NEUROENDOCRINE REGULATION OF GASTRIC ENDOCRINE CELL FUNCTION

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Abstract

The last decade has seen major advances in the understanding of diseases of the proximal gastrointestinal tract. Not least of these has been the recognition of the significance of *Helicobacter pylori* (*H. pylori*) gastritis in the pathogenesis of peptic ulceration, chronic atrophic gastritis and gastric carcinoma. In addition the development of proton pump inhibitors (PPI) has been heralded as a major therapeutic advance. However both *H. pylori* gastritis and PPI therapy elevate plasma gastrin concentrations and this has led to increasing interest in the physiological role of the antral hormone gastrin.

In this thesis, the role of intrinsic antral neurons in the regulation of gastrin release in response to a variety of stimuli has been examined. In addition, the effects of antral denervation on gastric emptying were clearly apparent and prompted investigation of the relationship between antral neurons and the cholecystokinin-dependent mechanisms that have been well described as regulators of gastric emptying in the intact rat. It was found that antral neurons are essential for normal gastric emptying but are not required for the CCK-mediated inhibition of gastric emptying.

In addition, following antral denervation there was elevated fasting and meal stimulated plasma gastrin, the post-prandial rise in gastrin release occurring by a non-gastrin releasing peptide mediated mechanism. Moreover, non-nutrient

distension of the stomach was an adequate stimulus for gastrin release following antral denervation, suggesting that in the intact rat, antral neurons normally inhibit gastrin release to non-nutrient distension.

The effects of antral denervation on the endocrine cells of the stomach were also studied as were the effects of hypergastrinaemia on the Enterochromaffin-like (ECL) cells of the gastric corpus. Of particular relevance was the demonstration by Ashahara et al., (1996) of the *reg* gene in the ECL cells of the rat. Expression of this gene has been associated with pancreatic endocrine cell growth and differentiation. Data is presented that indicates that expression of this gene is regulated by gastrin and that it may play a role in promoting the differentiation of endocrine cells of the proximal gastrointestinal tract. Mutations of *reg* were associated with the development of gastric carcinoid (ECL cell) tumours in patients with hypergastrinaemia.

Overall it is clear that the antral innervation plays a central role in the control of gastrin release and that loss of the normal innervation of the G-cell leads to fasting and meal-stimulated hypergastrinaemia. The effects of persistently elevated gastrin on other cell populations of the stomach are likely to be of increasing importance with respect to understanding the mechanisms of tumour development.

The work presented in this thesis has appeared in part in the following publications:

Papers

Higham A.D., Vaillant C., Yegen B., Thompson D.G., Dockray G.J.: Relationship between CCK and antral innervation in the control of gastric emptying in the rat. *Gut* 41:24-32 1997

Higham A.D., Noble P., Thompson D.G., Dockray G.J.: Increased sensitivity of gastrin cells to gastric distension following antral denervation in the rat. *J Physiol* 503(1):169-175 1997

Higham A.D., Dimaline R., Varro A., Attwood S, Armstrong G, Dockray G.J., Thompson D.G.: Atrophic gastritis and large carcinoid nodules: Octreotide suppression test predicts beneficial outcome from antrectomy. *Gastroenterology* (*In press*).

Abstracts

Higham, A.D. Thompson, D.G., Dockray, G.J.: Antral denervation by benzalkonium chloride leads to gastric retention of solids in the rat. *Gut* 36(1):A52 1995.

Higham, A.D. Thompson, D.G., Dockray, G.J.: Peptidergic afferent and intrinsic innervation of the rat stomach following antral denervation. *Neurogastroenterol Mot* 7:9 1995.

Higham, A.D. Valliant, C. Thompson, D.G., Dockray, G.J.: Complete loss of peptidergic afferent fibre innervation of the rat stomach following antral denervation. *Reg Peptides* 57:199 1995.

Higham, A.D., Thompson, D.G., Dockray, G.J.: Post-prandial gastrin release after antral denervation: no role for gastrin-releasing peptide. *J Physiol* 487:34P 1996.

Higham A.D., Noble P.J., Thompson D.G., Dockray G.J. Increased gastrin release following distension after antral denervation. *J Physiol* 499: 104P 1997.

Higham A., Thompson D.G., Dimaline R. & Dockray G.J.: Increased *Reg* gene expression in the rat gastric corpus following omeprazole treatment. *Reg Peptides*. 71:55 1997.

Chapters and Invited Reviews:

Higham A.D.: Clinical endocrinology of gastric function. *Surgery* (in press) 1997.

Statement of Originality

All of the data in this thesis that is presented as results has arisen wholly from my own work apart from the following contributions from colleagues within the Department of Physiology, University of LIverpool. The tissue culture of AR42J and HIT cells was performed by Dr Lisa Bishop as was the permanent transfection of the HIT cells. The plasma CCK assay was performed by Mrs C McLean. On occaision, the preparative surgery of the gastric fistula rats was performed by myself in conjunction with P J Noble.

Abbreviations

BAC	Benzalkonium chloride
CAG	Chronic atrophic gastritis
CCK	Cholecystokinin
CGRP	Cacitonin gene-related peptide
CRF	Chronic renal failure
EC	Enterochromaffin
ECL	Enterochromaffin-like
EGF	Epidermal growth factor
GRP	Gastrin-releasing peptide
HGF	Hepatocyte growth factor
LOH	Loss of heterozygosity
MEN1	Type I multiple endocrine neoplasia syndrome
NO	Nitric oxide
PA	Pernicious aneamia
PAP	Pancreatitis associated protein
PPI	Proton pump inhibitor
PSP	Pancreatic stone protein
PTP	Pancreatic thread protein
SP	Substance P
TGF-α	Transforming growth factor alpha
VIP	Vasoactive intestinal polypeptide
ZE	Zollinger Ellison syndrome

Contents

Chapter 1

Introduction

Historical review of gastrointestinal peptide physiology

At the beginning of this century two schools of thought were prominent with regard to the mechanisms that controlled visceral function. Langley provided evidence that responses to feeding were mediated by nervous reflexes (Langley, 1898) and his conclusions were supported by the work of Sherrington (1899) and Pavlov (1910). Set against this were the experiments of Bayliss and Starling (1902) and Edkins (1905) who proposed that blood borne agents (named secretin and gastrin respectively) were responsible for stimulating digestive secretions from the pancreas and stomach. Although Bayliss and Starling (1899) previously had reported the reciprocal effects of the vagal and splanchnic innervation on gastric motility, it took almost forty years before it was considered that the nervous and endocrine systems might interact closely to regulate visceral functions such as gastric acid secretion (Uvnas 1942).

Whilst the work of Bayliss and Starling was readily accepted, Edkins' proposal that an antral hormone stimulated gastric acid secretion remained unconfirmed until the work of Komarov in the late 1930's and of Grossman *et al.,* a decade later (1948). Meanwhile several groups attempted to purify to homogeneity secretin, the hormone proposed by Bayliss and Starling to stimulate pancreatic secretion in response to a meal. This was achieved in 1961 by Jorpes and Mutt who isolated secretin and at the same time Gregory and Tracy isolated and characterised the peptide hormone gastrin (Gregory and Tracy, 1964). Within a few years it was shown conclusively that gastrin was produced by and localised within a defined

population of endocrine cells in the gastric antral mucosa (McGuigan, 1968). Not suprisingly at the time, gastrin was considered to be strictly a gastrointestinal hormone. Furthermore, the similarities between gastrin and secretin seemed to confirm the prevailing view that peptides acted as classical hormones.

Almost coincidentally however, Burnstock *et al.,* (1963) described neurotransmission in the gut that was mediated by a nonadrenergic and noncholinergic agent. Morphological studies of the myenteric plexus suggested the existence of peptide-containing nerve fibres (Baumgarten *et al.,* 1970; Cook and Burnstock 1976). Subsequently, considerable evidence has been provided that has generated widespread support for the idea that peptides act as nuerotransmitters in neurons of the enteric, sympathetic, parasympathetic and central nervous systems.

Although von Euler and Gaddum as early as 1931, had suggested that agents with identical biological activity were present in extracts of both gut and brain, the work of Vanderhaeghen *et al.,* (1975) raised again the idea that a peptide previously considered to be a classic gut hormone (gastrin) might be present within neurons of the central nervous system. The technical and analytical advances in the last two decades have now established fundamental new concepts in the distribution of regulatory peptides. It is now clear that any one peptide may have a specific function at a specific site, determined by the cell type responsible for its synthesis, mode of release and subsequent tissue distribution, but the same peptide may occur at another independent site within the body and have a separate function and mode of action determined again by the local environment. Hence peptides such as

cholecystokinin (CCK) and gastrin, previously considered as hormones may act elsewhere as neurotransmitters or paracrine mediators, the converse also being true.

With the general acceptance of similarity between the spectrum of peptides that function as hormones and as neurotransmitters, it is perhaps less surprising that there is such close interaction between neurons and endocrine cells in the regulation of visceral functions such as gastric acid secretion. The main objective of the studies described in this thesis was to determine the role of antral neurons in the control of antral G-cell responses to physiological stimuli. The experimental model of antral denervation that was developed to investigate the the role of intrinsic neurons in the regulation of the G-cell produced chronic hypergastrinaemia and delayed gastric emptying. This prompted further studies on the role of antral neurons in the regulation of gastric emptying and the effects of sustained elevations in plasma gastrin on endocrine cell gene expression with particular relevance to enterochromaffin-like (ECL) cell growth and carcinoid tumour development.

The following account will describe the normal gastric innervation and its role in regulating G cell function and gastric emptying, review the physiology of gastrin and the pathophysiology of hypergastrinaemia in particular the development of ECL cell tumours, review the evidence for the role of gastrin in endocrine cell growth and differentiation and finally introduce putative regulators of cell growth and differentiation in gastroentero-pancreatic endocrine cells.

Innervation of the Stomach

Langley (1921) first recognised the importance of the intrinsic nervous system of the gut, considering it a separate division of the autonomic nervous system in addition to the sympathetic and parasympathetic divisions. Considerable evidence in support of this has been presented (Cooke, 1987; Furness and Costa, 1987; Wood, 1987) and it is clear that processes such as absorption, secretion and coordinated motor activity may be regulated wholly by the enteric nervous system. The extrinsic innervation of the gut interacts with enteric neurons exerting an overriding influence when necessary so that gut function may be integrated with the prevailing needs of the body as a whole. Extrinsic neuronal inputs are supplied by vagal and spinal nerve trunks each providing both afferent and efferent nerve fibres.

i) Vagal innervation of the stomach

The majority of nerve fibres in the vagi are afferent fibres having their nerve cell bodies in the nodose ganglia. These afferent fibres have their peripheral endings within the muscle and mucosal layers of the gut wall and act as mechano-, thermo- and chemo-receptors conveying signals to the central nervous system (Andrews, 1986; Blackshaw and Grundy, 1990; El Ouazziani and Mei, 1979; 1891). Almost all vagal afferent fibres are unmyelinated (Andrews, 1986) but a small population of nerve fibres within the vagus are small diameter myelinated fibres believed to be of sympathetic origin (Lundberg *et al.*, 1976).

Using retrograde tracing and immunohistochemistry it has been shown that vagal afferent fibres projecting from the anterior wall of the stomach have their cell bodies in the left nodose ganglion and those that project from the posterior gastric wall have their cell bodies in the right nodose ganglion (Dockray *et al.,* 1988; Green 1988). A number of peptides have been identified in vagal afferent fibres including substance P (SP) (Katz and Karten, 1980; Lundberg *et al.,* 1978; 1983; 1985) CCK (Mantyh and Hurt, 1984), vasoactive intestinal polypeptide (VIP) (Lundberg, 1978; Said and Rosenberg, 1976) and gastrin (Dockray *et al.,* 1981). Despite this variety, the majority of peptide-containing vagal nerve fibres project to the thoracic viscera (Lundberg, 1983; Mulderry 1985; Cadieux 1986) such that less than 10% of gastric vagal afferent fibres are peptidergic (Green, 1988).

The vagus also carries efferent fibres to the stomach but these account for only 10% of the total number of vagal fibres. The majority of vagal efferent fibres are excitatory cholinergic fibres that are involved with the normal regulation of gastric tone as well as secretion of gastrin and gastric acid. In addition however, there are also inhibitory pathways such as those causing release of nitric oxide (NO) (Desai *et al.,*, 1991) and VIP (Grider *et al.,*, 1985).

ii) Spinal afferent innervation of the stomach

Similar to the vagal innervation of the stomach, spinal afferent neurons for the most part are unmyelinated (Kuo *et al.,*, 1982). Gastric spinal afferents have their nerve cell bodies in the thoracolumbar dorsal root ganglia (Green and Dockray, 1988). Conversely however, there is no lateralisation of innervation such that

retrograde tracing from one surface of the stomach labels equal numbers of nerve cell bodies in the right and left dorsal root ganglia. The spinal efferent innervation of the stomach is approximately 95% adrenergic and projects from cell bodies contained within the coeliac ganglion (Macrae *et al.,* 1986).

Several different peptides have been identified within spinal afferent neurons but the most important are calcitonin gene-related peptide (CGRP) (Gibson *et al.*, 1984; Lee *et al.*, 1987; Rosenfeld *et al.,* 1983) and substance P (Hokfelt *et al.,* 1976; Price 1985; Seybould and Elde, 1980). CGRP is of primary interest because of its specific distribution within the stomach and is relevant to the work contained within this thesis. A brief review of the physiology of CGRP is given below before describing the intrinsic innervation of the stomach.

Discovery and distribution of CGRP:

The existence of CGRP was predicted on the basis of the discovery of a novel cDNA sequence corresponding to an mRNA transcript that was shown to arise from alternative splicing of the gene encoding calcitonin (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Subsequently a related peptide β-CGRP was isolated (Amara *et al.,* 1985; Steenbergh *et al.*, 1985) whose primary gene does not encode for calcitonin. The distribution of CGRP within the stomach has important implications. CGRP-immunoreactive fibres may be found in all layers of the stomach (Clague *et al.*, 1985; Green and Dockray 1988; Sternini *et al.*, 1987) but there are no intrinsic neurons or endocrine cells that contain CGRP in the rat stomach. In fact in rodent stomach almost all CGRP is contained within spinal

afferent extrinsic nerve fibres (Green and Dockray, 1988; Sternini *et al.*, 1987; Varro *et al.*, 1988). This makes CGRP a useful marker for an intact spinal afferent gastric innervation.

Biological actions of CGRP in the stomach:

It is well established that CGRP acts as a peripheral neurotransmitter from spinal afferent nerve terminals (Holzer, 1992). Much of the available experimental evidence points to a role for CGRP in gastric mucosal protection. Hence, installation of noxious chemicals into the gastric lumen results in increased mucosal damage if the spinal afferent innervation has been lesioned (Holzer and Sametz, 1986) or the action of CGRP is blocked by immunoneutralisation (Forster and Dockray, 1991). Convesrely, stimulation of CGRP release protects against mucosal damage (Holzer and Lippe, 1988; Holzer *et al.,* 1989; 1990) induced by installation of noxious chemicals.

The gastroprotective effect of CGRP acts partly by increasing gastric mucosal blood flow possibly by the release of NO (Lambrecht *et al.*, 1993). In addition however, CGRP has been shown to be a potent inhibitor of gastric acid secretion (Tache *et al.*, 1984; Pappas *et al.*, 1986; Helton *et al.,* 1989), a factor that also contributes to the gastroprotective effects of this peptide. The inhibition of acid secretion is mediated in part by release of somatostatin from D cells of the gastric corpus (Dunning and Taborsky, 1987; Inui *et al.,* 1991). In addition it has been shown that CGRP regulates somatostatin synthesis by corpus D cells at the level of gene transcription (Sandvik *et al.*, 1993).

iii) Intrinsic innervation of the stomach

The intrinsic nerve supply of the gastrointestinal tract, collectively termed the enteric nervous system, comprises two main plexi of nerves described first by Meissner (1857) and Auerbach (1864). Meissner described a submucous plexus and Auerbach a plexus of nerves lying between the circular and longitudinal muscle layers generally referred to as the myenteric plexus. The structure and distribution of the enteric nervous system were described in detail by Gaskell (1886) and the functional significance of the enteric nervous system as a separate branch of the autonomic nervous system was propsed later by Langley (1921).

Studies of the anatomy of intrinsic gut neurons were facilitated greatly by the techniques of myotomy and myomectomy coupled with immunohistochemistry first described by Furness and Costa (1979). The variety and colocalisation of neuropeptides present in enteric neurons, their projections and putative actions have received much interest over the past two decades (for review see Dockray 1994; Furness and Costa 1987). Generally speaking, the tachykinin/substance P family of peptides are present in intrinsic excitatory neurons (Katayama and North, 1978; Bartho *et al.*, 1982; Bartho and Holzer, 1985; Holzer, 1989) and the VIP/peptide histidine isoleucine family is present in intrinsic inhibitory neurons (Brookes *et al.*,, 1991; Ekblad *et al.*,, 1987; Shultzberg *et al.*,, 1989; Grider, 1989). Gastrin-releasing peptide (GRP) is present in secretor motor neurons and this is of particular relevance as GRP-containing neurons stimulate the release of gastrin from antral G

cells (Bunnett *et al.*, 1985; Kovacs *et al.*, 1995; Varner *et al.*, 1991). In the lesioning experiments described later in this thesis, substance P, VIP, GRP and CGRP were assayed as markers of different classes of intrinsic or extrinsic neurons to enable confirmation of denervation. There follows a brief review of substance P, VIP and GRP in relation to the enteric nervous system.

Substance P

Substance P (SP) was the first gut neuropeptide to be discovered. Although extracts of horse intestine had been shown to stimulate atropine resistant contraction of rabbit ileum as early as 1931 (von Euler and Gaddum, 1931), SP was isolated and sequenced first from the bovine hypothalamus (Chang and Leeman, 1970; Chang and Leeman, 1971). The identical peptide was shown later to be present in small intestine (Carraway and Leeman, 1979). Evidence for its neuronal localisation was suggested by Ehrenpreis and Pernow (1953) who reported reduced levels of SP bioactivity in the aganglionic segment of colon affected by Hirschsprung's disease. Lembeck (1953) suggested that SP was located within the primary afferent neurons on the basis of findings of higher concentrations of peptide in dorsal compared with ventral spinal roots. Although there is compelling evidence that a subset of primary afferent neurons contain both SP and CGRP (Gibbins *et al.*, 1985; Green and Dockray, 1988), lesioning experiments using capsaicin, have shown that in the rat stomach and small intestine the majority (>90%) of SP is present in intrinsic neurons (Holzer *et al.*, 1980).

The presence of SP within primary afferent neurons has prompted studies that suggest that SP released from these neurons may act as a central neurotransmitter of pain and may play a role as a peripheral transmitter during inflammatory reactions in the gut (Dockray, 1994; Holzer, 1988; Sharkey, 1992). The predominant localsiation of SP within intrinsic gut neurons has directed several groups towards identifying its possible role within the gut. There is evidence that SP directly stimulates intestinal smooth muscle contraction (Taylor and Bywater, 1986) and that SP release is involved in the contraction that occurs on the oral side of a distending bolus as part of the peristaltic reflex (Bartho *et al.*, 1982; Holzer, 1989; Yokayama and North, 1983). In the rat colon, SP release appears to mediate only the noncholinergic contraction that occurs with high pressure distension as this could be inhibited by immunoneutralisation (Grider, 1989b).

In addition to the direct effects of SP on smooth muscle, there is also evidence that release of SP may stimulate other myenteric plexus neurons (Yan and Youther, 1982; Fosberg *et al.*, 1984). Regardless of the precise physiological role of SP in the control of motility, intrinsic SP neurons generally can be considered to be excitatory and SP is the most useful marker of this class of neuron.

Vasoactive intestinal polyptide

Vasoactive intestinal polypeptide (VIP) was discovered by Said and Mutt by screening of extracts of gut and lung for vasodilator activity (Said and Mutt, 1970; 1972). Vasodilatation induce by VIP is only one manifestation of its ability to relax smooth muscle. In the gut VIP is localised to a population of myenteric neurons that

project distally and innervate the circular muscle (Brookes *et al.,* 1991; Ekblad *et al.*, 1987), and to a population of submucosal neurons that project to the mucosa (Schultzberg *et al.*, 1980; Costa and Furness, 1983). There is evidence from immunoneutralisation studies that VIP may mediate the descending relaxation of the peristaltic reflex (Grider and Makhlouf, 1986; Grider *et al.,* 1985; Grider, 1989a). In addition, VIP may be one of the final mediators of the vagovagal, non-adrenergic, non-cholinergic relaxation of the stomach (Faherenkug *et al.*, 1978; Ito *et al.*, 1988), and play a role in the control of gastric emptying (Forster *et al.*, 1991; D'Amato *et al.*, 1992).

