al.* 2000). Data from gastrin-deficient and transgenic mice also provide further support for the trophic effect of gastrin on the ECL cell (Wang &Brand 1992; Wang *et al.* 1996; Koh *et al.* 1997; Wang & Dockray, 1999). Therefore, there is a causal connection between hypergastrinemia and ECL cell hyperplasia and ECL cell carcinoids (review, Bordi *et al.* 1995; review; Waldum *et al.* 1992; Waldum *et al.* 1998c; review, Dayal 1998).

PPIs are highly effective gastric antisecretory agents with long duration (review, Larsson *et al.* 1988). They are intensively used to treat acid related disease, and are nowadays prescribed even for children (review, Israel & Hassall 1998). As we know, potent acid inhibitory drugs induce achlorhydria with secondary hypergastrinemia. Hypergastrinemia, even in short-term or to a moderate degree, may increase the risk of ECL cell gene mutations (review, Waldum *et al.* 1993; review, Bordi *et al.* 1995; review, Waldum & Brenna 2000). In rodents, it has been shown that long-term inhibition of acid secretion will induce carcinoids in some individuals (Havu 1989; 1990). However, in the rodent mastomys (*praomys natalensis*), a moderate degree of hypergastrinemia induced by blockade of the H_2 receptor causes the development of gastric carcinoids (review, Nilsson *et al.* 1992; Nilsson *et al.* 1993; Modlin & Tang 1996). On the other hand, a reduced bone mineral density has been reported after gastrectomy both in man and animals (Kaplan *et al.* 1977; Filipponi *et al.* 1990; Persson *et al.* 1993; Klinge *et al.* 1995). A gastric factor released from the stomach was postulated to be involved in this pathgenesis (Kaplan *et al.* 1977). This factor (gastrocalcin) was believed to be localized to the ECL cell and regulated by gastrin (Persson & Håkanson 1991). Long-term acid inhibition treatment may therefore result in a change in bone mineral density (Håkanson *et al.* 1990b; 1990c).

Ciprofibrate, one of perioxisome-proliferators, used as a hypolipidaemic agent, has been associated with hypergastrinemia and the development of ECL carcinoids (Eason *et al.* 1988a; Spencer *et al.* 1989). It was believed that the hypergastrinemic effect of these compounds was due to an inhibitory effect of acid secretion (Eason *et al.* 1988b). But, recent study from our laboratory showed that ciprofibrate did not increase gastric pH (Martinsen TC *et al.* 1996). The mechanism behind the gastrin releasing effect of ciprofibrate is not known. However, the fact that ciprofibrate induces ECL cell carcinoids, demonstrates that hypergastrinemia and not hypoacidity causes ECL cell hyperplasia and carcinoids (Waldum *et al.* 1998b). ECL cell hyperplasia can be reduced by somatostatin analogues like octreotide (Moldin *et al.* 1992; Reubi *et al.* 1992). These observations suggest that ECL carcinoids can be induced by hypergastrinemia alone without hypo-/anacidity as in ciprofibrate treated rats (Bakke *et al.* 2000).

In summary, gastric acid secretion is controlled by a variety of hormones, neuropeptides and paracrine substances (Schubert & Makhlouf 1992). It is now generally accepted that gastrin is the most important physiological stimulus, and that the ECL cell plays a central role in the regulation of gastric acid secretion, being influenced by both stimuli and inhibitors (Waldum *et al.* 1992, review, Waldum & Sandvik 1989; Waldum *et al.* 1991a; review, Håkanson *et al.* 1994a). Furthermore, it has become apparent that the ECL cell also may play a role in gastric carcinogenesis (review, Modlin & Tang 1996; review, Waldum *et al.* 1998c), especially in chronic hypergastrinemic conditions. Therefore, it should be taken into account that long-term potent inhibition of gastric acid secretion may induce an increased risk of gastric carcinomas, particularly in young individuals (review, Waldum *et al.* 1993b; review, Waldum & Brenna 2000).