Submucosal VIP-containing neurons that project to the mucosa stimulate secretion from enterocytes (Cooke, 1992). In terms of gastric endocrine cell function, VIP has been shown to inhibit gastric acid secretion by stimulating the release of somatostatin from mucosal D cells (Saffouri *et al.*, 1984). From the above description therefore, in contrast to SP, VIP can be considered as a marker of inhibitory myenteric plexus neurons.

Gastrin-releasing peptide

In 1971, Espramer and Anastasi isolated bombesin from amphibian skin (Anastasi *et al.*, 1971). A major effect of this peptide was stimulation of acid secretion that was shown in the dog to be mediated by gastrin (Bertaccini *et al.,* 1974). The mammalian counterpart of bombesin was identified using C-terminally directed antibodies to the amphibian peptide (Walsh *et al.*, 1979). Coincidentally, McDonald and co-workers discovered that extracts of the non-antral porcine

stomach were a potent stimulus to gastrin release (McDonald *et al.,* 1978; McDonald *et al.*, 1979). They proposed that the peptide responsible was gastrin-releasing peptide (GRP) and subsequently the C-terminal sequence of GRP and bombesin have been shown to be almost identical. However, GRP is not strictly the mammalian equivalent of amphibian bombesin as amphibia have separate genes encoding GRP and bombesin (Nagalla *et al.*, 1992).

In the mammalian gut, GRP-immunoreactivity is found exclusively within neurons (Dockray *et al.*, 1979). The majority of GRP immunoreactive fibres are present in the mucosa with some fibres present in the circular muscle and myenteric plexus. (Dockray *et al.*, 1979). Most GRP-containing neurons have their cell bodies in the myenteric plexus and project their axons distally (Costa *et al.*, 1984; Ekblad *et al.*, 1984). There is good evidence that the major physiological role of this neuropeptide in the stomach is to stimulate gastric acid secretion via release of gastrin from antral G cells (Bunnett *et al.*, 1985; Hildebrand *et al.*, 1991; Hirschowitz and Molina, 1983; Kovacs *et al.*, 1995).

A schematic representation of the major classes of neuron present within the gastric antrum is shown in Figure 1.1.

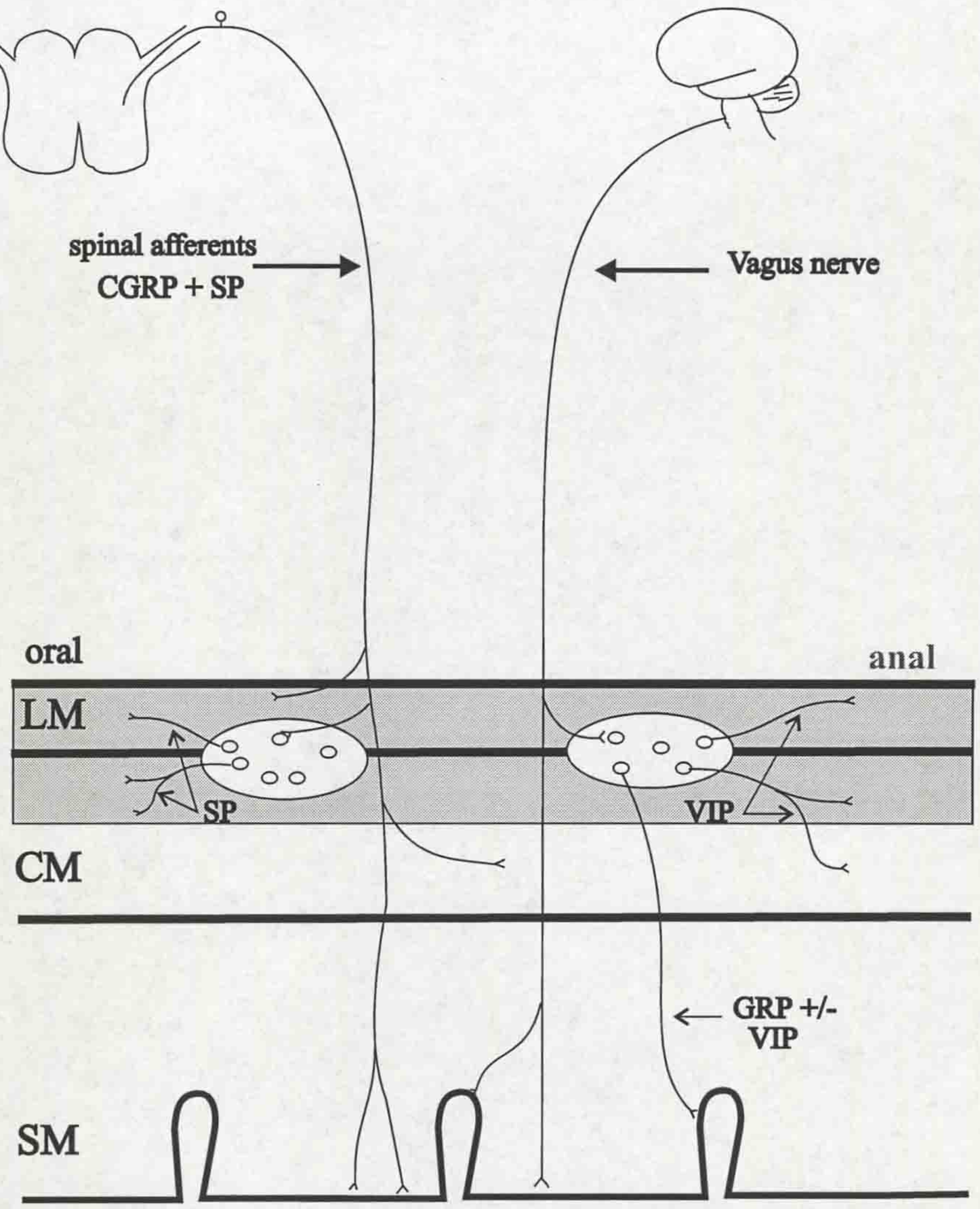


Figure 1.1: Schematic representation of the major classes of intrinsic and extrinsic neurons that innervate the gastric antrum in the rat and are susceptible to lesioning by serosal application of the neurotoxin BAC. The shaded area depicts the likely depth of penetration of BAC. LM - longitudinal muscle; CM - circular muscle; SM - submucosa. Excitatory (SP) and inhibitory (VIP) myenteric neurons are shown and receive inpputs from spinal afferent (CGRP) and vagal nerve fibres. GRP is present predominantly in secretomotor nerves that supply the mucosa (see text for full details).

Neuroendocrine regulation of gastric emptying

In recent years a substantial body of evidence has emerged detailing the interactions between the intestinal hormone cholecystokinin (CCK) and vago-vagal reflexes that together mediate the delayed gastric emptying in response to fat- and protein-rich meals (Dockray, 1988; Mayer, 1994). Exogenous CCK inhibits gastric emptying in both rat (Conorver *et al.*, 1988; Green *et al.*, 1988) and man (Chey *et al.,* 1969; Liddle *et al.*, 1986). Protein -rich meals are known to stimulate secretion of endogenous CCK (Liddle *et al.*, 1984; 1986) and delay gastric emptying (Green *et al.*, 1988; Moran and M^{c}Hugh, 1981), an effect that is reversed by specific CCK-A receptor antagonists (Green *et al.*, 1988; Liddle *et al.*, 1989). In addition there is good evidence that vagal afferent fibres express CCK-A receptors (Corp *et al.*, 1993; Moriarty *et al.*, 1997) and that the CCK-mediated delay in gastric emptying is initiated by stimulation of vagal afferent fibres (Raybould and Tache, 1988; Forster *et al.*, 1990; Forster *et al.*, 1991; Holzer *et al.*, 1994). Furthermore, the efferent limb of the reflex that results in relaxation of the gastric body also requires an intact vagus nerve (Raybould and Tache, 1988; Raybould *et al.*, 1987). Vagal efferent relaxation of the gastric corpus is mediated by non-adrenergic and non-cholinergic neurotransmission that probably involves both NO (Desai *et al.*, 1991; Forster *et al.*, 1991; D'Amato *et al.*, 1992) and VIP (Forster *et al.*, 1991; D'Amato *et al.*, 1992).

In addition to the CCK-vagal pathway that regulates gastric relaxation in response to feeding, gastric emptying may also be affected by spinal afferent innervation, since lesioning of spinal afferent neurons by capsaicin pre-treatment

reduces the emptying rate of liquid test meals (Forster et al, 1990; Forster and Dockray, 1992).

Overall the rate of gastric emptying depends on the pressure gradient between the duodenum and stomach and the resistance to flow across the pylorus and antrum (Mayer, 1994). The content of the ingested meal determines the rate of emptying and CCK-mediated vagovagal reflexes play an important role in delaying gastric emptying by relaxation of the gastric corpus. The importance of antro-pyloric motility in regulating gastric emptying has received less attention although the pulsatile manner of delivery of gastric content to the duodenum can be attributed to the phasic motor pattern of the antrum (Houghton *et al.*, 1988; Malbert *et al.*, 1991; Prather *et al.*, 1993). There is some evidence that CCK may act on the distal stomach both by a direct action on smooth muscle and indirectly via release of neurotransmitters (Scheurer *et al.*, 1983; Yamagishi and Debas, 1978). However the relationships between CCK and antral neurons in the regulation of gastric emptying has not been examined previously in any detail and represents one of the objectives of the studies presented in this thesis.

Neuroendocrine Regulation of Gastric Acid Secretion.

The G cells, D cells and ECL cells of the stomach together with the extrinsic and intrinsic innervation of the stomach, provide an intricate regulatory mechanism for the control of gastric pH (Fig 1.2).

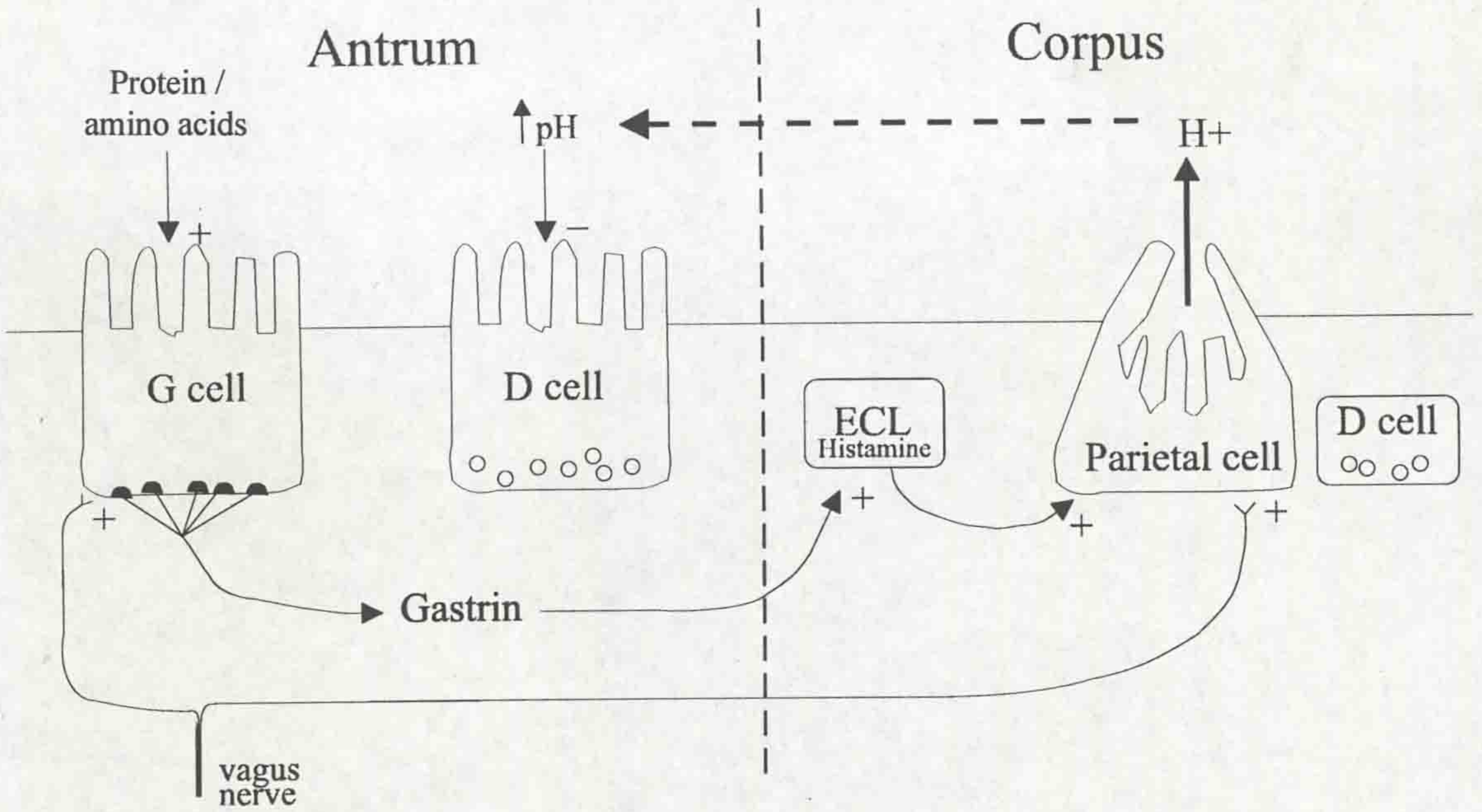


Figure 1a

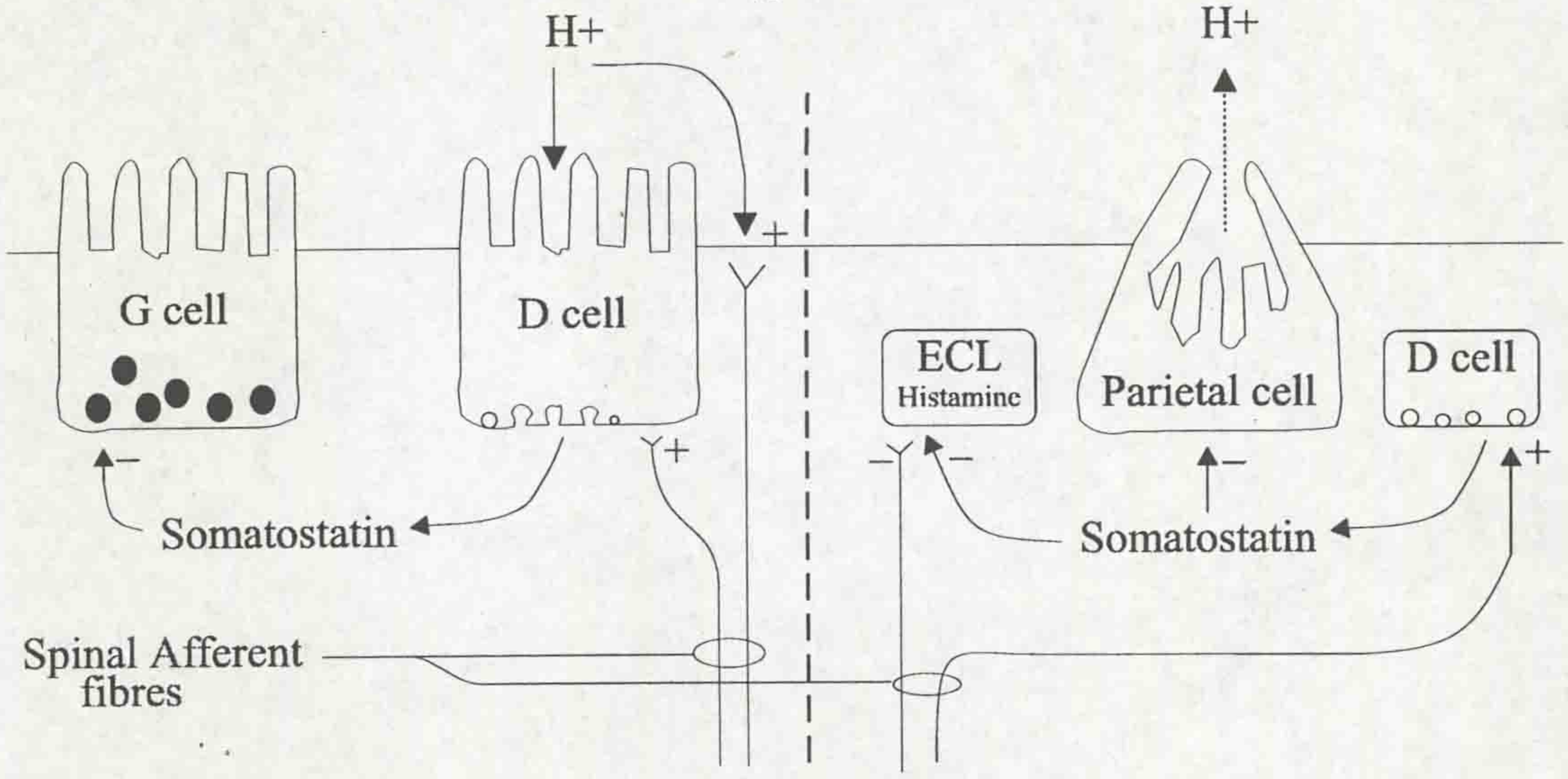


Figure 1b

Figure 1.2: Following meal ingestion, intraluminal pH rises and somatostatin (SOM) release from the D cell is inhibited. The G cell secretes gastrin that leads to histamine release from the ECL cell and H^+ release from the parietal cell. Vagal neurons also stimulate the parietal cell (1.2a). As luminal pH then falls, antral D cells release SOM that inhibits further gastrin release. Spinal afferent neurons regulate SOM release from corpus D cells. SOM inhibits the ECL cell and parietal cell (1.2b). See text for full description.

G cells

These cells are present in the antrum of the stomach and are also found in lesser numbers in the proximal duodenum (M^{c}Guigan, 1968; Solcia *et al.*, 1975). Their primary function is to synthesise, store and release the amidated 17 amino acid peptide gastrin. G cells process the prohormone precursor peptide progastrin by sequential steps invoving sulfation, phosphorylaton, cleavage, C-terminal amidation or glycine extension and further cleavage (for review see Dockray *et al.*, 1996). The acid secretory activity of gastrin is dependent on the presence of the amidated caboxyl terminus (Soll *et al.*, 1984; Takeuchi *et al.*, 1980). Alternative forms of gastrin, extended at their C-terminus by glycine also have been reported to possess bilogical activity other than stimulation of acid secretion, namely stimulation of expression of the gene encoding the H^{+}-K^{+}-ATPase present in parietal cells (Kaise *et al.*, 1995). Furthermore, recent studies suggest that amidated gastrin, glycine extended gastrin and progastrin itself are all putative growth factors (Seva *et al.*, 1994; Singh *et al.*, 1996; Wang *et al.*, 1996).

G cells have a luminal brush border and secrete gastrin in response to the presence of protein and amino-acids within the gastric lumen (Dockray and Gregory, 1989; Walsh, 1994). In keeping with these observations are the reduction in gastrin mRNA reported with fasting and increases in gastrin mRNA abundance seen with feeding (Wu *et al.*, 1991). In addition G cells respond to changes in luminal pH although this may be indirect, being mediated by changes in somatostatin release from adjacent antral D cells (Karnik *et al.*, 1989). G cells

express several different receptors on their basolateral membrane including receptors for gastrin releasing peptide (Vigna *et al.,* 1990; Weigart *et al.,* 1992).

ECL cells

These cells are present only in the gastric corpus and their main function is to synthesise histamine by the decarboxylation of the amino acid histidine (Rubin and Schwartz, 1979). This reaction is catalysed within the cell cytosol by the enzyme histidine decarboxylase (Kubota *et al.,* 1984). Histamine is then transported into secretory vesicles probably by the vesicular monoamine transprter $VMAT_2$ (Merickel and Edwards., 1995). Unequivocal evidence for the stimulaton of acid secretion by histamine was provided by the discovery of H_2 receptor antagonists and their ability to block acid secretion (Black *et al.,* 1972). Gastrin stimulates the ECL cell to release histamine (Chuang *et al.*, 1992) and if the gastrin stimulus is sustained, ECL cells increase their production and storage capacity of histamine, in part by upregulating HDC and $VMAT_2$ gene expression (Dimaline and Struthers, 1996; Swarovsky *et al.*, 1994). The secretion of histamine by the ECL cell can be inhibited by release of somatostatin from corpus D cells (Chuang *et al.*, 1993). Finally ECL cells increase in number in response to continued gastrin stimulation (Hava, 1986; Poynter and Selway, 1991; Wangberg *et al.,* 1995).

D cells

D cells are present in both the antrum and corpus of the stomach. In both sites, their function is to synthesise, store and release somatostatin (Dockray *et al.,* 1996). Antral D cells have a luminal brush border and respond to changes in pH in

the stomach (Gustavsson and Lundquist, 1978; Holst *et al.*, 1987; Schubert *et al.*, 1988). In contrast, corpus D cells do not have a luminal brush border and somatostatin mRNA abundance in corpus D cells is much less affected by changes in luminal pH that occur with feeding or treatment with omeprazole (Sandvik *et al.*, 1993). It appears that corpus D cell somatostatin gene transcription is regulated primarily by neurohumoral influences of which the most significant may be those of the CGRP-containing spinal afferent neurons (Sandvik *et al.*, 1993).

Regulation of acid secretion

The parietal cells are responsible for the release of acid (H^+) into the gastric lumen. They can be stimulated to release H^+ by cholinergic neurons and by histamine (Black *et al.*, 1972; Black and Shankley, 1987; Kabekar *et al.*, 1969; Main and Pearce, 1982; Soll, 1978). Gastrin receptors have been demonstrated on parietal cells (Soll *et al.*, 1984) suggesting that gastrin also may act directly on these cells to induce acid secretion. As intragastric pH rises (such as with ingestion of a meal) antral G cells release gastrin in response to a variety of subsequent stimuli. These include rising luminal pH (Dockray *et al.*, 1991), increased cholinergic stimulation (Kabekar *et al.*, 1969; Main and Pearce, 1982), release of GRP from non-cholinergic neurons (Saffouri *et al.*, 1984; Schubert *et al.*, 1992), the presence of intraluminal protein and amino-acids (Chiba *et al.*, 1980; Saffouri *et al.*, 1980; Walsh, 1994), and a reduction in somatostatin release from adjacent antral D cells that are themselves inhibited by the rising luminal pH (Gustavsson and Lundquist, 1978; Holst *et al.*, 1987; Schubert *et al.*, 1988).

Gastrin passes via the circulation to the ECL cell and stimulates these cells to release histamine (Chuang *et al.,* 1992). ECL cells may also respond to the increased cholinergic drive that accompanies meal ingestion (Maine and Pearce, 1982; Soll, 1982). Histamine released from ECL cells acts via parietal cell H_2 receptors causing H^+ release (Black *et al.,* 1972, Black and Shankley, 1987). This co-ordinated response provides the acid pH required for gastric digestion by pH dependent enzymes and as digestion and gastric emptying proceeds, returns gastric pH to its normal basal level (Fig 1.2a).

As the gastric pH falls, antral D cells respond by releasing somatostatin that in turn inhibits gastrin release from G cells (Gustavsson and Lundquist, 1978; Holst *et al.,* 1987; Schubert *et al.,* 1988). As a reslt of reduceded intraluminal pH, gastrin levels fall, permitting somatostatin release from corpus D cells which in turn prevents further secretion of histamine from the ECL cell and H^+ from the parietal cell (Fig. 2b). The spinal afferent innervation of the corpus may also stimulate corpus D cell somatostatin release as it has been shown to functionally regulate corpus rather than antral D cell somatostatin gene expression (Sandvik *et al.,* 1993).

It may be readily appreciated from the above description that disorders of gastric acid secretion are likely to be associated with elevated plasma gastrin levels. Hence, primary disorders of the G cell or ectopic gastrin secretion that result in hypergastrinaemia will result in increased acid output. Conversely, decreased acid output from whatever cause will result in elevated plasma gastrin as antral G cells

respond to the high intragastric pH. There follows a discussion of conditions associated with hypergastrinaemia and the related pathophysiology of the ECL cell .

Clinical significance of chronic hypergastrinaemia

From a clinical viewpoint the major conditions of the stomach that are associated with altered endocrine cell function are encompassed by the conditions associated with hypergastrinaemia (Figure 1.3). The commonest of these are the conditions associated with *H pylori* infection with or without concurrent treatment with a proton pump inhibitor (PPI). However, Zollinger Ellison sydrome (ZE) and pernicious anaemia are the conditions associated with the highest and most sustained elevations in plasma gastrin. In these conditions ECL cell (carcinoid) tumours have been described (see below) and it appears that it is the trophic action of gastrin on the ECL cell that is of pathophysiological importance. The elevations of plasma gastrin in *H pylori* or with PPI therapy are of lesser magnitude and rarely sustained and ECL cell tumours have not been reported. However, *H pylori* infected patients treated with a PPI may develop significant elevations in plasma gastrin and ECL cell hyperplasia has been documented in up to a third of such patients taking a PPI for five years (Eiselle *et al.,* 1997). Although hyperplasia precedes dysplasia and neoplasia, the time course of this progression is not known and it may take several years before carcinoid tumours develop. Hence carcinoid tumours developing in patients rendered hypergastrinaemic by H *pylori* and PPI therapy remains a remote possibility.

Associated Condition	*Representative Range of Plasma Gastrin* (pM)*	*Causal mechanism for hypergastrinaemia*
Pernicious Anaemia (PA)	200 - >1000	Atrophic gastritis leads to achlorhydria and secondary rise in plasma gastrin
Zollinger Ellison (ZE) Syndrome	Normal - >1000	Functioning gastrin secreting tumour usually of the duodenum or pancreas causes increased acid output.
Chronic Renal Failure. (CRF)	Normal - 600	Kidneys are the major site of breakdown of circulating gastrins.
Proton Pump Inhibitor Treatment (PPI) #	Normal - 350	Reduces acid output by direct action on the parietal cells' H^+/K^+ ATPase.
H. pylori infection	Normal - 200	a) antral gastritis reduces antral somatostatin with loss of inhibition of gastrin release by gastric acid. b) Corpus gastritis leads to atrophy and hypochlorhydria

Figure 1.3: Conditions associated with hypergastrinaemia and the primary causal mechanism for the elevated plasma gastrin.

* Amidated gastrin; normal fasting <30pM; normal post-prandial <90pM.

depends on duration of treatment and presence of *H pylori*. (6-8 week treatment with PPI raises plasma gastrin up to twofold).

ECL cell (Carcinoid) tumours and Hypergastrinaemia.

Chronic atrophic gastritis (CAG) with or without pernicious anemia (PA) is the commonest cause of ECL cell hyperplasia and in 5-10% of patients there is a progression to gastric carcinoid tumors (Borch *et al.*, 1985; Bordi *et al.,* 1991; 1995). The achlorhydria of CAG leads to hypergastrinemia which is generally accepted to be a stimulus to ECL cell growth (Bordi *et al.,* 1995; Creutzfeld, 1988; Hakanson and Sundler, 1990; Solcia *et al.,* 1986). The natural history of these tumors in man has been extensively reviewed (Modlin *et al.,* 1995; Rindi, 1995; Solcia *et al.,* 1991; Thomas *et al.,* 1994), prompted at least in part by the demonstration that in rodents hypergastrinemia secondary to long-term treatment with H_2-receptor antagonists or proton pump inhibitors, may lead to gastric carcinoid tumors (Hava, 1986; Poynter and Selway, 1991; Wangberg *et al.,* 1995).

Enterochromaffin-like cell tumors associated with CAG are usually small (<1cm), may be single or multiple, are most often present incidentally in patients over the age of 60, and rarely progress to metastatic disease (Bordi *et al.,* 1995; Solcia *et al.,* 1991; Modlin *et al.,* 1995). This has led to conservative management for the majority of patients with or without endoscopic screening. Some authors have advocated twice daily subcutaneous injections of the long acting somatostatin analogue octreotide (Ferraro *et al.,* 1996; Bordi *et al.,* 1993). This therapy has been shown to reverse ECL cell hyperplasia in patients with CAG without ECL cell tumors, and to inhibit gastrin release from the antral gastrin (G-) cell (Ferraro *et al.,* 1996; Bordi *et al.,* 1993). Surgical antrectomy to remove G-cells is also effective in

reducing plasma gastrin levels and is reported to result in complete resolution of small ECL cell tumors (Hirschowitz *et al.,* 1992; Modlin *et al.,* 1995; Scully *et al.,* 1997). In contrast, the management of large ECL cell tumours (>2cm) associated with CAG is less well defined. Antrectomy has been advocated since it may induce tumor regression, or at least halt metastatic spread (Eckhauser *et al.,* 1988; Scully *et al.,* 1997) However, reports of a metastasis rate of >20% for primary tumours that are >2cm in diameter, together with the demonstration that in a few cases the tumors fail to regress following antrectomy, has persuaded others to recommend total gastrectomy (Borch *et al.,* 1985; Thomas *et al.,* 1995).

Although all PA patients are hypergastrinaemic and the majority show ECL cell hyperplasia some other factor(s) must also be involved in the progression of hyperplasia to neoplasia as the incidence of carcinoid tumours in PA patients is 5-10% (Borch *et al.,* 1985; Bordi *et al.,* 1991; 1995). Azzoni has suggested that the expression of BCL-2 by hyperplastic ECL cells may be of importance as it may prolong exposure to other oncogenic factors (Azzoni *et al.,* 1996). However relatively little is known of the underlying genetic risk factors for ECL cell tumour development in CAG.

In the sporadic form of ZE the incidence of carcinoid tumours is similar to that in PA. In contrast patients with ZE as part of the type I multiple endocrine neoplasia syndrome (MEN1) have a much higher incidence of carcinoid tumour development (up to 35%) presumably due to an inherited susceptibility to endocrine cell tumour development (Lehy *et al.,*, 1992). The recent positional cloning of the

MEN1 gene (Chandrasekharappa *et al.*, 1997) has facilitated the study of abnormalities of this gene in the development of carcinoid tumours. Loss of heterozygosity (LOH) at the MEN1 locus could be demonstrated in the majority of carcionid tumours arising in patients with ZE as part of the MEN1 syndrome (Debelenko *et al.*, 1997). However very few gastric carcionids arising from CAG had associated LOH at the MEN1 gene locus suggesting other genes may be important in the pathogenesis of these tumours. Data is presented in this thesis that is in favour of a potential candidate gene, *reg,* being of importance to the development of carcinoid tumours. Before reviewing the available information on the *reg* family of genes, the role of gastrin in the differentiation and growth of endocrine cells of the stomach and pancreas is discussed.

Gastrin, enodcrine cell growth and differentiation in the stomach and pancreas.

In addition to the well established role of amidated gastrin in regulating gastric acid output, there is an increasing body of evidence to suggest that gastrin also regulates the differentiation of some gut endocrine cells and the growth and overall numbers of certain endocrine and epithelial cell populations within the stomach.

The first direct evidence of the trophic effect of amidated gastrin came from the observation that pentagastrin administration to rats increased the functioning

parietal cell mass thereby contributing to the long term increase in acid output. (Crean *et al.,* 1969). Prior to this, gland atrophy following antrectomy had been reported (Lees and Grandjean, 1958), indirectly suggesting a trophic role for gastrin in maintaining the acid secreting mucosa. Gastrin also stimulates the growth of ECL cells (Bordi *et al.,* 1995; Tielemans *et al.,* 1990), a subject that has received much interest due to the theoretical risk of ECL cell tumour development in patients receiving long term pharmacological acid suppression.

More recent studies using transgenic mice clearly demonstrate the importance of amidated gastrin acting via the CCK-B receptor in the maintenance of normal cell populations within the gastric mucosa. Hence, overexpression of amidated gastrin leads to increased proliferation of precursor cells in the proliferative zone of the gastric mucosa and increased parietal and ECL cell numbers (Wang *et al.,* 1993). Conversely, targeted disruption of the CCK-B receptor produced marked gastric mucosal atrophy with reductions in the numbers of parietal and ECL cells (Langhans *et al.,* 1997; Nagata *et al.,* 1996). Similarly, targeted disruption of the gastrin gene resulted in a marked reduction in ECL cell and parietal cell number but did not affect the proliferative index in the stomach (Koh *et al.,* 1997). The latter study is of particular interest as it highlights the importance of gastrin in regulating the differentiation of gastric parietal and ECL cells independent of the degree of stem cell proliferation.

There is also good evidence that gastrin is responsible for the development in the embryo of endocrine cells of the pancreas (Wang *et al.,* 1993). In the case of

pancreatic endocrine cell development candidate genes have been proposed to be involved in the growth and differentiation of islet β-cells. (Terazono *et al.,* 1988; Otonkoski *et al.,* 1994; Watanabe *et al.,* 1994). The *reg* gene was first isolated by Terazono and colleagues in 1988 by differential screening of a rat regenerating pancreatic islet cDNA library. This gene has been renamed *reg* Iα, following the discovery of several other homologous genes that appear to comprise a multigene family.

The *reg* gene family

The rat *reg* Iα cDNA contained a single open reading encoding for a 165 aminoacid protein, of which the first 21 aminoacids appeared to constitute a signal peptide. The human homologue of rat *reg* Iα was identified by screening of a human pancreatic cDNA library with a 60 base pair complement of the rat cDNA (Terazono *et al.,* 1988). Human *reg* Iα encodes a 166 aminoacid protein with a 22 aminoacid signal peptide. Human *reg* Iα normally is expressed in the pancreas, gastric mucosa and kidney; ectopic expression of *reg* has been described in tumours of the colon and rectum (Watanabe *et al.,* 1990). The homology between rat and human *reg* cDNA was 75% (68% in AA sequence) including similar homology in the 5' untranslated region. Perhaps of significance was the observation that the position of all seven cysteine residues within the predicted protein were conserved. Subsequent cloning of the respective genes indicated another common feature, each consisting of 6 exons and 5 introns. These two features are common to all members of the *reg* gene family.

Following the cloning and sequencing of *reg* it became clear that the previously described pancreatic stone protein (PSP) was a truncated product of the same gene (Watanabe *et al.,* 1990). In fact PSP consists of 144 aminoacids (23-166 of the parent *reg*) and has been renamed PSP_I. A shorter variant also has been identified and is 11 amino acids shorter (34-166 of the parent *reg*). This protein is identical to the pancreatic thread protein (PTP) also referred to as PSP_{II} (Watanabe *et al.,* 1990).

Other members of the human *reg* family that have been identified so far, include *reg* Iβ (Moriizumi *et al.,* 1994), *reg* related sequence (Watanabe *et al.,* 1990) and pancreatitis associated protein (Iovanna *et al.,* 1991; Morizumi *et al.,* 1993). Confusingly, pancreatitis associated protein (PAP) has an identical aminoacid sequence to peptide 23 (Katsumata *et al.,* 1995) and HIP, a protein that was first identified by its expression ectopically in primary hepatocellular carcinoma (Lasserre *et al.,* 1992). These members of the gene family are tandemly ordered in a 95kbp DNA region of human chromosome 2p12 as determined by fluorescence in situ hybridisation (Miyashita *et al.,* 1995).

The murine *reg* gene family has also been studied but reveals unexpected differences. The murine homologue of *reg* Iα has been identified and localised to chromosome 12 (Unno *et al.,* 1993). A second member of the family *reg* II has also been cloned. Although the gene consists of 5 introns and 6 exons with conservation of all cysteine residues, the whole sequence is <60% homologous to *reg* I, has no human homologue and maps to mouse chromosome 3 (Unno *et al.,* 1993). Further

members of the mouse *reg* gene family have recently been described. Hence murine *reg* IIIα, *reg* IIIβ and *reg* IIIγ bear reasonable homology to each other but represent a separate gene group and are localised on Chromosome 6 (Narushima *et al.,* 1997). Unlike other members of the *reg* family, the *reg* III genes are preferentially expressed in the intestine and are expressed only at low levels in regenerating pancreatic islets.

The available evidence suggests that members of the *reg* gene family are expressed within endocrine cells of the stomach and pancreas. In particular, *reg* Iα (*reg*) expression appears to be markedly upregulated during regeneration following surgical resection (Terazono *et al.,* 1988;) or mucosal ulceraton induced by waster immersion stress (Ashahara *et al.,* 1997) or treatment with non-steroidal anti-inflammatory drugs (Kawanami *et al.,* 1997). However, the potential relationship between gastrin and expression of the *reg* gene has not been explored.

Reg gene expression and endocrine cell growth and differentiation

The *reg* gene was first proposed as a regulator of pancreatic islet cell growth following its isolation from regenerating islets (Terazono *et al.,* 1988). In mature adult pancreas, *reg* expression is limited to acinar cells. The Reg protein is secreted into pancreatic juice where it has been suggested to act as an inhibitor of pancreatic stone formation (Patard *et al.,* 1996; Bertrand *et al.,* 1996). During islet cell regeneration the Reg protein also appears to be produced by endocrine cells as it has been shown to colocalise almost exclusively with insulin in β-cell secretory granules (Terazono *et al.,* 1990).

Other models of islet cell growth such as hyperplasia following surgical wrapping of the pancreas (Zenilman *et al.,* 1996) and islet cell regeneration following growth suppression by insulinoma (Miyaura *et al.,* 1991), have shown a consistent relationship between expression of *reg* and increasing islet cell number. None of these studies attempted to determine whether *reg* may act as a growth factor or as a promoter of differentiation.

Otonkoski and colleagues (1994b) proposed that *reg* expression was a marker of proliferation rather than differentiation based on observations using nicotinamide to stimulate differentiation and hepatocyte growth factor (HGF) to stimulate proliferation in 7day cultures of human foetal pancreatic cells. *Reg* expression was significantly increased by HGF. However, the effects of HGF are not purely trophic. In fact, the in vitro mitogenic effect of HGF is limited to the first three to four days of culture (Beattie *et al.,* 1996). Thereafter, HGF has been shown to increase the formation of islet cell clusters in 6day cultures of human foetal pancreatic cells and increase their insulin content (Otonkoski 1994a). Thus HGF appears to stimulate both growth and differentiation in this model. HGF also stimulates the differentiation of AR42J cells into insulin secreting cells (Mashima *et al.,* 1996). The pancreatic cell line AR42J has recently been shown to express *reg* (Zenilman *et al.,* 1996) and can be manipulated by appropriate culture conditions to differentiate towards either acinar or endocrine cell phenotype (Christophe, 1994; Mashima *et al.,* 1996). Interestingly, differentiation towards an acinar cell phenotype is associated with reduced *reg* expression (Zenilman *et al.,* 1997).

In 1994, Watanabe *et al.,* described the amelioration of diabetes (induced surgically in rats by 90% pancreatectomy) by daily administration of recombinant rat Reg protein. Administration of Reg protein led to a significant increase in islet cell size, insulin positive β-cell number and amelioration of diabetes. They also demonstrated a threefold increase in the incorporation of ^{3}H-thymidine into cultured rat islet cells exposed to recombinant rat Reg protein. Autoradiography demonstrated a limited increase in the proliferative index of insulin positive cells from 0.1% in controls to 0.2% in Reg treated cultures. Based on these experiments, Watanabe *et al.,* claimed that the trophic effects of Reg protein explain its ability to stimulate β-cell regeneration and ameliorate diabetes.

In contrast, Smith *et al.,* (1994) argue against *reg* playing a role in islet cell growth from experimental evidence employing a model of selective β-cell growth induced by glucose infusion for 96hrs. Such a procedure produces a 50% increase in the rat islet β-cell mass with a fivefold increase in the proliferative index of islet β-cells (Bonner-Weir *et al.,* 1989). Using this model Smith *et al.,* (1994) could find no evidence of increased *reg* expression during or after the glucose infusion. Isolated rat islets in culture however, do respond to glucose exposure, both by increased *reg* expression and increased ^{3}H-thymidine incorporation (Francis *et al.,* 1992).