At present, some interesting points in this research field remain to be further investigated, for instance:

1. It has been well demonstrated that gastrin is not only the principal physiological stimulus of gastric acid secretion via a histamine dependent way (review, Waldum and Sandvik 1989), but that it also has a general trophic effect on the oxyntic mucosa (Koh & Chen 2000). The ECL cell in the oxyntic mucosa is both functionally and trophically controlled by gastrin. The precusors of gastrin, such as non-amidated gastrin has formerly been thought to be without any biological significance, but have recently been reported to have both a secretory and a trophic effect (Dickison *et al.* 1990; Seva *et al.* 1994; Higashide *et al.* 1996; Sandvik & Dockray 1999; Chen *et al.* 2000b). There is no present agreement concerning the receptors involved in this effect by non-amidated gastrin.
2. Anaesthetized animal models have been intensively used to study gastric acid secretion. However, it is evident that anaesthetic agents may affect gastric acid secretion (Lee *et al.* 1967; Albinus *et al.* 1978; Barrett *et al.* 1978; Yang *et al.* 1990; Graffner *et al.* 1991). Moreover, the mechanisms behind this action have not been fully elucidated.
3. Neuropeptides are involved in the regulation of gastric acid secretion. It is postulated that this effect is achieved via a modulation of histamne release from the ECL cell. However, there are many neurons present in the enteric nervous system of the stomach, and a variety of neural peptides have been identified in these neurons (review, Sundler *et al.* 1991). Thus, due to this complex net formed by these peptides, the regulatory effects of the different peptides have not been clarified.
4. Attention has been paid to possible extragastric effects of gastrin. Apart from its key regulatory effect on gastric acid secretion, the ECL cell is also claimed to play a role in bone metabolism via release of a putative peptide to act on the calcium uptake (Persson & Håkanson 1991). However, there is still a problem that this putative peptide has not been indentified. Therefore, the exact physiological significance of the ECL cell in bone metabolism remains to be shown.

5. The risk of hypergastrinemia for gastric carcinogenesis has been considerated and well studied in adult rats, but not in younger ones.
6. Some hypergastrinemia related ECL omas in rodents were originally thought to be gastric adenocarcinomas before they were correctly classified (Kawase & Ishikura 1995; Waldum *et al.* 1999). It would therefore be of interest to study other animal models with dedifferentiated gastric adenocarcinomas.
7. The role of *H. pylori* infection in the development of ECLomas has been an area of recent investigation (Hirayama *et al.* 1999). Experimental data have demonstrated that *H. pylori* infection stimulates histamine release from isolated rat ECL cells as well as ECL cell proliferation in vitro (Kidd *et al.* 1999; 2000). It indicates that abnormal function of the ECL cells may play a role both in the *H. pylori* infection-related diseases and gastric carcinogenesis (Wang *et al.* 2000).
8. Beside histamine, the ECL cell also contains calbindin, pancreastatin and Reg protein (Fukui *et al.* 1998), but their physiological relevance is unclear at present.

The aims of the present study

1. To evaluate the stimulatory mechanisms of the main gastrin precursor glycine-extented gastrin on gastric acid secretion
2. To evaluate the effect of PACAP on gastric acid secretion
3. To study further the hypergastrinemia related ECLomas in Japanese cotton rats (*Sigmodon hispidus*)
4. To study the effects of long-term omeprazole treatment on the stomach and bone metabolism in young male rats
5. To investigate the effect and possible mechanisms of different anaesthetic agents on gastric acid secretion

General discussion

Here, I will focus on material and methodological considerations, as well as on the main results.

Material and methodological considerations

Animals

In papers I, II, V, male Wistar rats were used to establish the totally isolated vascularly perfused stomach and chronic fistula models. The gastric content and fasting time influence the plasma gastrin level, histamine release and HDC activity and subsequently gastric acid output (Dimaline *et al.* 1993). So, it is very important to select a suitable fasting time in order to obtain an empty stomach. A 36 hour fasting period was found to be ideal.

Young male Sprague-Dawley rats were chosen in paper III to study the effects of long-term omeprazole treatment on bone mineral density and the stomach. This choice was due to the fact that young rats are growing more rapidly, making them susceptible to changes in food uptake reflected in bone and body weight gain. Otherwise, male rat body weight increases with time, while females do not. Moreover, as developing animals, young rats may have a different response to the chronic hypergastrinemia induced by potent acid inhibitors.