Reg expression in endocrine cells of the stomach

In 1996, Ashahara and colleagues reported the presence of *reg* in the endocrine cells of the rat stomach. In fact, by using the techniques of in situ

hybridisation and immuno-histochemistry they clearly demonstrated that the large majority of *reg*-positive cells were the ECL cells. Furthermore, *reg* mRNA abundance was upregulated during the period of mucosal repair following stress ulceration induced by water immersion. Plasma gastrin concentrations and acid output were not measured. Nevertheless the presence of *reg* in ECL cells and its upregulation during ECL cell regeneration suggest that *reg* may play a role in the growth and/or differentiation of the gastric ECL cell.

In summary, *reg* Iα is a major member of the *reg* family and is expressed during pancreatic islet cell regeneration and in the rat gastric ECL cell. The protein product of the gene stimulates the formation of mature endocrine cells in the regenerating pancreas. Expression of *reg* is associated with growth and differentiation of endocrine cells but its exact function as trophic factor or promoter of differentiation remains to be determined. The ectopic expression of *reg* and related family members in tumours of the gastrointestinal tract and liver justifies the further studies required to elucidate the exact role of the *reg* gene and its protein product. The possibility that the *reg* gene is involved in the development of gastric carcinoid tumours in patients with hypergastrinaemia is examined in this thesis.

Aims and Objectives

1. To develop a model of localised denervation of the gastric antrum in the rat in order to study the role of intrinsic antral neurons in the regulation of gastric function.

2. To examine the effects of lesioning of antral denervation of on gastric emptying in the rat to test the hypothesis that intrinsic neurons of the gastric antrum regulate gastric emptying and mediate the actions of extrinsic reflexes already known to influence gastric emptying.

3. To examine the effects of antral denervation on the control of gastrin release in the rat in order to test the hypothesis that antral neurons regulate the reponses of the G-cell to feeding and gastric distension.

4. To investigate *reg* gene expression in the pancreatic cell line AR42J in order to test the hypothesis that gastrin and EGF may regulate *reg* expression

5. To determine the relationship between *reg* expression and hypergastrinaemia in both rat and man to test the hypothesis that gastrin stimulation of ECL cell function and growth are associated with upregulation of *reg* expression.

6. To investigate the possibility that mutations of the *reg* gene (a putative regulator of endocrine cell differentiation) are associated with carcinoid tumour development as a consequence of chronic hypergastrineamia.

Chapter Two

Materials and Methods

Materials

All laboratory chemicals that can be considered as in routine use were obtained from either BDH chemicals, Poole U.K., or from Sigma Chemicals limited, Poole, U.K..

The materials used for routine molecular biology experiments that were not available from the above companies were obtianed from either Gibco GBRL, Renfrewshire, Scotland, or from Severn Biotech, Worcester, U.K.. All radioactive materials were obtained from Amersham Life Sciences, Buckinghamshire, U.K.. Other specific items are listed below.

Drugs/Chemicals:

Benzalkonium chloride	Sigma Chemicals Ltd.
Bovumin	First link, West Midlands, U.K.
BW2258U89	Dr J Leban, Burroughs Wellcome, U.S.A.
Capsaicin	Sigma Chemicals Ltd.
Diazepam (Valium)	Roche products Ltd., Derbeyshire U.K.
Enrofloxacin	Bayer Health Buisness Group, Suffolk, U.K.
Fentanyl/Fluanisone (Hypnorm)	Janssen Animal Health, Buckinghamshire, U.K.
L-740093	Merk Sharp and Dohme, Harlow, U.K

Peptides:

Gastrin	Penninsula Laboratories, St Helens,UK.
porcine GRP_{14-28}	Bachem (uk) Ltd., Saffron Walden, U.K.
rat $Tyr^0\alpha$-$CGRP_{28-37}$	Bachem (uk) Ltd., Saffron Walden, U.K.
Substance P	Penninsula Laboratories, St Helens,UK.
porcine VIP	Pennsula Laboratories, St Helens,UK

Restriction endonucleases:

Apa I	Promega, Southampton, U.K.
Not I	Gibco GBRL, Renfrewshire, Scotland
Nru	Gibco GBRL, Renfrewshire, Scotland
Spe I	Gibco GBRL, Renfrewshire, Scotland
Xba	Promega, Southampton, U.K.

Cloning Vectors:

pGEMT-Easy Vector	Promega, Southampton, U.K.
pcDNA3.1 ZEO	Invitrogen, De Schelp 12, Netherlands

Methods

Animals

Adult Wistar rats (approximately 250g) were kept on a 12hr light/dark cycle and fed standard laboratory rat chow. Rats were fasted overnight before operations and refeeding experiments but allowed free access to water. Rats were anaesthetized by i.m. injection of a mixture of fentanyl citrate (0.32 $mg.kg^{-1}$), fluanisone (4 $mg.kg^{-1}$) and diazepam (2$mg.kg^{-1}$). Post-operatively, rats were given buprenorphine (0.1$mg.kg^{-1}$) for analgesia, antibiotic prophylaxis (enrofloxacin 5$mg.kg^{-1}$) and 10ml of saline by subcutaneous injection.

Benzalkonium chloride treatment

The stomach was exteriorised through a midline upper abdominal incision. The serosal surface of the region to be denervated was covered with a layer of gauze cut to size. Benzalkonium chloride (0.02%, 0.1%, or 0.5% w/v) dissolved in 0.9% NaCl was painted onto the gauze, which was kept moist with the BAC solution for 3 minutes, and then removed. The serosal surface of the stomach was then rinsed thoroughly with normal saline and returned to the peritoneal cavity and the incision closed in layers. The two faces of the stomach were treated separately; in some experiments only the anterior face of the antrum was treated. A similar method was used for denervation of the corpus employing a strip of gauze (approximately 5mm wide) placed over the corpus alongside the border of the glandular and non-glandular regions. In order to selectively lesion the afferent innervation of the stomach, the same experimental approach was employed using

the sensory neurotoxin capsaicin (1% w/v in olive oil) in place of BAC. Control animals underwent a sham operation with serosal application of normal saline (or olive oil). Animals were allowed to recover for at least 11 days before experiments.

Refeeding experiments

Control and BAC-treated rats were fasted for 24hrs on wire-bottomed cages, and were then either maintained as fasted controls or allowed to refeed for 30 minutes. In some experiments rats were injected subcutaneously 30 minutes before refeeding with either a GRP antagonist BW2258U89 at a dose of 2mg.kg^{-1} (Moody *et al.,* 1995) or with vehicle (0.14M saline). Rats were killed by cervical dislocation, decapitated and trunk blood was taken for assay of plasma gastrin. Samples of antral and corpus mucosa were taken for mRNA extraction (see below) and the stomach content was removed. Gastric retention was determined by the dry weight of solid material in the stomach.

Gastric fistula rats

Rats were treated by application of either BAC or saline to the antral serosa as described above and a small stainless steel Gregory cannula was installed in the corpus as previously described (Dimaline *et al.,* 1986). The cannula was exteriorised through a midline stab incision and the incision closed in layers. Rats began training in Bollman cages on the tenth post operative day.

Gastric emptying studies

Gastric fistula rats were allowed at least 11 days to recover from surgery before experiments. Prior to experiments the stomach was washed with warm 0.14M NaCl to remove solids. On the experimental day, the emptying of test solutions (saline, 50mM HCl, 4.5 % peptone, containing phenol red, 60mg.l^{-1}) instilled into the stomach via the gastric fistula, was determined as previously described (Forster *et al.,* 1990).

Gastric distension studies

Prior to experiments the stomach was washed extensively via the gastric cannula with warm 0.14M NaCl to remove solids. Rats were left for at least 90 minutes before experiments began. Plasma gastrin responses were determined after gastric distension with the following test solutions: 2% (w/v) methyl cellulose (2%MC); 2%MC at pH6 (50mM ammonium acetate); 2% MC with 50mM HCl; 4.5% w/v peptone. In each case the stomach was distended by connecting the cannula to a reservoir of test solution and elevating the reservoir to 5cm H_2O pressure for 30mins. Immediately following distension, rats were killed by cevical dislocation and decapitation and 1.5ml of trunk blood was then taken for gastrin radioimmunoassay. Samples of antrum from control and BAC-treated rats were taken to confrim denervation.

Measurerment of acid output

Rats fitted with a gastric cannula were trained in Bollman cages to permit the required handling of the cannula before acid output could be measured. Prior to experiments, rats were fasted overnight. On the day of experiment the stomach was

washed extensively with warm 0.14M NaCl to remove solids. Rats were left for at least 90 minutes before experiments began. Gastric acid output was determined by collecting gastric secretion over four sequential 15 min periods and titrating to pH 7.0 with 20mM NaOH using a pH autotitrator as previously described (Dimaline, *et al.,* 1986).

Omeprazole treatment

Rats were given omeprazole (400µmol.kg^{-1}) by gavage under light Halothane anaesthesia. Rats were fed *ad libitum* and received omeprazole daily for five days. On the fifth day rats were killed by cervical dislocation and decapitation and 1.5ml of trunk blood was taken for gastrin radioimmunoassay. Samples of gastric corpus were taken for total RNA extraction (see below).

Radioimmunoassay

The neuropeptide content of tissues was determined after extraction with boiling water (0.1 g.ml^{-1}), homogenizing and centrifuging and re-extraction of the pellet with 0.5M acetic acid, as previously described (Hutchison *et al.,* 1981). Water or acid extracts were assayed at the appropriate dilution for the following peptides: Gastrin releasing peptide (GRP) was assayed using antibody 1078, with porcine GRP 14-28 as standard and iodinated Tyr^{1} COOH-terminal decapeptide of bombesin as label, as previously desribed (Dockray *et al.,* 1979). Substance P was assayed with antibody L83, using undecapeptide substance P as standard and ^{125}I-Bolton Hunter labelled substance P as label. Vasoactive intestinal polypeptide (VIP) was assayed using antibody L25, with porcine VIP as standard and ^{125}I labelled VIP as label as previously described (Hutchison

et al., 1981). Calcitonin gene related peptide (CGRP) was assayed with antibody L273, using rat Tyr^0a-CGRP 28-37 as standard and the same peptide labelled with ^{125}I as label as previously described (Varro *et al.,* 1988). The concentration of amidated gastrins in plasma gastrin was assayed using antibody L-2, using synthetic human non-sulphated G-17 as standard and for labelling with ^{125}I as previously described (Dockray *et al.,* 1991). The standard conditions for each assay are shown in Figure 2.1

Extraction of RNA

Extraction of Tissue RNA:

Tissue mRNA was extracted and quantified as described previously (Dimaline *et al.,* 1991). Samples of antral or corpus mucosa were homogenised in 3mls of 4M guanadinium isothiocyanate buffer containing 25mM sodium acetate pH 6.0 and 0.84% (vol/vol) β-mercaptoethanol and frozen at -80°C until required. After thawing at room temperature, samples were pipetted onto a 5.7M caesium chloride cushion (1.3ml) in an ultracentrifuge tube. Samples were centrifuged overnight at 41,100 rpm to pellet the RNA. The mRNA pellet was dissolved in 300µl of Tris-EDTA (TE) buffer with 0.2% SDS and preciptated with 0.3M sodium acetate under 100% ethanol overnight.

Extraction of Cell RNA

RNA extraction from cultured cells was performed using the Quickprep™ total RNA extraction kit (Phaarmaciaa Biotech, Herts, U.K.) according to the manufacturers instuctions. The media (2ml) was removed from cultured cells and replaced by 900µl of a mixture of lithium chloride buffer, β-mercaptoethanol and extractoin buffer. Cells were

Peptide Assay Antibody/Dilution	BUFFER	INCUBATION	SEPARATION Ch:Dx:MPr*
GASTRIN **L2 - 1:100,000**	Na^+Bicarbonate pH 8.4, 0.5% Bovumin, 10mM Na^+ Azide	72hrs : 4 °C	10 : 1 : 0.5
CGRP **L271 - 1:80,00**	50mM PO_4, pH 7.4 0.5% Bovumin, 0.25% EDTA, 0.14M NaCl	72hrs : 4 °C	10 : 1 : 1
GRP **1078 - 1:80,000**	Na^+Bicarbonate pH 8.4, 0.5% Bovumin, 10mM Na^+ Azide	48hrs : 4 °C	10 : 1 : 0.5
SUBSTANCE P **L-83 - 1:400,000**	50mM PO_4, pH 7.4 0.5% Bovumin, 0.14M NaCl, 10mM Na^+ Azide	48hrs : 4 °C	10 : 1 : 0.5
VIP **L-25 - 1:40,000**	20mM PO_4, pH 6.0 0.4% BSA, 0.1% EDTA 10mM Na^+ Azide 0.025% w/v polybrene	48hrs : 4 °C	10 : 1 : 0.5

Fig 2.1 Conditions for each radioimmunoassay are provided in the table. *For separation of the assay a mixture of charcoal dextran and milk protein (Marvel) in the above ratio (g/100ml) was added to each tube.

resuspended in the solution by scraping and then pipetted into a universal container. The cells were then homogenised and the lysate transferred to a 1.5ml eppendorf containing 600µl of caesium trifluoroacetate solultion and left to stand on ice for ten minutes. RNA was pelleted by centrifugation at 15000rpm for 15 minutes in a microcentirfuge. The RNA pellet was washed thoroughly, allowed to dry and resuspended in 10-20µl of diethyl pyrocarbonate-treated (DEPC) water before quantification. Any unused RNA was preciptated with 0.3M sodium acetate under 100% ethanol and stored at -80°C.

RNA electrophoresis

Stored precipitated RNA was pelleted by centrifugation at 13000rpm for 15 minutes, washed twice in 70% ethanol/DEPC H_2O and once in 100% ultrapure ethanol. Pellets were air dried at room temperature and dissolved in 10-50µl of DEPC H_2O. RNA samples were quantified by spectrophotometer and 10µg aliquots added to denaturing buffer (formamide: 37%formaldehyde: 10 x MOPS (3-(4-Morpholino) propane sulfonic acid) in ratio 4:1.75:1 and heated to 55 °C for 15 minutes. Samples were then placed on ice and 2µl of RNA loading buffer added to each sample. The denatured RNA was then electrophoresed in a 1x MOPS/2% formaldehyde 1% agarose gel.

Northern Blot and Hybridisation

After electrophoresis, RNA was electroblotted to nylon membranes (Nytran 0.5) overnight in 0.5% TAE buffer (containing 0.2M *Tris* base, 1M acetate and 0.1MEDTA) at 4 °C. RNA was crosslinked to the nylon membrane by ultraviolet light (UV Cross Linker, Hoeffer scientific intstruments, San Fransisco, USA). Membranes were placed in

hybridisation buffer (50% formamide containing 5 x Denhardts solution, 5 x sodioum chloride sodium phosphate EDTA buffer (SSPE), 0.5% SDS and sonicated salmon sperm DNA (200μg.ml^{-1}) and warmed to 65 °C for 4 hours prior to the addition of ^{32}P-labelled cRNA probe (2x10^6 cpm.ml^{-1}). Membranes were hybridised overnight and then washed twice with 2xSSPE containing 0.1% SDS at room temperature for 20min, and once with 0.1xSSPE/0.1% SDS at 65^{0}C for 20min. Signals were removed from membranes using boiling 0.1% SDS and membranes rehybridized with alternative specific probes as required. Signals were quantified using a Phosphor Imager.

Reverse transcription/PCR

Reverse transcription of RNA was performed by first annealing 1μl oligo dTs (500μg.ml^{-1}) to ≈50μg of total RNA by heating to 55^{0}C for 5minutes and cooling slowly. cDNA was generated by incubating the following reaction at 42°C for 1 hour.: oligo dT and RNA (10μl), 2μl DTT(100mM), 1μl dNTPs (25 mM), 1μl RNAsin, 4μl 5x AMV RT Buffer and 2μl AMV reverse transcriptase. The cDNA generated was purified by phenol/chloroform extraction, precipitated with 0.5M ammonium acetate and washed with 100% ethanol. The cDNA was then resuspended in 100μl of TE. 5μl of this solution was used as template for PCR.

Human *reg* cDNA was generated by reverse transcription of total RNA extracted from gastric mucosal biopsies taken from either normal corpus or from carcinoid nodules. Two different size products were generated by separate pairs of primers chosen to include either a 297bp central portion of the coding region

containing an *EcoR* I restriciton site, or the full length coding sequence. The smaller fragment was used as template for riboprobe synthesis (see below) and the larger fragment (557bp) used for cloning and sequencing (see below). Primers were chosen based on the full coding sequence of the published human *reg* mRNA (Terazono *et al.,* 1988) as follows: The 297 bp fragment: sense - TCTCCTGCCTGATGTTTCT GTCTC antisense - GGCGGTTCTTTTTGGGGTCAT, The 557bp fragment: sense GATTGTTGATTTGCCTCTTA, antisense - TCCAGCTGCCTCTAGTTTTTGAA. Rat *reg* cDNA was generated by reverse transcription of total RNA extracted from rat pancreas. PCR primers were chosen to include the full coding sequence of the published rat *reg* mRNA (Terazono *et al.,* 1988) as follows: sense - AGCCTGC AGAGATTGTTGACTTG; antisense - AGGGGGTTGACTTTGCTTTTGATA and gave a predicted product size of 651bp (Figure 2.2). Restriction digestion of the 651bp PCR product with *Eco*R1 cleaved the product into 253 & 398bp fragments.

Polymerase chain reaction was performed under standard conditions in a final volume of 100µl containing 10x *taq* polymerase buffer (10µl), MgCl (1.5mM), dATP (0.2mM), dCTP (0.2mM), dGTP (0.2mM), dTTP (0.2mM), template cDNA (5µl), sense and antisense primers (5ng. μl^{-1}) and *taq* polymerase (0.5µl). 60µl of mineral oil was layered over the reaction mixture which was then placed into a thermal cycler (Hybaid, Middlesex UK). After a 5minute denaturation at 95°C, thirty cycles consisting of annealing (1 minute at 58 °C), extension (2 minutes at 72°C) and denaturation (1 minute at 95°C) were performed. A final extension step (72 °C) was performed for ten minutes before determining the outcome by agarose gel elecrophoresis.

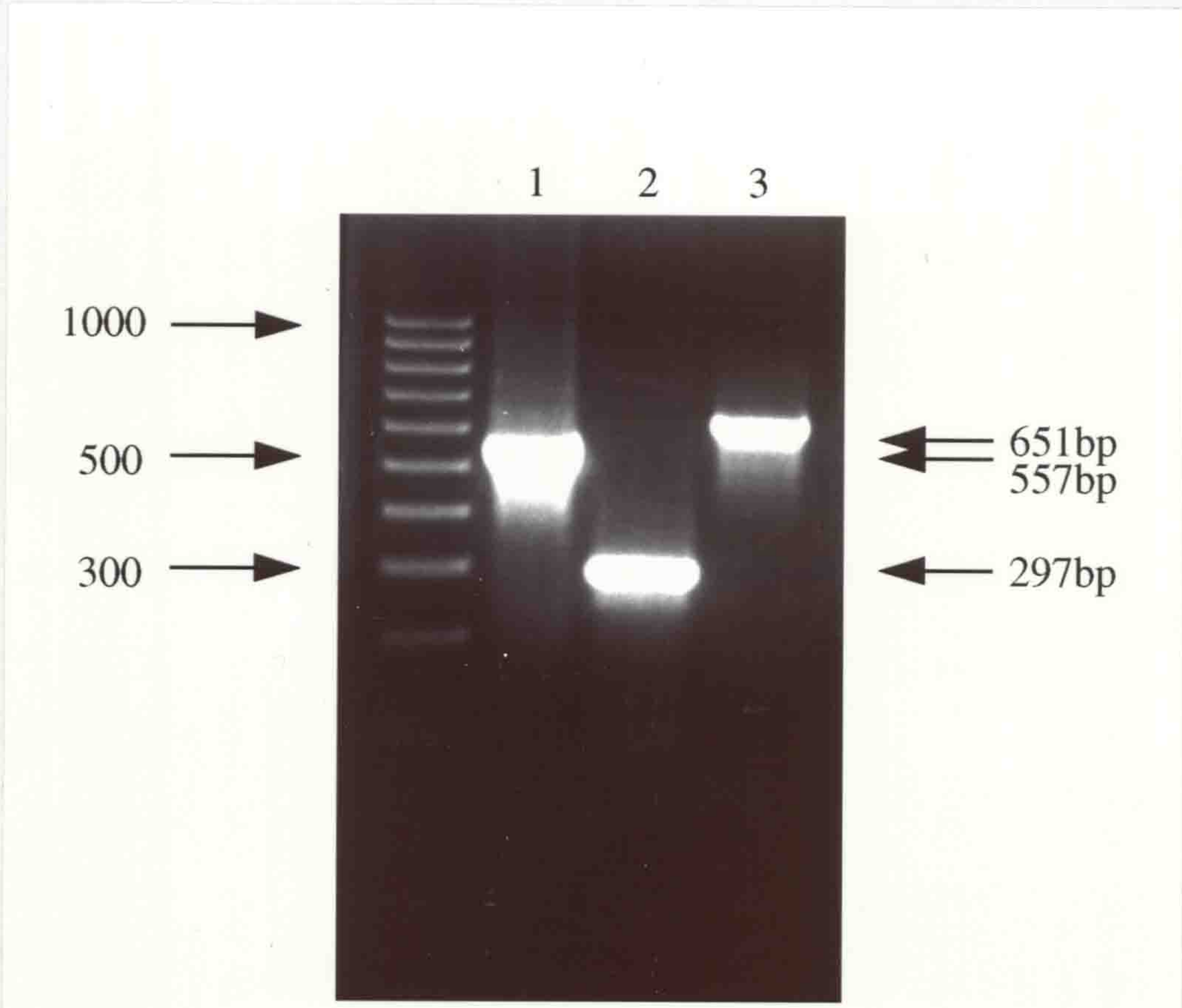


Figure 2.2 PCR products of human and rat *reg* cDNA using human carcinoid tumour RNA and rat pancreatic RNA as template for reverse transcription. The predicted sizes of the human PCR products were 557bp (lane 1) and 297bp (lane 2) depending on the primers used. The predicted size of the rat PCR product was 651bp (lane 3). See text for full details.