Studying rodent models can give valuable information in delineating the role of ECL cells in the development of gastric neoplasia. However, spontaneous gastric ECLomas in laboratory animals are extremely rare. Female Japanese cotton rats (*Sigmodon hispidus*) were found to have a high incidence of spontaneous gastric carcinomas (Kawase & Ishikura 1995). Formerly they were thought to be adenocarcinomas, and later they were reclassified as malignant ECL omas secondary to hypergastrinemia (Waldum *et al.* 1999).

The totally isolated vascularly perfused rat stomach, chronic fistula rat and isolated gastric cells

A variety of in vitro methods have been developed to study the regulation of gastric acid secretion. These methods include the use of the totally isolated vascularly perfused rats stomach (Kleveland *et al.* 1986) or luminally perfused mouse stomach (Schubert *et al.* 1988), mucosal sheet mounted in a chamber (Schubert & Makhlouf 1987b), perfused mucosal segments (Wilkes *et al.* 1988) and cultured segments (Harty *et al.* 1981), isolated glands (Berglind & Öbrink, 1976; Chew & Hersey 1982) and dispersed mucosal cells (Soll *et al.* 1979; Brenna & Waldum, 1991; Prinz *et al.* 1993; Brenna *et al.*1994; Bakke *et al.* 2000).

The totally isolated vascularly perfused rat stomach was used in papers I, II and V. The intact ECL-parietal cell axis, paracrine regulatory pathways, the polarity of the endocrine cells such as the antral G cell, D cell, the oxyntic ECL cell, the receptors and the structure of these cells are well maintained in this preparation. Also the test conditions are almost fully controlled without endogenous hormone (particularly gastrin) background influence, substances can be added to the vascular perfusate of the stomach at different concentrations, the effect can be easily detected in the perfusates collected from the portal vein and lumnen. The model has been widely used to determine the release of histamine from the ECL cell stimulated by gastrin, gly-G, neuropeptides, paracrine regulatory pathways, CCK-2 receptor agonist and antagonist as well as histamine itself (Kleveland *et al.* 1986; 1987; Sandvik *et al.* 1987; 1988; 1989a, 1989b; 1991b). The results obtained have established this model as an ideal one to study the regulation of ECL cell and the role of the ECL cell in the regulation of gastric acid secretion in vitro.

Chronic fistula rats were used in paper II to assess the stimulatory effect of PACAP on gastric acid secretion. The results are consistant with those in isolated stomachs. The advandages of this model are that the inervation is intact and it can be used repeatedly. It is, however, easily influenced by endogenous factors, and histamine concentration can only be measured in peripheral blood.

In paper II, the direct effect of PACAP on isolated parietal cells was assessed by the aminopyrine method. This method is very useful for examining the receptor function in parietal cells. Formely, different PACAP receptor types have been found in different types of isolated gastric mucosal cells (Läuff *et al.* 1997; Zeng *et al.* 1998; 1999b). The limitations are mainly due to changes in the receptors introduced by the isolation procedure, along with the lack of secondary paracrine mechanisms.

IHC

The entire endocrine cell population of the oxyntic mucosa can be disclosed by immunohistochemical staining for general neuroendocrine markers like CgA, synaptophysin and CgA-derived peptides like PTC (Tab 1). IHC is used in papers III and IV to assess endocrine cell changes both in antral and oxytic mucosa of the stomach. The ECL cell is argyrophilic and may be identified by histochemical methods like the Grimelius and Sevier-Munger methods (review, Wilander 1989).

The Sevier-Munger method is more specific for the ECL cell than the Grimelius method, which also stains cells belonging to the D1 and EC cell group. However, IHC using an antibody specific for an antigen expressed on the cell type is today the preferred and the most specific method to identify different cell types. Histamine taking place in the regulation of

acid secretion is synthetised, stored and secreted by the ECL cell in the oxyntic mucosa. Antibodies for histamine may therefore be used to detect ECL cells (Håkanson *et al.* 1986a). However, histamine is also found in mast cells and histamine positivity is therefore not completely specific for ECL cells. However, the histamine forming enzyme HDC, which is the rate limiting enzyme for histamine production, is only found in ECL cells. Thus, antibodies for HDC is the preferred and most frequently used specific ECL cell marker (Dartsch *et al.* 1999a; 1999b). CgA is a general neuroendocrine marker (Cetin *et al.* 1989) and in the rat oxyntic mucosa the ECL cells make up from 65 to 70 % of the entire CgA positive cells (Cappella *et al.* 1991). In hypergastrinemic situations the ECL cell demonstrates an even higher percentage of the neuroendodcrine cells. Antibodies for CgA or PTC are sometimes used to assess ECL cell densities (Håkanson *et al.* 1995; Norlén *et al.* 1997). The ECL cell also contains calbindin and VMAT-2 (Furness *et al.* 1989; Giorgio *et al.* 1996; Zhao *et al.* 1997). Antibodies for VMAT-2 has been claimed to be specific for ECL cells (Rindi *et al.* 2000), but it has subsequently become clear that also the A like cells express VMAT-2. When studying the density of ECL cells in different species, it should be taken into account that the density is higher in rodents, particularly in the rat, than in man (Capella *et al.* 1991; Solcia *et al.* 2000). In our rat studies we therefore used CgA as a general endocrine cell marker and HDC as a specific ECL cell marker in our immunohistochemical studies.

In paper III we did double immunohistochemical staining to identify proliferative ECL cells. In this immunohistochemical study with double staining the ABC method was applied in both cycles but with different chromogens in the final step.

Histological indices

Density and volume density were used as indices in counting the positive immunoreactive cells of the stomach in paper III. The former is simple and easy to do under the light microscopy, however, it is influenced by mucosal thickness and cell size (D'adda *et al.* 1990). On the other hand, the volume density is not influenced by such factors. Combining both methods can give more accurate results. No discrepancy was found between these two methods in our paper. It seems therefore that they are both suitable as indices for gastric endocrine cells.

Bone mineral metabolism indices

In paper IV, we used DXA to assess the effect of long-term omeprazole treatment on bones and body composition, BMC, BMD, fat and lean mass in young growing male rats.

In previous studies concerning the effect of omeprazole on bones, ash weight has been used as an indicator for BMC (Håkanson *et al.* 1990b; Persson *et al.* 1993). However, since its introduction DXA has become the predominant method (Mazess *et al.* 1990). The two monoenergic peaks are provided by an X-ray generating DXA, and the K-edge filter has been shown to be a precise method for assessing body composition. It provides a three-compartment model of body composition: fat, lean tissue mass and BMC and has the advantage of a minimal radiation dose, and accurate regional values. Formely, DXA has been used in assessing bone metabolism in rats femurs by our group (Syversen *et al.* 1999), showing that this procedure is precise with a very good correlation to calcium content and ash weight of the femurs. Morever, it is simple and repeatable, it is therefore an ideal method for assessing calcium metabolism in small animals.

2. Main results and discussion

Does Gly-G stimulate gastric acid secretion?

It is known that gastrin is the most important physiological stimulus for gastric acid secretion. Gastrin stimulates acid secretion by increasing histamine release from the ECL cell via CCK-2 receptors. Gastrin is synthesized as precursor prohormones that require post-translational processing for bioactivation (Merchant *et al.* 1994). Previously, it was believed that processing intermediates were without physiological relevance. Indeed, the main precusor Gly-G is much less potent than amidated gastrin in stimulation gastric acid secretion. However, the precusor Gly-G-17 has recently been reported to stimulate acid secretion and cell growth in rodents (Dickison *et al.* 1990; Seva *et al.* 1994; Higashide *et al.* 1996; Sandvik & Dockray 1999; Chen *et al.* 2000b). Gly-G-17 was suggested to have a direct enhancing effect on H^+, K^+, ATPase gene expression via a novel receptor on parietal cells (Kaise *et al.* 1995). Formerly, work at our laboratory has shown that Gly-G-17 stimulates histamine release from the ECL cell at a low potency, an effect that could be prevented by a specific CCK-2 receptor antagonist (Sandvik &Dockray 1999). This supports that Gly-G-17 binds to the CCK-2 receptor and is a weak gastrin agonist without physiological importance in the regulation of acid secretion. The present study also clearly shows that Gly-G-17 does not stimulate gastric acid secretion or histamine release at low concentrations (0.52 and 5.2 nM). However, at very high concentrations (520 nM) Gly-G-17 elicited histamine release and acid secretion comparable to G-17 at its maximal effective concentration of 0.52 nM. The acid output concentration-response curve parallels that of histamine release during Gly-G-17 stimulation of the isolated stomach. The increase in acid output could be completely prevented by the H_2 receptor blocker ranitidine at a dose of 10 nM. Thus, the acid stimulatory effect of Gly-G-17 at very high concentrations can be completely explained by stimulation of histamine release from the ECL cell, and it is not neccessary to suggest direct action on the parietal cell. So, from our previous and present studies, it is unlikely that Gly-G-17 plays a physiological role in the regulation of gastric acid secretion.