DNA Ligation

The PCR fragments of human and rat *reg* cDNA were ligated into the pGEM-T Easy vector by incubating approximately equimolar concentrations of PCR product and vector in the presence of DNA ligase at 4°C overnight according to the maufacturers instructions.

Cloning and Sequencing

Bacterial Transformation: Chemically competent JM109 bacterial cells were stored at -80°C. Prior to transformation, cells were thawed on ice for 20minutes before addition of 2µl of 8.7M β-mercaptoethanol and 1µl of DNA ligation mixture. Incubaton on ice was continued for a further 30 minutes. The cells were then heated to 42 °C for 45 seconds and immediately placed on ice for a further 2minutes. Thereafter, 450µl of SOC medium (containing 2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM Kcl, 10mM $MgCl_2.6H_2O$ and 20mM glucose) was added and the reaction incubated at 37°C for one hour with constant gentle agitation.

After this incubation a 100µl aliqout was spread onto petri dishes containing 20mls of LB/agar (1% Tryptone, 0.5% Yeast extract, 1.0% NaCl, 1.5% agar) that had been treated previously with 100µl isopropyl-β-D-thiogalactosidase (IPTG 0.1 M), 30µl 5-bromo-4-chloro-3indolyl-β-D-galactosidase (XGAL 0.2 M) and 200µl of ampicillin ($10mg.ml^{-1}$). The remaining cell suspension was pelleted by centrifugation (5000rpm for 3 minutes) and the cells resuspended in 200µl of SOC medium. A further 100µl aliquot

of cells, now at a higher cell density, was plated onto a LB/agar plate. Plates were then incubated overnight at 37°C and selection of successfully transformed colonies was by colour selection and antibiotic resistance. Successfully transformed colonies (white) were sub-cloned onto a second LB/agar plate and into 3ml of LB medium containing ampicillin ($0.05mg.ml^{-1}$). Following a further overnight incubation at 37 °C, the amplified plasmid DNA was extracted and purified for restriction digestion (to confirm presence of the expected cDNA sequence insert) and sequencing.

Plasmid DNA extraction Small, medium and large scale plasmid DNA extraction was performed using commercially available kits according to manufacturers instructions (Wizard Minipreps, Midipreps and Maxipreps). The protocol is similar for each with adjustments for scale depending on the yield of plasmid DNA required. In general, small scale preperations were sufficient for sequencing purposes. Medium scale preparations were used to generate templates for cRNA probe synthesis and large scale preparations were used for generating linearised plasmid DNA for permanent cell transfection (see below).

Following overnight culture at 37 °C, bacterial cells were pelleted by centrifugation, the supernatant discarded and the cells resusupended in resuspension buffer. The cells were then lysed by additon of lysis solution (NaOH/SDS) and protein and bacterial DNA were precipitated by addition of a neutralising solution (potassium acetate). The precipitate was removed by centrifugation and the plasmid DNA extracted from the supernatant by additon of a high affinity resin. This resin with bound DNA was

immobilized to a column by vacuum suction. The column was then washed twice (80% ethanol) and the DNA eluted by addition of TE buffer (68 °C) followed by centrifugation with collection of the eluate. The DNA content and purity was measured by spectrophotometry.

Riboprobe synthesis

Templates for generating cRNA probes for gastrin, HDC, somatostatin and GAPDH were provided by Dr R. Dimaline and contained the appropriate template as described previously (Dimaline *et al.,* 1991; 1993a; 1994). The template for generating the cRNA probes for rat and human *reg* were prepared by restriction fragment digestion of midiprep purified pGEM t-Easy vector containing the full length rat *reg* sequence or the 253bp human *reg* sequence as described above. After sequenceing the vector to determine orientation of the insert, 10µg of each sample was digested with restriction enzyme *ApaI* in approriate enzyme buffer in a volume of 20µl for 3hours at 37^0C. Complete linearisation of plasmid DNA was confirmed by gel electrophoresis. 0.5µl of template was used for riboprobe synthesis. The reaction conditions were as follows: 2µl 5x SP6 buffer, 1µl DTT(10mM), 2.5µl DNTPs (100µM each ATP, UTP, GTP), 1µl CTP (10µM), 0.5µl RNAse, 0.5µl template DNA, 0.5 µl RNA polymerase (1-5units) and 2.5µl ^{32}P-CTP (≈ 5µM). The reaction mixture was incubated at 37^0C for 1 hour and then RNAse free DNAse added for a further 15 minutes. The synthesised probe was purified by size exclusion and 1µl of purified probe placed in 5ml of scintillation fluid and

incorporated radioactivity counted. Labelled probe was used at a concentration of $2x10^6$ cpm per ml of hybridisation buffer.

Preparation of DNA for permanent transfection

Two sequences of the human *reg* gene were chosen for permanent transfection into the hamster insulinoma tumour cell (HIT) line. These were the full length coding sequence of the normal (wild type) *reg* and the sequence in which the first methionine residue (initiator methionie) had been mutated to a valine (ATG→GTG). Representative clones contaning each sequence were prepared by small scale DNA extraction (Miniprep). The purified plasmid DNA (>600ng in 15µl) was then restriction digested with the combined endonucleases *Spe*I and *Not* I at 37 °C for 3hours. The restriction fragment containing the *reg* sequence was then purified by gel electrphoresis (1.5% agarose). The DNA band of correct size (≈ 600bp) was cut from the gel and DNA removed from the gel onto a column by centrifugation (13,000rpm for 60seconds), and eluted from the column by the additon of 20µl sterile water followed by centrifugation at 13,000rpm for 20seconds. A small aliqout of the DNA was again gel electrophoresed to assess purity and concentration ($20ng.\mu l^{-1}$).

Each restricition fragments was then ligated into the mammalian expression vector pcDNA3.1 Zeo. The vector circular DNA was linearised by concomittant digestion with the restriction endonucleases *Xba* and *Not* I. (The *Xba* restriction leaves an identical sequence overhang to that of *Spe* I used to create the insert DNA). Complete digestion by both enzymes was presumed by serial examination of control vector digests

by each restriciton endonuclease separately. After 4hours, the linearised vector was gel purified and eluted. The concentration of vector and its purity was estimated by gel elctrophoresis.

Ligation of the wild type and mutant *reg* inserts was acheived by additon of equimolar concentrations of insert and vector DNA in the presence of DNA ligase and appropriate buffer. The ligation reaction was incubated overnight at 14 °C. In addition a control ligation reaction was also performed to ensure that complete digeston by both endonucleases occurred whilst preparing the vector DNA. This consisted of linearised vector without any insert. As the *Xba* and *Not* I restriciton site overhangs are incompatible religation should not occur without the presence of insert DNA. Hence no colonies should form with cloning of the control ligation. Conversely, any colonies present from the *reg* insert ligations should all be positive for insert DNA. This selection process was required as the pcDNA3.Zeo vector does not incorporate the lac operon gene that allows for colour selection.

Following cloning into JM109 cells, colonies from each of the wild type and mutant *reg* transformations were selected for small scale DNA preparation. The presence of a *reg* insert was shown by restriction digestion with *EcoR*I and then confirmed by sequencing. Positive clones for each sequence were then processed for large scale DNA preparation to yield ≈ 300µl of concentrated DNA (3-5µcg.µl^{-1}). For each permanent transfection, 10µg of linearised DNA(in <20µl) was required. Therefore the appropriate volume of each vector containing wild type and mutant *reg* inserts were linearised by

digeston using the restriction endonuclease *Nru*. Linearised DNA was incorporated into cultured HIT cells by electroporation (see below).

Tissue culture and permanent transfection

The hamster insulinoma cell line HIT T15 was grown in RPMI 1640 medium supplemented with 11mM glucose, 10% v/v foetal calf serum, 2.5ug.ml^{-1} fungizone, 100U.ml^{-1} penicillin and 100µg.ml^{-1} streptomycin (all from GIBCO BRL, Life Technologies, Paisley, Scotland). Stably transfected cell lines expressing wild type or mutant human *reg* were produced by electroporation of 5x10^{6} cells in 0.8ml at 200V and 1070µF in incomplete medium containing 10µg of linearised pcDNA *reg* construct. Cells were grown in complete RPMI in 10cm dishes for 48 hours prior to selection with zeocin (750µg/ml^{-1}) (Invitrogen). Six colonies expressing each construct were cloned and screened for *reg* expression by northern blot.

The rat pancreatic cell line AR42J was grown in RPMI 1640 medium supplemented with 10% v/v foetal calf serum. For experiments, cells were plated out at 1.5x10^{6} cells in 5ml culture medium / 60ml dish and grown at 37°C/ 5% CO2. Following a 24 hour growth period, the cells were washed and their media replaced with incomplete RPMI with the following stimulii or appropriate vehicle (control). Human gastrin-17 (HG17), rat gly-extended gastrin-9 (G-Gly) in 50mM ammonium bicarbonate, wortmannin, mouse salivary gland extract (EGF) in RPMI, L740093 in 1x10^{-6}% DMSO. In all cases except for wortmannin, the cells were incubated for 24

hours at 37°C / 5%CO_2 prior to RNA extraction. In the case of wortmannin, the cells were incubated for 4 hours prior to RNA extraction.

Statistical analysis

The statistical test used for each experiment is indicated in the appropriate figure legend. In general, for the experiments involving denervation, the data was analysed using Mann Whitney U test and for other analyses Student's t test was used.

Chapter Three

Chemical denervation of antral neurons

Introduction

The gastric emptying of liquids is dependent on the pressure gradient between the lumen of the stomach and proximal duodenum, and the resistance to flow between the two (Mayer *et al.,* 1994). It has long been known that the rate of emptying depends on the nature of the luminal contents; thus, acid, hyperosmolal solutions, high viscosity, protein- and fat-rich meals all delay gastric emptying (Mayer *et al.,* 1994). The emptying of liquids is pulsatile in nature and this can be attributed to phasic contraction of the pyloric antral region of the stomach (Houghton *et al.,* 1988; Malbert *et al.,* 1991; Prather *et al.,* 1993). Alterations in the flow of gastric content across the antrum can be effected by changing either resistance to flow generated by antral contractions or the overall tone of the gastric body and the duodenum. There is a significant body of evidence to indicate the importance of vagal afferent innervation of the proximal gut (Raybould *et al.,* 1988; Forster *et al.,* 1990; Holzer *et al.,* 1994) and vagal efferent innervation of the gastric corpus (Forster *et al.,* 1991; D'Amato *et al.,* 1992) in regulating delayed gastric emptying by inducing gastric corpus relaxation in response to hyperosmolal, protein-rich or fat-rich solutions in the duodenum. However, much less is known of the role of intrinsic antral neurons in the control of pyloric flow.

Antral neurons however are known to innervate both the muscle layers of the antrum and the mucosal epithelial cells including endocine cells (Gabella, 1987; Furness and Costa, 1987; Ekblad *et al.,* 1987). The clearest example of this and the most relevant in terms of digestive physiology of the stomach is the innervation of G-

cells of the gastric antrum by GRP-immunoreactive neurons that have their cell bodies in the myenteric plexus (Dockray *et al.,* 1979; Miller *et al.,* 1989). Systemically administered GRP has been shown to stimulate gastric acid output by the release of gastrin from G-cells in both animals and man (Varner *et al.,* 1981; Bunnett *et al.,* 1985). Further evidence that GRP exerts physiologically regulates the G-cell comes from the demonstration that G-cells express GRP receptors (Vigna *et al.,* 1990) and GRP is capable of sitmulating the release of gastrin from G cells in primary culture (Giraud *et al.,* 1987).

The experiments described in this chapter were undertaken to define the anatomical lesion induced by chemical denervation of the rat antrum. This would then allow potential study of the role of the antral innervation in gastric emptying and the regulation of mucosal endocrine cell function. To this end I used the detergent benzalkonium chloride (BAC) which has already been used to lesion small intestinal myenteric neurons (Fox *et al.,* 1983; Dahl *et al.,* 1987). In keeping with the observations of others (Fox *et al.,* 1983; Dahl *et al.,* 1987) I was able to demonstrate that BAC application to the rat stomach produces a highly specific, reproducible and permanent loss of the all neurons in the treated region. Following antral denervation, the intrinsic innervation of the corpus is spared, but there was loss of the spinal afferent innervation of the gastric corpus. Conversely, when a band of proximal gastric corpus was denervated, intrinsic and extrinsic antral innervation was preserved.

Methods

All operations were performed on Wistar rats. For antral denervation rats were starved overnight, but for corpus denervation rats were fed *ad libitum*. Following exposure of the stomach, BAC (0.02%, 0.1%, or 0.5% w/v) dissolved in 0.9% NaCl was painted onto gauze, which was kept in contact with the region to be treated and kept moist with the BAC solution for 3 minutes, and then removed. The two faces of the stomach were treated separately; in some experiments only the anterior face of the antrum was treated. In order to selectively lesion the afferent innervation of the stomach, the same experimental approach was employed using the sensory neurotoxin capsaicin (1% w/v in olive oil) in place of BAC. Control animals underwent a sham operation with serosal application of vehicle (normal saline or olive oil). Animals were allowed to recover for at least 11 days before experiments. Denervation was assessed by radioimmunoassay of neuropeptides in extracts of BAC-treated and adjacent tissues.

Results

Dose-Response Relationship.

Initial experiments were directed at determining the optimal dose of BAC for antral denervation. The tissue concentrations of GRP, CGRP, substance P and VIP measured by radioimmunoassay of antral extracts were reduced in a dose dependent manner 11 days after prior administration of BAC (Fig 3.1). At a concentration of 0.02% BAC had no effect; 0.1% BAC produced a 25% loss of CGRP, but following 0.5% w/v BAC all neuropeptides were depleted. The concentrations of substance P, VIP and GRP in extracts of the gastric corpus were not affected by prior application of BAC to the antrum (Fig 3.2). However, the concentration of CGRP in the corpus was significantly reduced by application of 0.5% BAC to the antrum. (Fig 3.2)

Time Dependency of Responses

In order to determine the duration of responses to 0.5% BAC, neuropeptide concentrations were examined in extracts of tissues sampled 11 to 25 days after treatment. All neuropeptides assayed were depleted in antral extracts 11 days after BAC, and remained substantially reduced at all times up to 25 days (Fig 3.3). Similarly there was loss of CGRP in both antrum and corpus in rats treated with BAC on the antrum at all times examined (Fig 3.4).

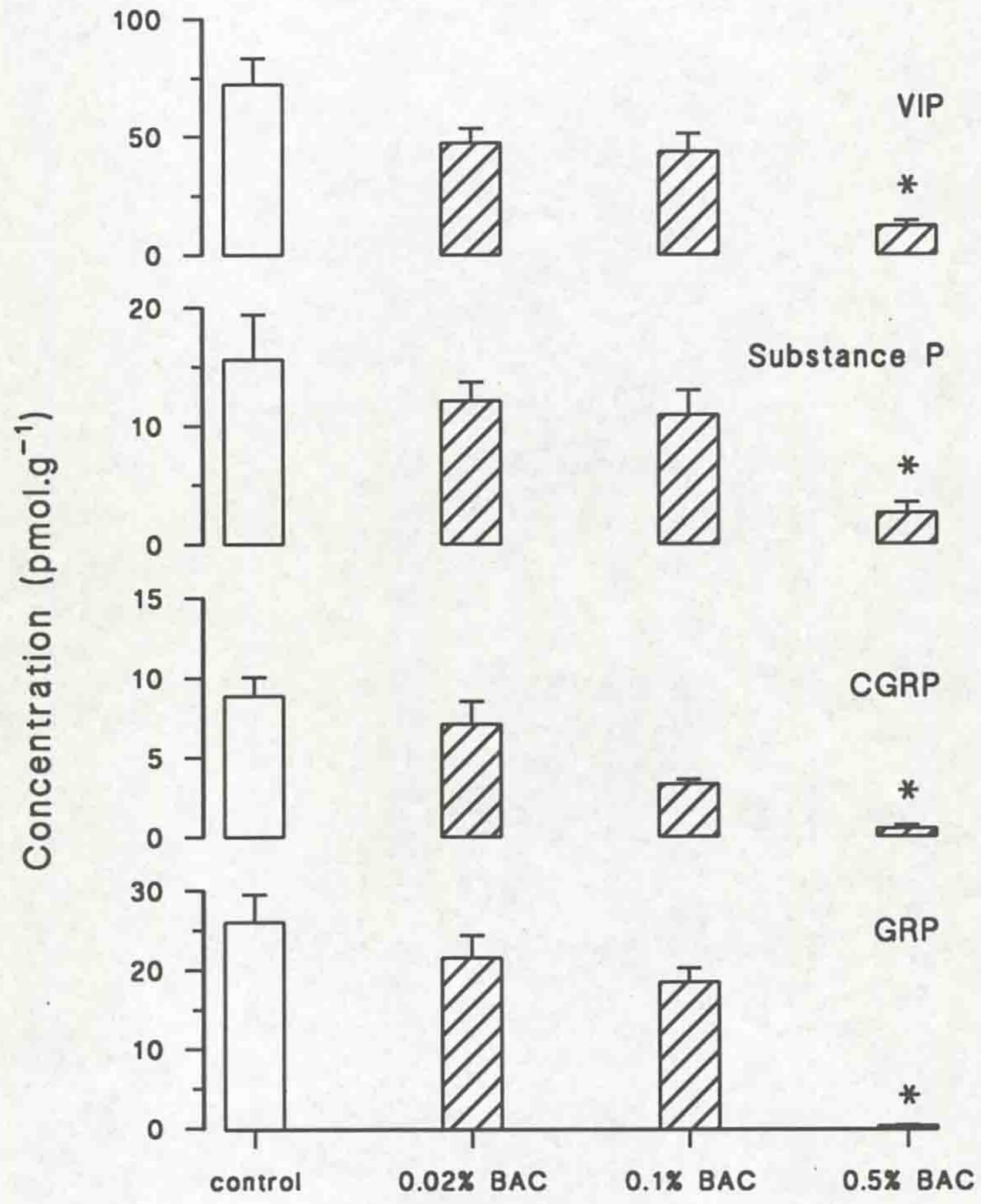


Fig 3.1 Neuropeptide concentrations (CGRP, GRP, substance P and VIP) in extracts of antrum following serosal application to the antrum of BAC or vehicle (control). Only at 0.5% w/v BAC was there a significant loss of neuropeptides derived from both intrinsic and extrinsic afferent neurons of the antrum. (n = 6 all groups; * $p<0.05$, compared with control, Mann Whitney U test; in this and subsequent figures, values are means ± SEM).