Vagal-ECL cell axis

The stomach is innervated both by extrinsic, mainly vagal fibers, and intrinsic neurons. The intrinsic neurons contain various substances known to influence the G- and D-, as well as the ECL cell, and thus to regulate gastric acid secretion indirectly (Ekblad *et al.* 2000). The term vagus-ECL cell axis has been introduced to describe an important relationship between vagal nerves and ECL cells (Chen *et al.* 1999). Although, acetylcholine has been shown to increase histamine release from isolated ECL cells (Prinz *et al.* 1993), we have not found an increased histamine release during perfusion with the cholinergic agent carbachol to the isolated stomach. The stimulation of acid secretion by carbachol in the isolated stomach could be explained by its direct effect on M3 receptor on parietal cells (Sandvik *et al.* 1998). Thus, acetylcholine seems not to play a role in the vagal ECL cell axis. PACAP is a neuropeptide originally extracted from ovine hypothalami by Miyata and collaborators (Miyata *et al.* 1989). PACAP belongs to the VIP superfamily and exists as two biologically active peptides - PACAP-38 and PACAP-27. Several recent investigations have shown that PACAP stimulates histamine release from isolated ECL cells (Sandor *et al.* 1996; Lindström *et al.* 1997; Zeng *et al.* 1998b; 1999). Also, PACAP 1 receptor has been found on the ECL cell and claimed to respond to the stimulation of PACAP (Läuff *et al.* 1999; Zeng *et al.* 1999). But, in contrast to the findings in vivo, Li et al. reported that PACAP inhibits acid secretion possibly via a stimulation of somatostatin and PGE_2 release in the totally isolated rat stomach (Li *et al.* 2000). Another study indicates that PACAP in intact animals has no effect on basal acid secretion, but that it inhibits maximal acid secretion stimulated by histamine or pentagastrin (Mungan *et al.* 1995). Furthermore, Healey et al. suggested that the parietal cell

also possesses a PACAP receptor (Healey *et al.* 1998). In paper II where we examined the effect of PACAP on acid secretion by using the totally isolated rat stomach, fistula rats and isolated parietal cells, we found that PACAP concentration-dependently stimulated histamine release and acid secretion in a parallel way, and that ranitidine completely abolished the increase in acid output. Furthermore, we failed to find any stimulation of aminopyrine uptake by PACAP in isolated parietal cells. These results support that PACAP is a potent stimulus of the ECL cell histamine release.

Is long-term potent acid inhibition safe for young individuals?

Potent acid inhibitory drugs like omeprazole are used extensively to treat acid-related diseases worldwide. Clinically, now even children are accepted for long-term potent acid inhibition. But, drug-induced achlorhydria is associated with hypergastrinemia, which is a trophic factor for the stomach, particularly the ECL cells (Waldum *et al.* 1993b). A causal relationship between sustained hypergastrinemia and gastric carcinod formation has been demonstrated in rodents (Havu *et al.* 1986, 1990). This event can be explained by the following sequence: acid blockade – hypergastrinemia - ECL cell hyperplasia - increased incidence of gastric ECL cell carcinoids (Håkanson & Sundler 1990a).

However, most studies have been performed in adult animals. The influence of potent acid inhibition and secondary hypergastrinemia on gastric mucosa in young animals is still not fully examined. The present result from young male rats dosed with omeprazole for 11 weeks, shows a marked hypergastrinemia and enlargement of oxyntic mucosa with hyperplasia of the ECL cell, whereas the antral G cell was hypertrophic and D- and EC cells in the antrum showed reduced density compared to controls. On the other hand, bone mineral content and density were reduced (see below). Although no gastric carcinoids were found in

these young male rats during the 11 weeks treatment period, it is possible that this early hypergastrinemia can increase the risk of gastric tumours later in life.