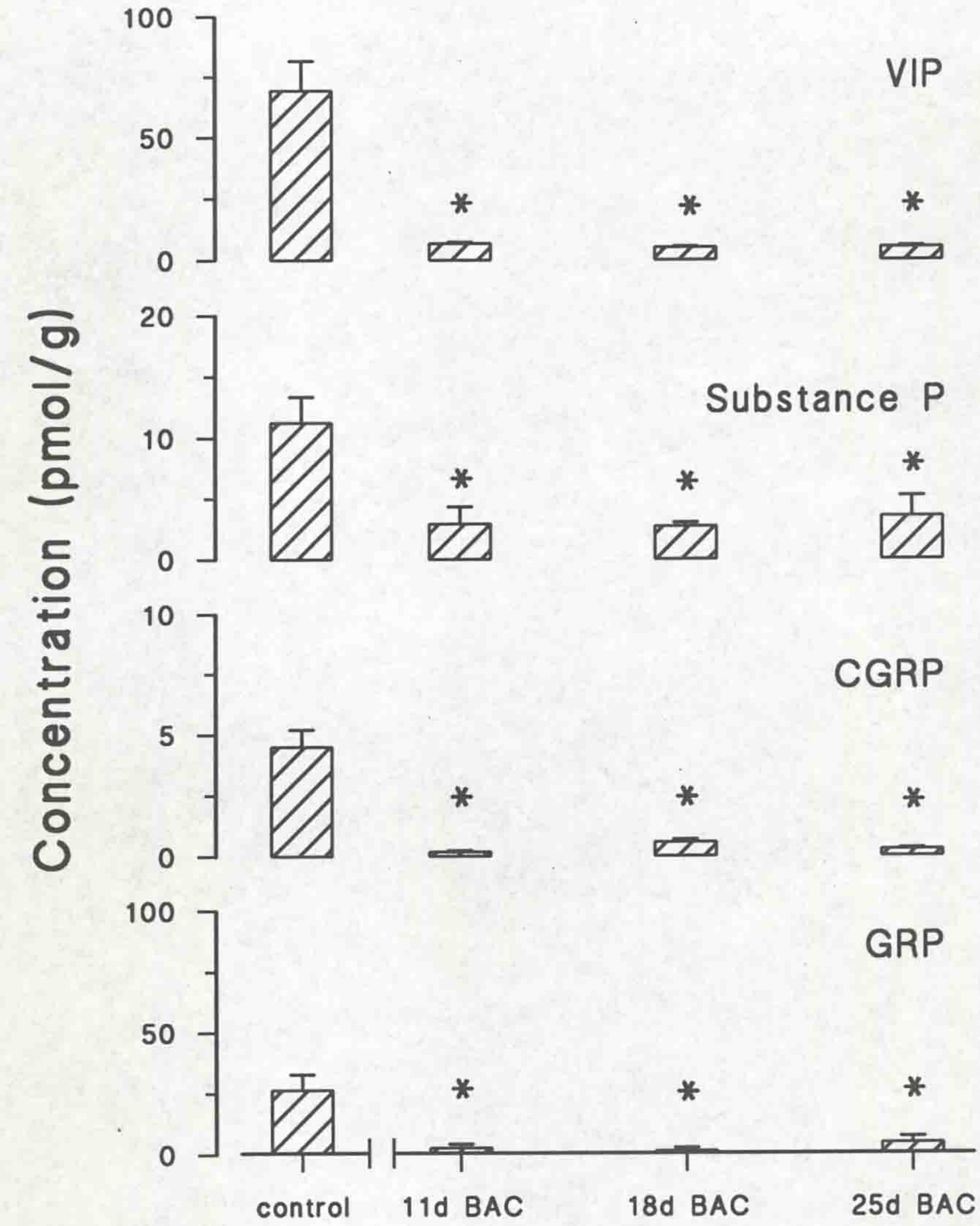


Fig 3.2 Neuropeptide concentrations (CGRP, GRP, substance P and VIP) in extracts of the antrum 11 (n=5), 18 (n=5) and 25 (n=3) days after application of 0.5% w/v BAC or vehicle (control, n=6) to the serosa of the antrum. The loss of all neuropeptides was complete at day 11 and unchanged thereafter. (* $p<0.05$, compared with control, Mann Whitney U test)

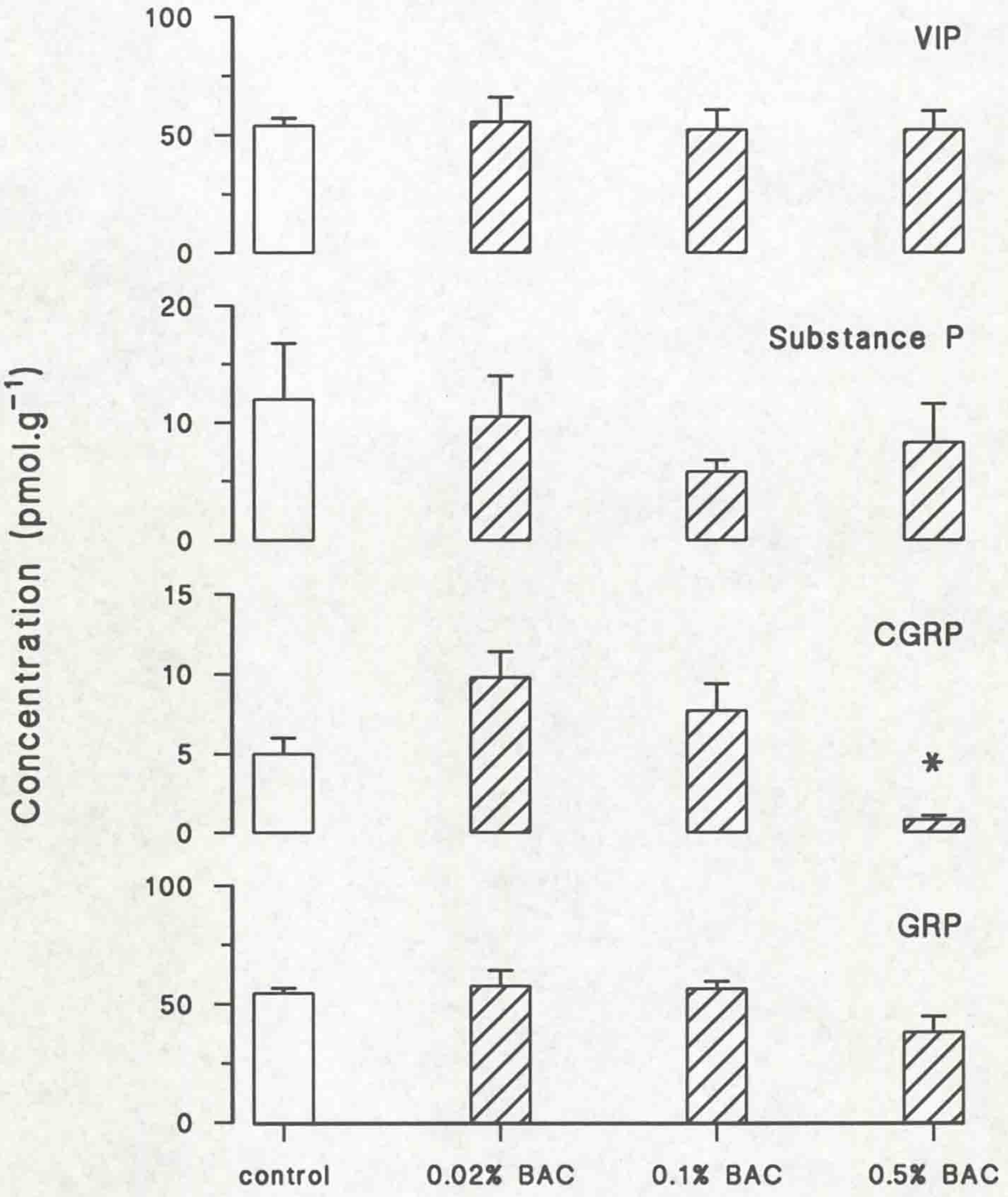


Fig 3.3 Neuropeptide concentrations (CGRP, GRP, substance P and VIP) in extracts of corpus following serosal application to the antrum of BAC or vehicle (control). Even at 0.5% w/v BAC there was no significant loss of neuropeptides derived from intrinsic neurons of the Corpus. However at 0.5% *w/v* BAC there is a significant loss of corpus afferent innervation as shown by the significant reduction in corpus CGRP levels. (n = 6 all groups; * $p<0.05$, compared with control, Mann Whitney U test)

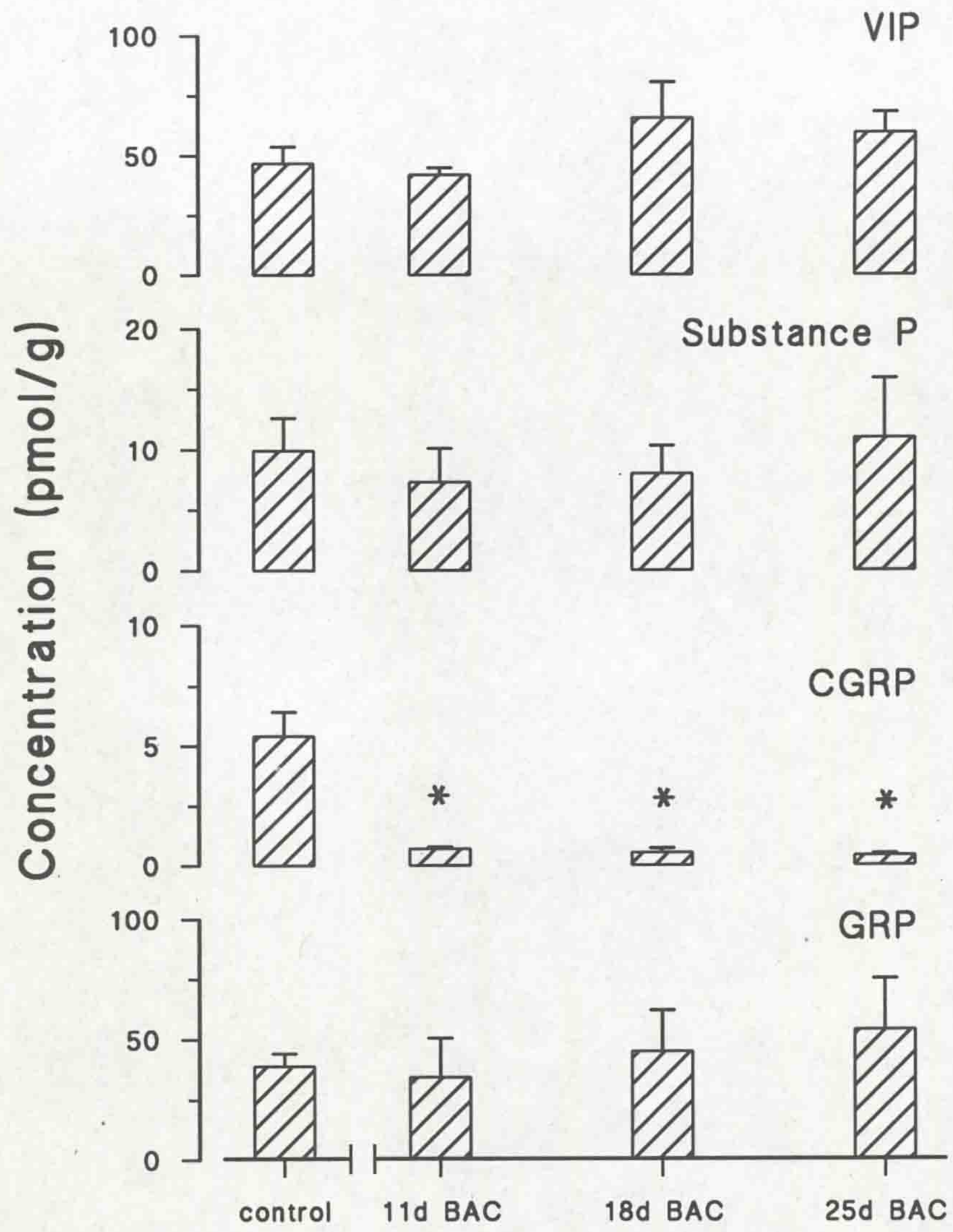


Fig 3.4 Neuropeptide concentrations (CGRP, GRP, substance P and VIP) in extracts of corpus 11 (n=5), 18 (n=5) and 25 (n=3) days after application of 0.5% w/v BAC or vehicle (control, n=6) to the serosa of the antrum. As with the results for the antrum, the selective loss of corpus CGRP after antral BAC was complete at day 11 and unchanged thereafter (* $p<0.05$, compared with control, Mann Whitney U test)

Specificity of Lesion

To examine the specificity of the effects described above, we studied the effect of BAC applied to a circumferential band (approximately 5mm) of the gastric corpus immediately distal to the non-glandular part of the stomach. In the BAC-treated region there was substantial depletion of all the neuropeptides studied (Fig 3.5). In the untreated corpus, and in the antrum, the tissue concentrations of VIP, substance P, CGRP and GRP were similar to control (Fig 3.5). It is worth emphasising that following BAC treatment of the corpus, antral CGRP was unaffected. Given that CGRP is present only in spinal afferent neurons, this result seemed to indicate a specific lesion in the corpus projections of these neurons induced by antral BAC treatment. To confirm this, the effects of the sensory neurotoxin capsaicin applied in a similar fashion to BAC, were examined as this neurotoxin is known to lesion only small diameter afferents leaving intrinsic innervation unaffected. Serosal application of 1% w/v capsaicin to the antrum, produced complete loss of CGRP in extracts of antrum and corpus (Fig 3.6). In contrast, neuropeptides found in intrinsic neurons (VIP and GRP) were not changed by treatment of the antrum with capsaicin (Fig 3.6).

Finally, in some experiments BAC was applied to a single side of the antrum. In this case, there was depletion of neuropeptides on the treated side as already noted, but the concentrations in the untreated side of the antrum were similar to controls (Fig 3.7).

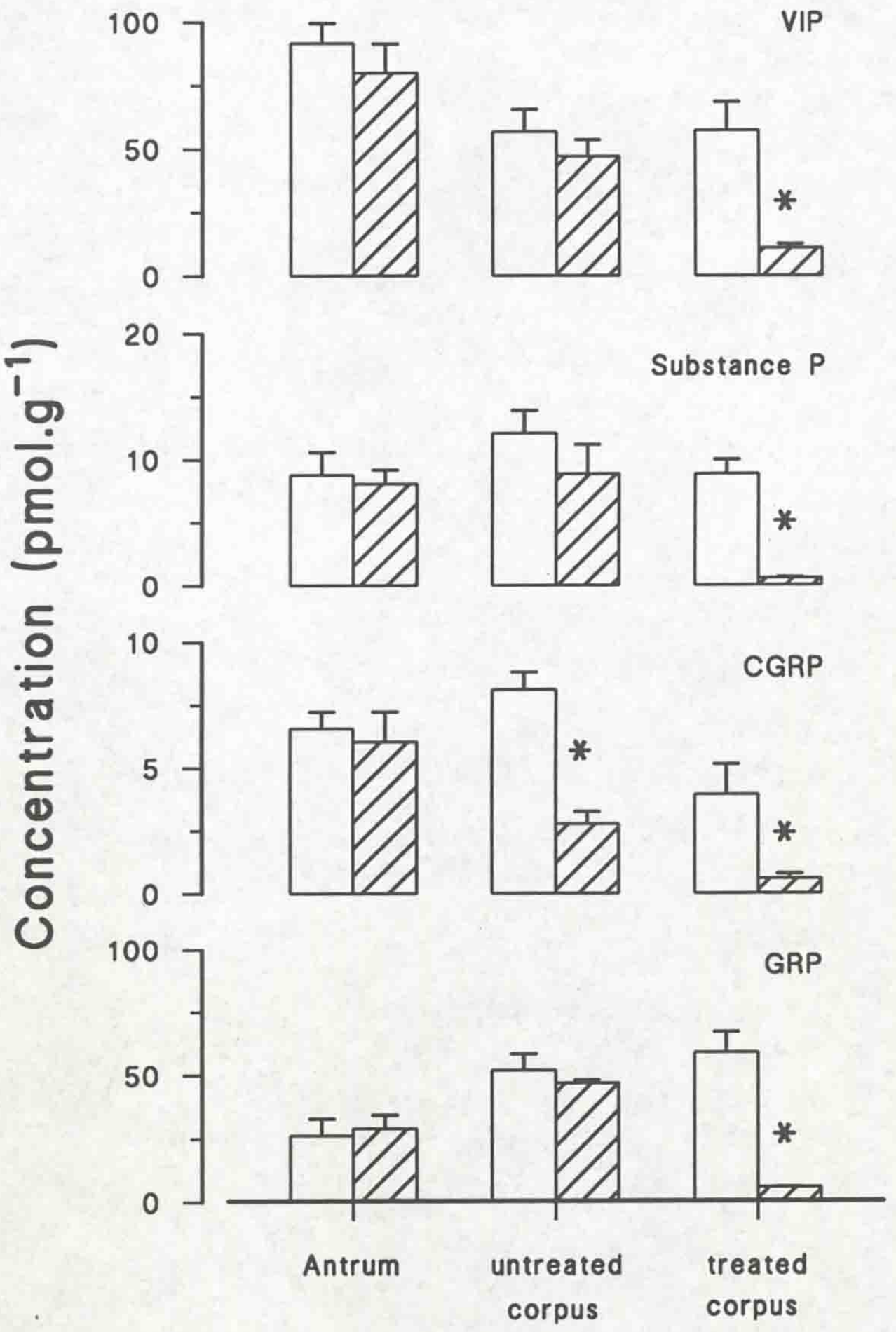


Fig 3.5 Neuropeptide concentrations (CGRP, GRP, substance P and VIP) in extracts of antrum, control corpus and a band of proximal corpus treated with either 0.5% BAC (hatched bars) or vehicle (open bars). There was complete loss of the neuropeptides in the band of corpus exposed to BAC but not in the remainder of the corpus, or the antrum. Note in particular the preservation of CGRP in the antrum. (* $p<0.05$, compared with vehicle, Mann Whitney U test; $n = 6$ all groups).

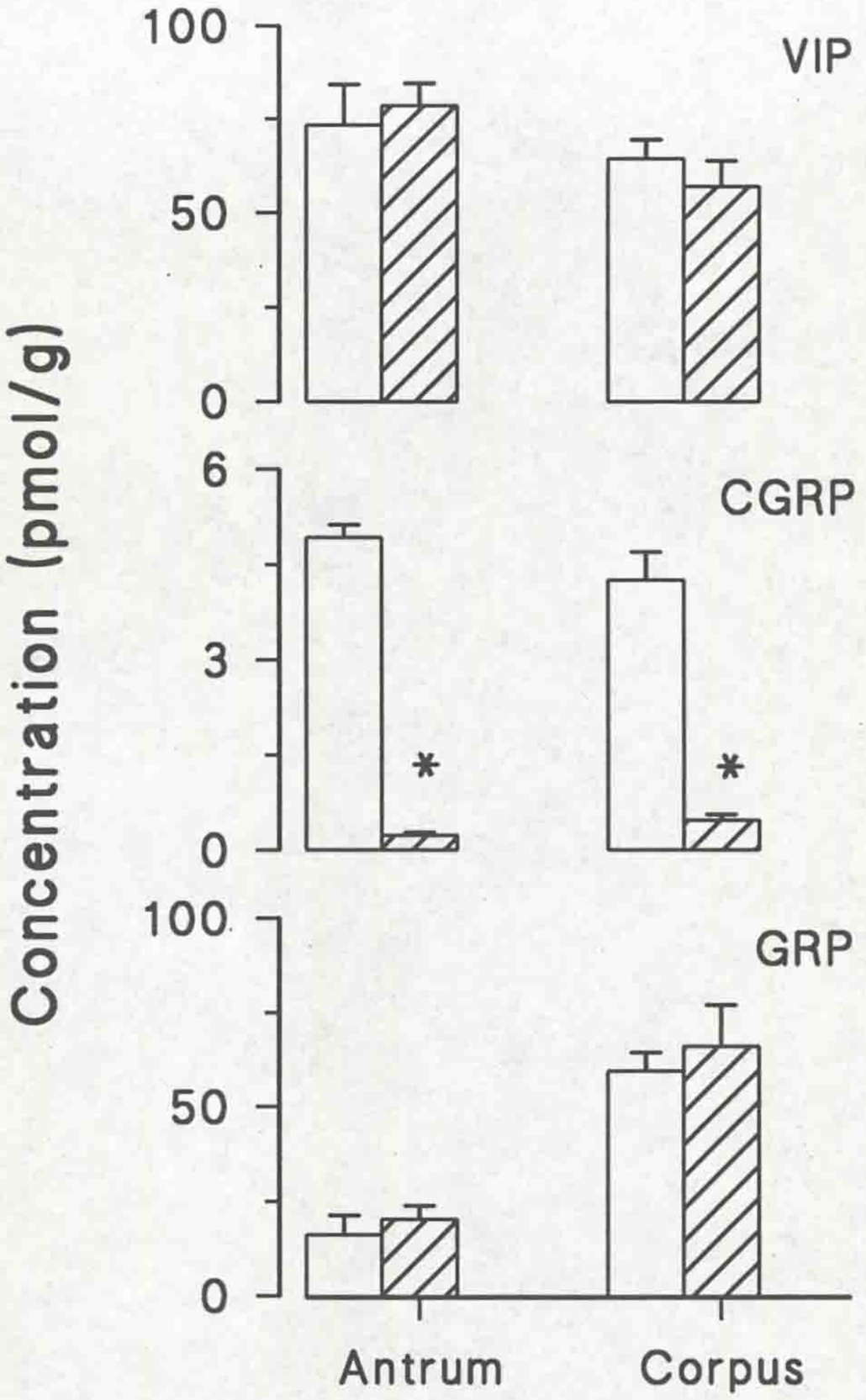


Fig 3.6 Neuropeptide concentrations (CGRP, GRP and VIP) in extracts of antrum and corpus following topical 0.1% w/v capsaicin (hatched bars), or vehicle (open bars), to the serosal surface of the antrum. The spinal afferent innervation of both the antrum and corpus was lost as shown by CGRP concentrations, while neuropeptides of the intrinsic innervation (GRP, VIP) were unaffected in both regions. (n = 4, both groups; * $p<0.05$, compared with vehicle, Mann Whitney U test).

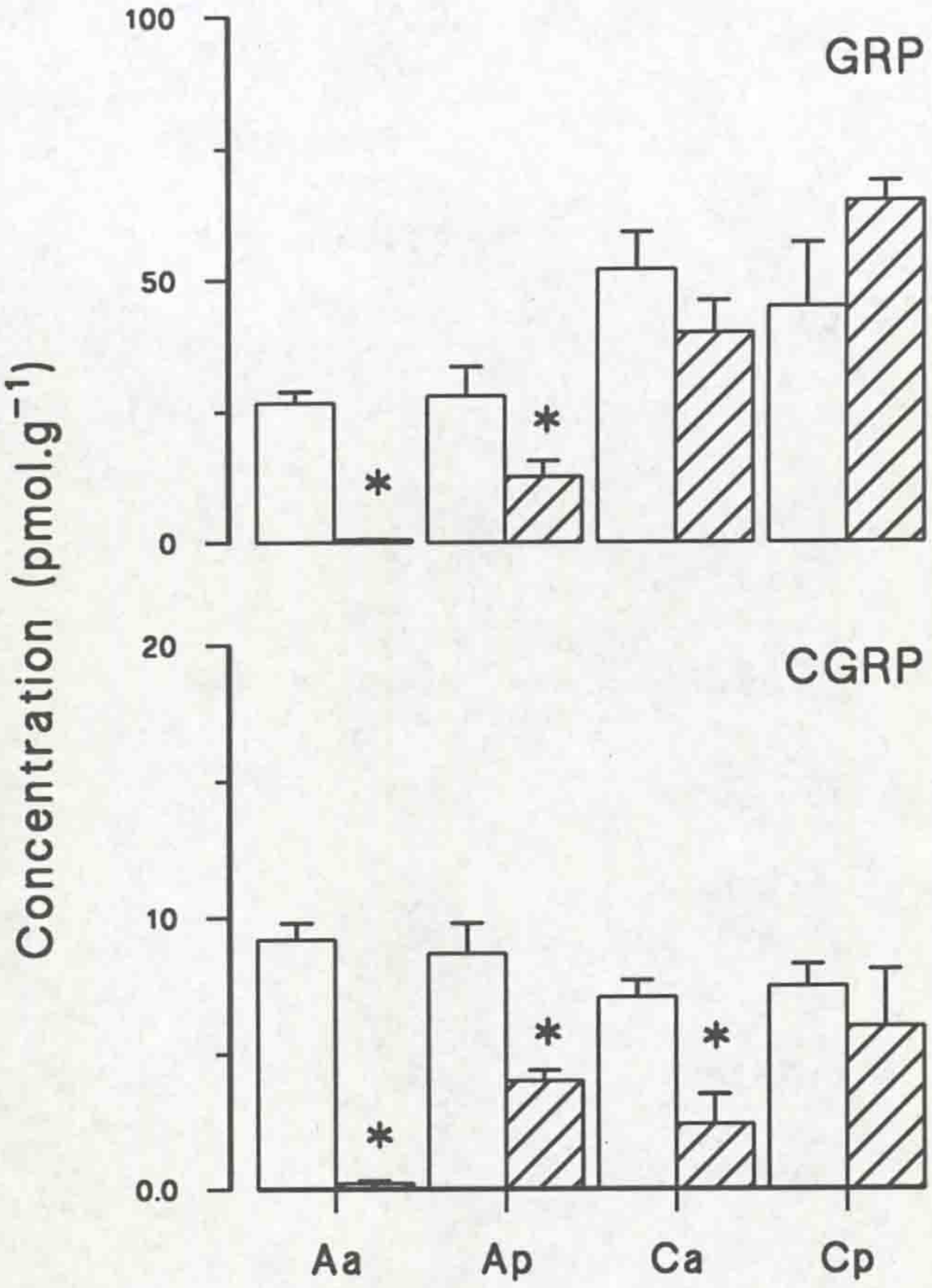


Fig 3.7 Neuropeptide concentrations (CGRP and GRP) in extracts of either the anterior (a) or posterior (p) surfaces of the antrum (A) and ipsilateral corpus (C) following topical 0.5% w/v BAC (hatched bars) or vehicle (open bars) to the anterior serosal surface of the antrum. Neuropeptides of the intrinsic innervation (GRP, VIP) were reduced only in the BAC treated region. The spinal afferent innervation of both the antrum and corpus was lost as shown by CGRP concentrations predominantly on the side of the stomach that received antral BAC (n = 4, both groups; * $p<0.05$, compared with vehicle, Mann Whitney U test).

Discussion

The experimental data obtained in these experiments provided substantial evidence that application of 0.5% w/v BAC to the serosal surface of the antrum for 5 minutes produced a chronic, profound and sustained loss of both the intrinsic and extrinsic afferent innervation. Although this is in keeping with the studies that have reported the effects of BAC on the rat small intestine (Fox *et al.,* 1983; Dahl *et al.,* 1987; Holle *et al.,* 1990; Jodal *et al.,* 1993), rat colon (Sato *et al.,* 1978, Sakata *et al.,* 1979) and opossum oesophagus (Gaumnitz *et al.,* 1993) the duration of exposure and concentration of BAC used was different. In the previous reports in the rat, the preferred method has been that described by Fox *et al.* (1983) requiring 0.062% w/v BAC and an exposure time of 30 minutes for each surface of the intestine. Others have used higher concentrations of BAC (0.2%) with the same exposure time (Zuculoto *et al.,* 1991). Fox *et al.* (1983) demonstrated clearly that the degree of denervation produced by BAC was concentration dependent. Extending these observations, See *et al.* (1988) confirmed that BAC induces myenteric denervation by disrupting the outer layers of the intestine following application to the serosa. The depth of damage to the intestine was dependent on the concentration used and the length of exposure time.

In order to reduce the time under anaesthetic, it seemed reasonable to use a higher concentration of BAC and to determine the appropriate dose by a dose response study. The effective dose to induce comprehensive denervation with a 5

minute exposure time was found to be 0..5% w/v BAC as this dose significantly reduced neuropeptide levels to less than 10% of control values.

The neuropeptides assayed were GRP, CGRP, VIP and substance P. These neuropeptides are representative markers of separate populations of intrinsic and spinal afferent neurons although there is a degree of overlap. Hence GRP is a good and specific marker in the rat antrum for intrinsic myenteric neurons that are secretomotor and stimulate release of gastrin from G cells (Dockray *et al.,* 1979). A proportion of these neurons also contain VIP which, in addition is also found separately in inhibitory motor neurons of the rat myenteric plexus (Ekblad *et al.,* 1987). CGRP is present almost exclusively in spinal afferent neurons (Varro *et al.,* 1988; Sternini *et al.,* 1986; Sternini *et al.,* 1987) in the rat although a proportion of these neurons also contain substance P (Green and Dockray, 1988). However the majority of substance P is found in intrinsic neurons of the myenteric and submucosal plexi (Holzer *et al.,* 1980).

The distribution of neuropeptides within the wall of the stomach explains to some extent the pattern of loss of as determined by radioimmunoassay. Thus, GRP present predominantly in myenteric neurons was reduced to undetectable levels. Similarly CGRP present in spinal afferent neurons that innervate the serosa and muscle layers of the gut also was reduced to undetectable levels. However VIP and substance P are known to be present also in submucosal neurons and the latter also

may be found in inflammatory cells. Although there was a significant reduction in VIP and substance P, these peptides were still detectable in tissue extracts.

The earliest time point that denervation was assessed was 11 days and the latest time point was 27 days. Antral denervation was complete at 11 days and there was no change at the later times studied. This observation is in agreement with previously published experiments on the rat small intestine that have shown complete denervation as early as 24 hours after exposure to BAC (See *et al.,* 1988) and persistence of denervation for up to 160 days (Zuculoto *et al.,* 1991; See *et al.,* 1988). In all the published studies on denervation induced by BAC it has been assumed that the effect was localised to the region of exposure. The data presented here indicate clearly that the spinal afferent innervation of the whole stomach is lesioned by antral BAC treatment. This was shown by loss of CGRP in antral and corpus tissue extracts following antral BAC.

Three lines of evidence suggest that this observation was not due to inadvertent exposure of the corpus to BAC. Firstly, other neuropeptide levels in corpus tissue extracts remained unaffected by antral BAC treatment. Secondly, in the reverse experiment lesioning a circumferential band of proximal corpus did not produce a loss of antral CGRP. Thirdly, exposure to BAC of only the anterior surface of the antrum caused a significant reduction of intrinsic neuropeptides only in tissue extracts of the anterior and not posterior antrum.

The validity of the observation that lesioning antral spinal afferents removes corpus spinal afferent innervation was confirmed by using the specific small diameter afferent fibre neurotoxin capsaicin. Again exposure of antral spinal afferents to capsaicin reduced CGRP levels in antrum and corpus leaving other neurotransmiters levels unaffected. Taken together these observations suggest that CGRP-containing nerve fibres that supply the gastric corpus also supply the antrum, either by passage over the antrum or by projection of branches to both regions.

Finally, confirmation of the effect of BAC on the gastric innervation described above were supported by immunohistochemical studies performed by Dr. C Vaillant. Samples of antrum and corpus were taken from rats that had either received antral BAC or BAC to a circumferential band of proximal corpus. The results of Dr. Vaillant's studies essentially confirm the data obtained by radioimmunoassay. Hence there was a complete loss of immunoreactive nerve cell bodies and fibres only in the treated region following exposure to 0.5% BAC. However, specific staining for CGRP demonstrated that following antral application of BAC there was loss of CGRP-immunoreactive fibres from both the antrum and corpus whereas denervation of a band of corpus with BAC resulted in complete loss of immunoreactive fibres in the treated region, but not in the untreated antrum (Higham *et al.,* 1997).

Summary

Antral BAC treatment effectively lesions all myenteric neurons and spinal afferent fibres of the antrum, as well as the spinal afferent innervation of the

remainder of the stomach. The intrinsic innervation of the proximal stomach is unaffected. Consequently there is a loss of innervation of the antral musculature and antral mucosal endocrine cells. The primary aim of developing such a model of complete antral denervation was to study the effects that this would have on gastric motility and antral endocrine cell function. The results of these studies are presented in the next two chapters.

Chapter Four

Antral denervation and Gastric Emptying

Introduction

For many years, antro-pyloric motility has been recognised to be an important factor controlling the rate of gastric emptying (Canon 1911). More recently, the pulsatile nature of antral contractions and the resting pressure of the pyloric sphincter have been shown to be significant factors governing the delivery of gastric content to the duodenum (Houghton *et al.,* 1988; Malbert and Ruckebusch, 1991; Prather *et al.,* 1993; Dent *et al.,* 1994; Mayer *et al.,* 1994). The likely role of the intrinsic antral innervation in determining the pattern of antral motor contractions has received little attention. This has been primarily because of the lack of available methodology to selectively lesion only antral neurons.

In contrast the extrinsic innervation of the stomach has been more amenable to study but have been shown to have little long term effects on the pattern of antro-pyloric motility (Quigley and Louckes, 1951; Stoddard *et al.,* 1973). Indeed the majority of studies on the extrinsic reflex control of gastric emptying have concentrated on duodeno-gastric reflexes that regulate gastric emptying by effecting changes in gastric corpus tone either through vagally mediated (Raybould and Tache, 1988; Raybould *et al.,* 1987; Holzer *et al.,* 1994) or spinal afferent pathways (Forster *et al.,* 1990; Forster *et al.,* 1991).

However, BAC has been shown to lesion myenteric neurons in a defined segment of rat jejunum and disrupt motor patterns that are dependent only upon an intact intrinsic innervation (Holle and Forth, 1990). In this chapter the data presented are from experiments that have used BAC to lesion the antral innervation to determine the role of antral innervation on the control of gastric emptying. Initially the retention of solid food was assessed and compared to the effects of the lesion produced by antral application of capsaicin. Finally the gastric emptying of a variety of liquid test meals was determined in control and BAC treated rats. The results demonstrate the requirement for an intact antral innervation for normal gastric emptying.

Methods

Antrally denervated and capsaicin treated rats were prepared and after each experiment denervation confirmed by radioimmunoassay of appropriate neuropeptide markers (see methods and chapter 3). Gastric emptying was determined either by studies of the retention of solid material in the stomach of fasted rats or by the determination of the emptying of liquids instilled into the stomach of rats with a gastric cannula. Gastric cannulas were placed at the time of antral denervation and all rats were allowed at least 11 days to recover after surgery.

For the determination of gastric emptying of solids, rats were fasted for 48 hrs on wire bottomed cages, killed by cervical dislocation and the stomach content weighed wet and again after prolonged drying at 50^0C. For the determination of gastric emptying of liquids in gastric fistula rats, the stomach was washed extensively with warm 0.14M NaCl to remove solids prior to experiments and then the emptying of test solutions (saline, HCl, peptone: containing phenol red, 60mg.l^{-1}) instilled into the stomach via the gastric fistula was determined.

Results

In fasted rats previously treated with 0.5% BAC on the antrum, there was a striking retention of solids in the stomach compared to controls (Fig 4.1). The wet and dry weight of gastric content was variable in BAC treated rats but all 0.5% BAC-treated rats exhibited overtly distended stomachs (by solid, gas or liquid) after a 48 hr fast. The retention of solid was not apparent after treatment with lower concentrations of BAC (Fig 4.1). However with 0.5% BAC the retention of solids was seen at all time points examined (Fig 4.2). In 48 hr fasted BAC treated rats that were allowed to refeed for 30 minutes, the dry weight of gastric content increased further (Fig 4.3). The dry weight of gastric content was similar to refed control rats.

Given that BAC causes loss of intrinsic and extrinsic afferent innervation whereas capsaicin lesions only extrinsic afferents (see chapter 3), the latter was used to determine whether the effects of BAC might simply reflect the loss of afferent innervation. Rats that had been pre-treated with capsaicin on the antrum did not exhibit gastric retention after fasting (Fig 4.4). To confirm the importance of the whole antrum in regulating gastric emptying the effects of denervation of a circumferential band of corpus and of one antral surface was also examined. Interestingly, these lesions had little effect on gastric retention of solid (Fig 4.4)

The emptying of several different types of test meal was significantly delayed in rats previously treated with 0.5% BAC on the antrum (Fig 4.5). It is worth noting

that in control rats, the emptying of HCl and peptone was inhibited compared with saline. However, in BAC-treated rats there was no significant difference in the emptying of saline, HCl and peptone, even though all of these emptied less rapidly than the corresponding rates in control animals.

It is known that in intact rats, prior administration of peptone just before an emptying trial with peptone (ie peptone plus preload) produces an exaggerated inhibition of emptying due to enhanced CCK release (Forster and Dockray, 1992). The same effect was observed in the present control rats; in addition, however, peptone plus preload produced a significant inhibition of emptying compared with saline in BAC-treated rats (Fig 4.5). This suggests that CCK might be mediating this effect in BAC-treated rats implying an intact vagally mediated duodeno-gastric reflex.

To ensure that the gastric retention in BAC-treated rats was not primarily due to enhanced CCK release, plasma CCK was measured in fasted BAC and control rats. The concentration in control rats (1.0 ± 0.1pM) was not significantly different from that in BAC-treated rats (1.2 ± 0.1 pM).

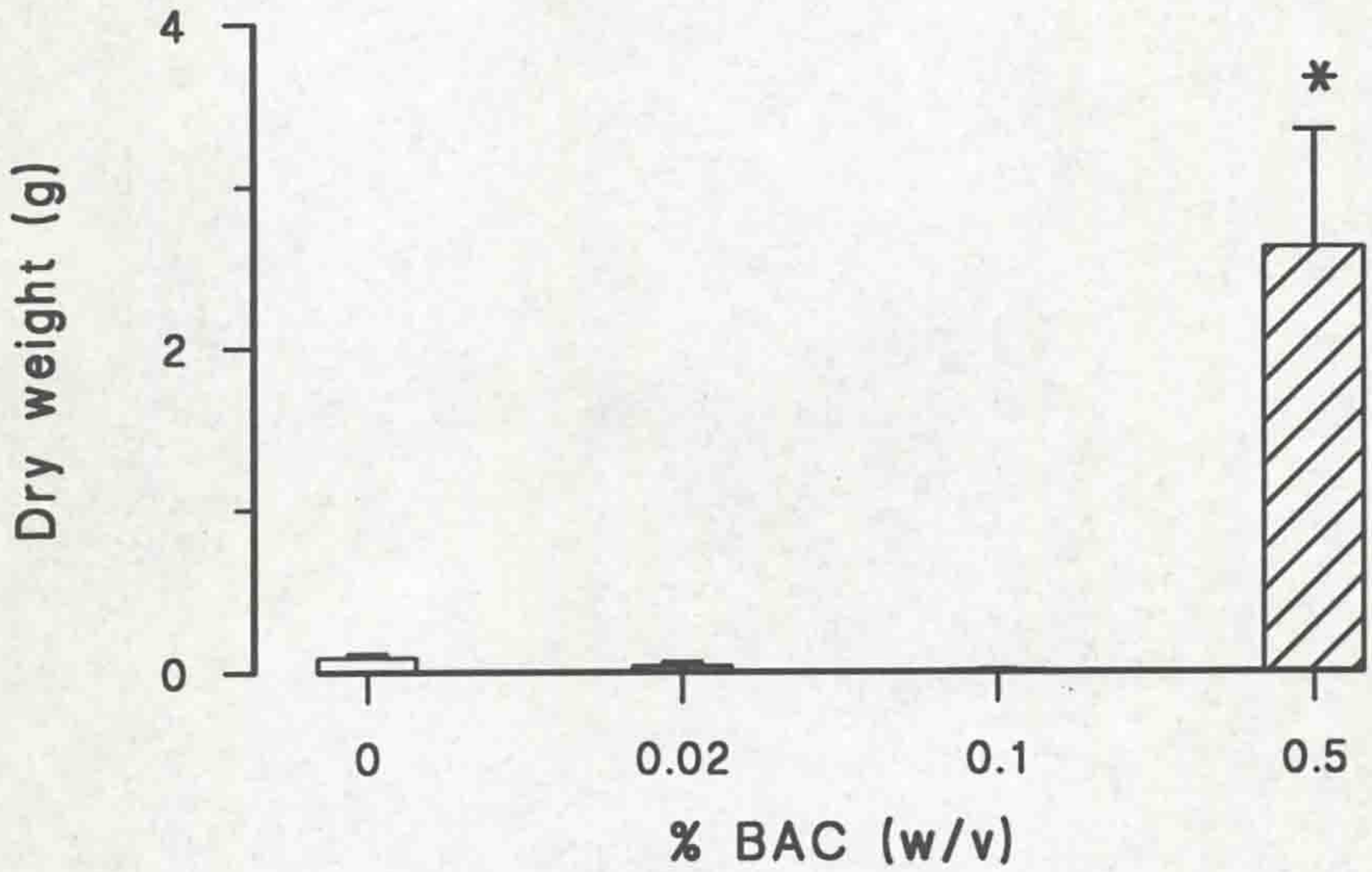


Fig 4.1 Gastric retention after a 48 hr fast in control rats (open bars, n=6) and in rats treated with increasing concentrations of BAC to the antrum (hatched bars, n=6). Only at the highest dose of 0.5% w/v BAC was there significant retention of solid in the stomach (*$p < 0.05$, Mann Whitney U test).