Does the ECL cell play a role in bone mineral metabolism?

Hypocalcemia can be observed in rats after gastrectomy (Kaplan *et al.* 1977; Filipponi *et al.* 1990; Fries *et al.* 1992, Muhlbauer *et al.* 1998) or fundectomy (Persson *et al.* 1993; Klinge *et al.* 1995; Lehto-Axtelius *et al.* 1998; Rumenapf *et al.* 1998). In the past it was considered to be the result of calcium and/or vitamine D deficiency. However, both oral and parenteral calcium supplementations have failed to prevent osteopenia after gastrectomy. The pathogenesis of osteopenia secondary to gastric resection is still unknown. Numerous investigations have been done as to the effect of gastric peptides on calcium homeostasis. A gastric factor has been postulated as being important for calcium absorption (Schulak & Kaplan 1974; 1975; Håkanson *et al.* 1990b; 1990c; Persson *et al.* 1989; 1991; Axelson *et al.* 1991;). More recently, the ECL cell in the oxyntic mucosa being under gastrin control was claimed to play a role in the regulation of bone mineral metabolism by secreting an anticipated peptide (gastrocalcin) with osteotrophic effect (Persson & Håkanson 1991; review, Håkanson et al. 1998; review, Chen *et al.* 1999). Long-term omeprazole treatment resulting in hypergastrinemia and gastric ECL cell hyperplasia may therefore result in a change in bone mineral metabolism (Håkanson *et al.* 1990b; 1990c). However, PPI treatment influences other gastric functions than acid secretion, like gastric emptying (Benini et al. 1996; Rasmussen et al. 1997), food intake (Campbell *et al.* 1991) and bacterial overgrowth (Thorens *et al.* 1996). Furthermore, gastrin, histamine and CCK have also been reported to result in hypocalcemia in rats (Norberg *et al.* 1976; Stulberg *et al.* 1976). It has been claimed that gastrin affects calcium metabolism by influencing parathyroid hormone (PTH) release (Gagnemo-Persson *et al.* 1994; 1997). However, at present nobody has succeeded in

purifying any regulatory peptide from the ECL cells. Thus, it is still doubtful whether there exists a gastrocalcin in the ECL cell.

We found in the present study that drug induced anacidity, hypergastrinemia and ECL cell hyperplasia induced osteopenia in young rats. Our findings are therefore not in agreement with the hypothesis that gastrin stimulates gastrocalcin release from ECL cells. Ghrelin is stored in the gastric A-like cells and may play a role in the development and growth of the body (Kojima *et al.* 1999). However, we did not find any change in A-like cell density or morphology in the young rats dosed with omeprazole, which makes it rather unlikely that the osteopenic effect of PPI dosing in young rats could be mediated by an interaction with ghrelin.

To conclude: The stomach probably plays a role in regulating bone metabolism, but the exact mechanism is still unknown.

Spontaneous animal ECLoma models

Previously, ECLomas in man were thought to be rare. However, a proportion of gastric carcinomas, particularly of diffuse type, may be ECLomas (Waldum *et al.* 1999; 1998d; Qvigstad *et al.* 1999). Animal models would offer a better opportunity to study the pathogenesis of neoplastic transformation of ECL cells in more detail and under controlled conditions.

Unfortunately, spontaneous gastric carcinoids in laboratory animals are very rare. The African rodent species *Mastomys* does, however, develop ECLomas spontaneously (Snell & Stewart 1969). Even reducing gastric acidity only moderately, leading to a slight

hypergastrinemia enhances the frequency of tumours in this species (Nilsson *et al.* 1993). As in normal ECL cells, mastomys ECLoma cells have CCK-2 receptors, and the tumor development may be prevented or retarded with the somatostatin analogue octreotide (Modlin *et al.* 1992). However, the gastric tumours occurring spontaneously in Japanese cotton rats (*Sigmodon hispidus*) (Kawase and Ishikura, 1995) have been reclassified to ECLomas (Waldum *et al.* 1999). These tumours occur predominantly in females. In the present study these tumours are further examined by histochemical, immunohistochemical and Northern blot procedures. In cotton rats with tumour, gastrin was greatly elevated, whereas serum total protein was lower than in healthy ones. Furthermore, these tumours occur only in the oxyntic mucosa. The tumours occur in an oxyntic mucosa greatly thickened and with ECL cell hyperplasia. Many of the neoplastic cells showed typical ECL cell differentiation by IHC. The number of parietal cells in the oxyntic mucosa was within the normal range, showing that the hypergastrinemia probably is not due to a reduction in gastric acid secretion caused by gastric oxyntic atrophy, but suggesting that the hypo-/anacidity may be due to a neutralisation of the gastric content by leakage of fluid from the luminal surface parallel to what occurs in patients with Ménétrier's disease. By Northern blot analysis, the expression of mRNA for gastrin in the antral, and HDC and CgA in the oxyntic mucosa, was much higher than in healthy cotton rats. The spontaneous gastropathy with secondary hypo-/anacidity and hypergastrinemia, and gastropathy resembling Ménétrier's disease and subsequent development of malignant ECLoma, may be a very useful model for studying the pathogenesis of gastric carcinogenesis in general.

Thus, gastric ECLomas occur spontaneously, but due to a gastropathy inducing hypo-/anacidity and hypergastrinemia in Japanese cotton rats, whereas in mastomys ECLomas occur even in normogastrinemic animals. In mastomys, however, the tumour insidence may

be increased by inducing hypergastrinemia. The basic defect in mastomys appears to be a mutation of the gastrin receptor, making it constitutively activated even at normogastrinemic levels.

Finally, ECLomas may of course be induced by life-long profound acid inhibition in normal rats (Havu 1986; Havu *et al.* 1990). Thus, activation of the gastrin receptor is the common pathogenetic factor for these three gastric animal tumour models.

Anaesthetics and acid secretion

Different anaesthetized animal models have been used to study the regulatory mechanism of gastric acid secretion. However, anaesthetics can affect gastric acid secretion (Lee *et al.* 1967; Albinus *et al.* 1978; Barrett *et al.* 1978; Yang *et al.* 1990; Graffner *et al.* 1991). Numerous experiments have been done, but the exact mechanism is still uncleared. It has been suggested that many factors such as vagal nerves, intramural neurones, gastric endocrine cells, and peptides are involved in the inhibitory mechanism. The ECL cell plays a key role in the regulation of gastric acid secretion, and anaesthetic agents have recently been described as inhibiting ECL cell histamine release (Norlén *et al.* 2000). The present study shows that to inhibit of anaesthetics on acid secretion can be mediated by a direct effect on parietal cells and/or an effect on the histamine release from ECL cells.

Conclusions

1. The main gastrin precusor Gly-G-17 is a weak gastrin agonist.

2. Neuropeptide PACAP exerts its stimulatory effect on gastric acid secretion via increasing histamine release from the ECL cell.

3. The gastric tumour occurring in Japanese cotton rats (*Sigmodon hispidus*) is a typical ECLoma with a hypoacidic/hypergastrinemic background. It offers unique possibilities to study ECLomas.

4. Young male rats, in response to the hypergastrinemia induced by long-term omeprazole treatment, display ECL cell hyperplasia and reduced bone and body weight gain which may have clinical implications for children receiving omeprazole treatment.

5. The suppression of gastric acid secretion by anaesthetic agents is mainly mediated by an inhibition of histamine release from the ECL cell.

6. From the present studies, the importance of the ECL cell in the regulation of gastric acid secretion both physiologically and pathologically is further strengthened.

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Table 1. The specificity of different histochemical and immunohistochemical staining methods to different gastric endocrine cells and mast cell

	Cell types						
	ECL	EC	D	G	A-like	D1/P	Mast
Grimelius	postive	positive		positive	positive	positive	
Masson		positive					
Fontana							
Sevier Munger	positive	positive				positive (D1)	
IR-CgA	positive	positive	positive	positive	positive	positive	
IR-Histamine	positive						positive
IR-HDC	positive						
IR-Calbindin	positive						
D28K							
IR-VMAT-2	positive				positive		
IR-PTC	positive		positive		positive		
Ghrelin					positive		