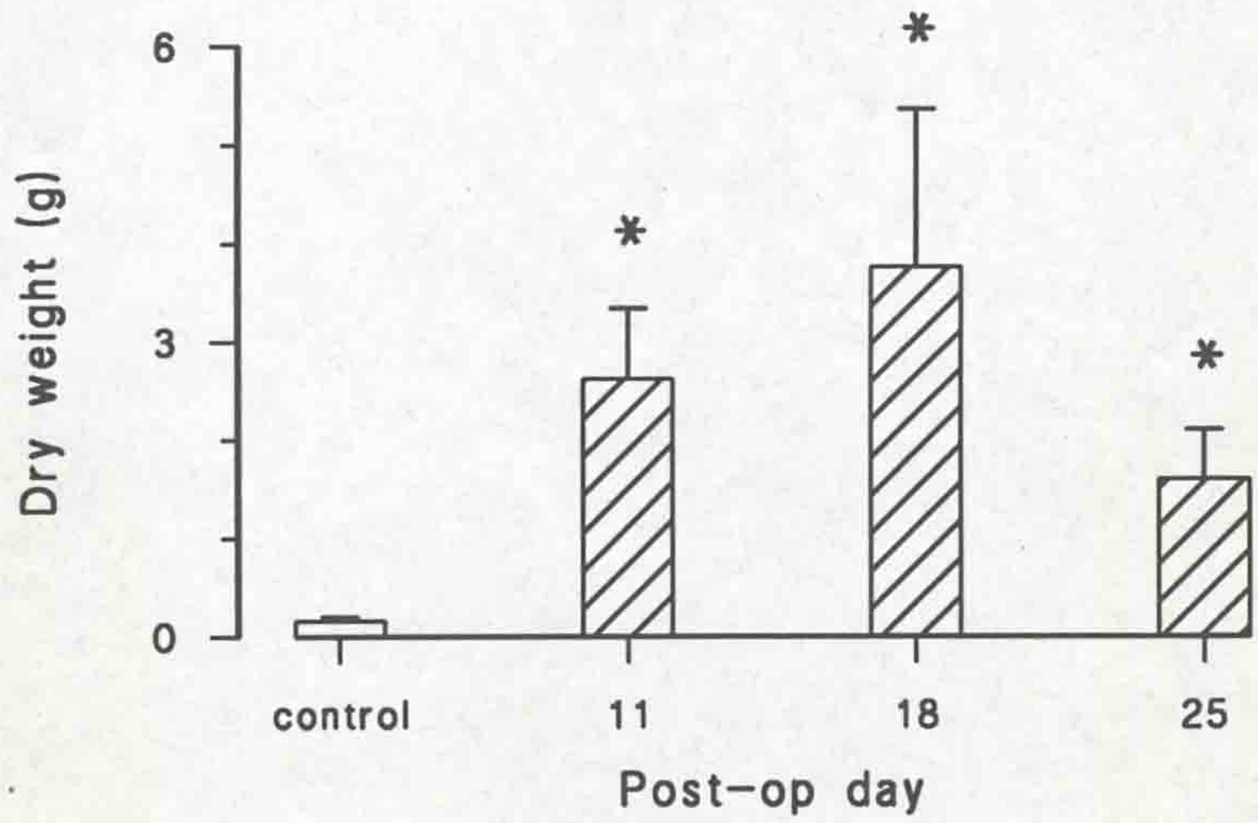


Fig 4.2. Gastric retention at various time points following antral BAC (hatched bars, n=6) or sham operation (open bars, n=6) and after a 48 hr fast. At all time points there was a significant retention of solid within the stomach only in rats treated with BAC to the antrum (*$p < 0.05$, Mann Whitney U test).

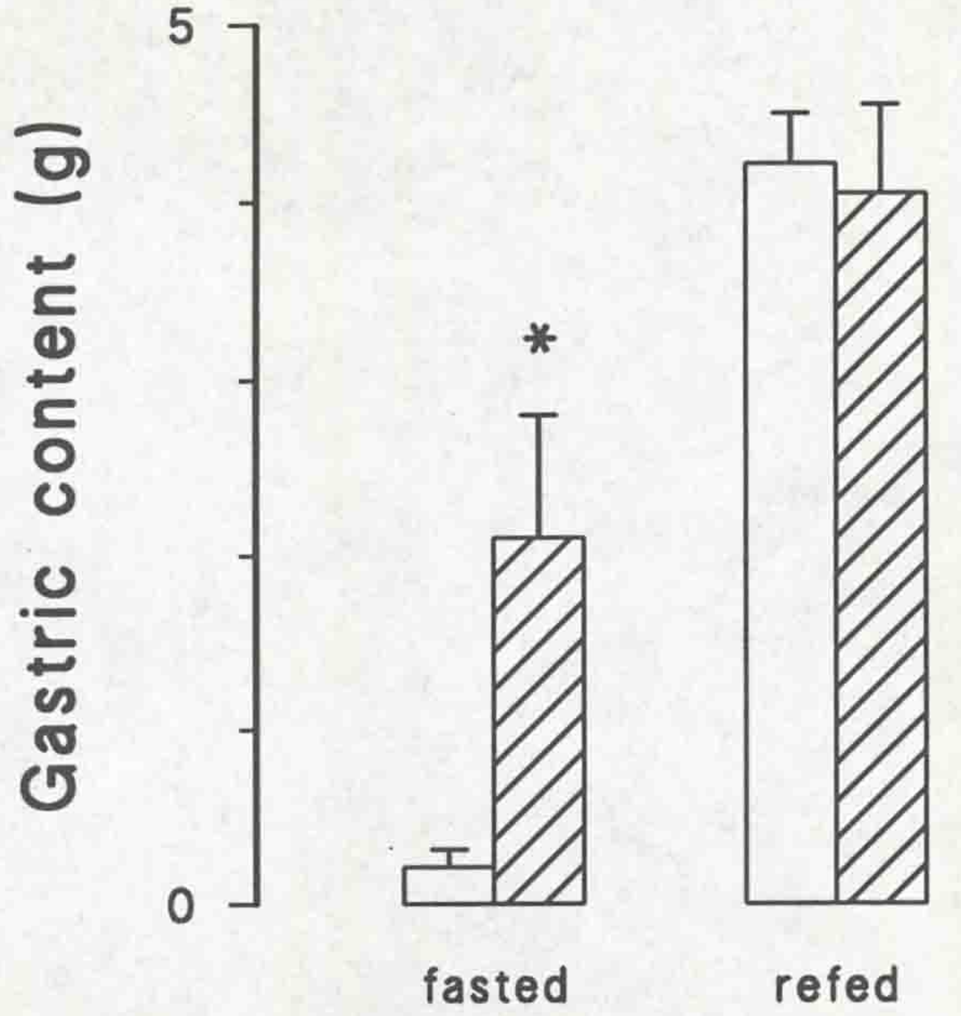


Fig 4.3 Gastric retention in control rats (open bars, n=6) and in rats treated with 0..5% w/v BAC to the antrum (hatched bars, n=6) after a 48 hr fast and following a 30 minute refeed. There was a significant retention of solid in the stomach of fasted BAC treated rats compared to controls but after refeeding the dry weigh of gastric content was similar in both groups (*$p < 0.05$, Mann Whitney U test).

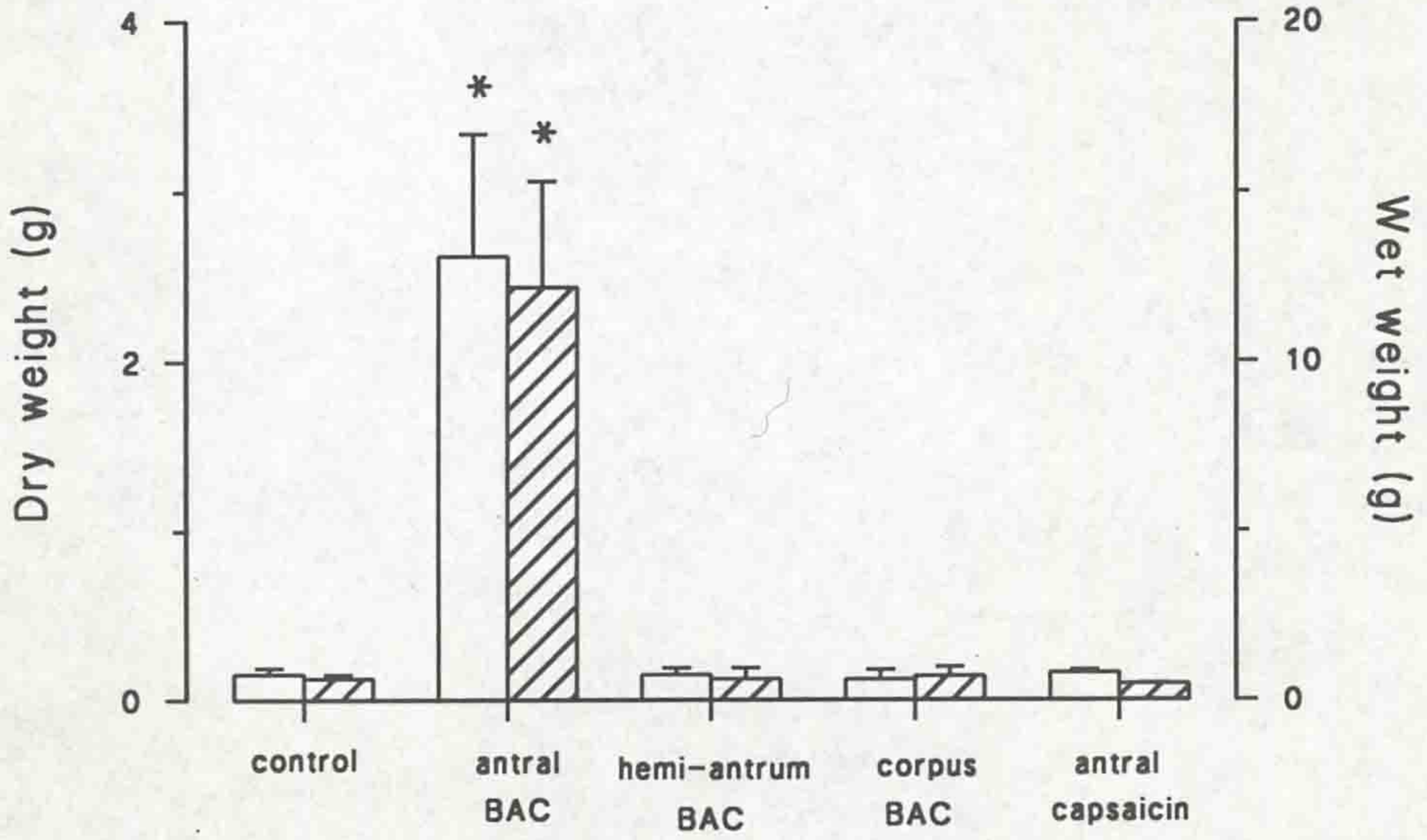


Fig 4.4 Gastric retention after a 48 hr fast in control rats and rats treated with BAC either to the whole of the antrum, or to the anterior surface of the antrum only, or to a band of corpus adjacent to the non-glandular stomach, or with capsaicin to the antrum. Data shown are for wet weight (hatched bars) and dry weight (open bars). Only BAC treatment of the whole antrum leads to gastric retention (*$p < 0.05$, Mann Whitney U test).

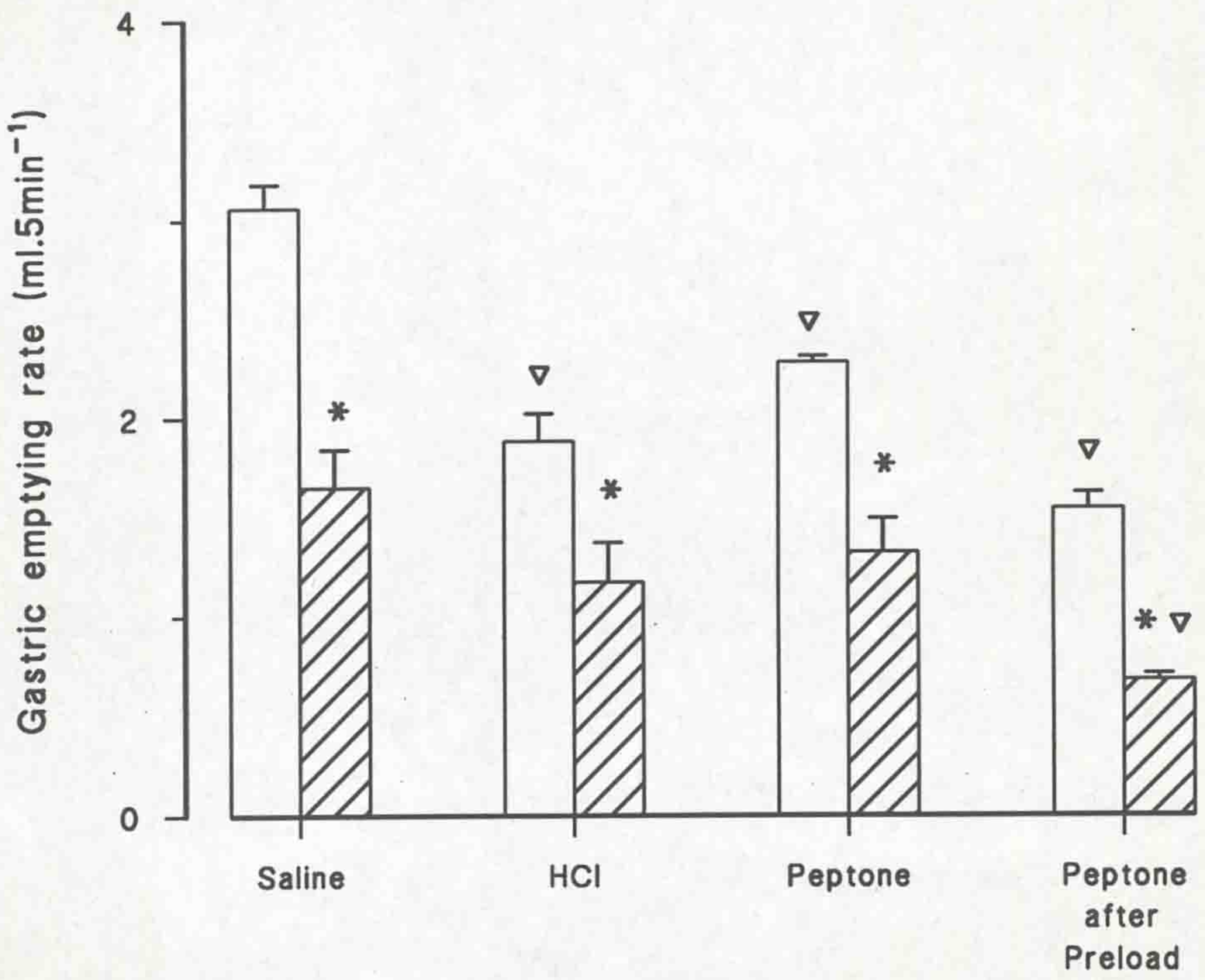


Fig 4.5 Gastric emptying rates for different liquids in gastric fistula rats pre-treated with either 0.5% w/v BAC (n=8, hatched bars) or vehicle (n=6, open bars). For all liquids shown emptying rates are slower in BAC treated rats. In control rats, peptone and HCl empty more slowly than saline, and peptone after a peptone preload empties more slowly than peptone alone. In BAC treated rats, saline, HCl and peptone empty at the same rate, but peptone after a peptone preload induces further delay in gastric emptying.

Discussion

The present data provides evidence that the integrity of the intrinsic innervation of the rat antrum is obligatory for the normal emptying of solids and liquids from the rat stomach. Specifically, it was found that the loss of intrinsic antral neurons caused by the detergent BAC resulted in retention of solids, and delayed emptying of both nutritive (peptone) and non-nutritive (saline, HCl) solutions. The retention of solids and liquids could not be attributed to changes in plasma CCK, which is the hormone best known for its ability to inhibit gastric emptying (Dockray 1988) since plasma CCK was normal. In a further series of experiments performed on antrally denervated gastric fistula rats, Dr B Yegen studied the effects of the specific CCK-antagonist (Green *et al.,* 1988) on the emptying of liquid test meals. In these experiments there was no significant difference in the emptying rates of saline, HCl and peptone after L-364,718 in BAC-treated rats compared to BAC rats that did not receive the antagonist. Interestingly, however, the delayed emptying of peptone after a preload was reversed and was comparable to the emptying of saline. It would appear therefore that the action of CCK in controlling gastric emptying can be exerted independently of the antral innervation.

The application of BAC to the antrum lesioned both the intrinsic and extrinsic afferent innervation; however loss of the latter cannot explain the retention of solids, since there was no retention in rats treated with capsaicin which lesions only extrinsic afferent fibres. Taken as a whole, therefore, the present data lead to the conclusion that

the antral innervation is essential for the gastric emptying of solid and of both nutritive and non-nutritive solutions, but not for the delaying actions of CCK on gastric emptying.

The neuroendocrine mechanisms that determine gastric emptying have attracted increasing attention in recent years. In the case of CCK such studies have suggested a primary site of action at receptors on vagal afferent nerve fibres, the activation of which evokes a vago-vagal reflex leading to relaxation of the gastric corpus (Raybould and Tache, 1988; Tache *et al.,* 1988; Forster *et al.,* 1990). The possible involvement of the distal stomach in mediating the effect of CCK on gastric emptying has been suggested by previous studies (Yamagishi and Debas, 1978) but little has been done to determine whether - in this case - CCK acts directly on smooth muscle or indirectly via the innervation. The findings here suggest that any effects of CCK on the innervation of the antro-pyloric region are not of central importance in its role as an inhibitor of gastric emptying.

The effect of antral denervation on emptying can be considered specific since denervation of a fully circumferential band of proximal corpus (which would also lesion vagal efferent fibres) did not lead to gastric retention. In part the retention of solids after antral denervation may be attributable to a decreased capacity of the antrum for trituration and therefore the reduction of solids to a form suitable for emptying. In addition, however, there is also plainly a marked delay in emptying of liquids. This effect is distinguishable from the reduced emptying of liquids seen after

lesioning of afferent neurons by capsaicin (Forster *et al.,* 1990; Forster *et al.,* 1991) since it is only in antrally denervated rats that there is a substantial delay in the emptying of isosmotic saline.

The pumping action of the antrum is well known, and contributes to the episodic delivery of gastric contents to the duodenum (Malbert and Ruckebusch, 1991; Dent *et al.,* 1994). We suggest that the co-ordination of this activity requires intrinsic antral neurons and possibly intact efferent vagal neurons and that in the absence of these neurons the antrum generates an unremitting high-resistance to the passage of both liquids and solids. In view of the non-specific effect of BAC in lesioning all neuron populations within the area of exposure (excluding the submucosal neurons) it is not possible to speculate which if any neuron population is essential to maintain normal gastric emptying. It is interesting to note however that targeted disruption of the neuronal nitric oxide synthase gene leads to a phenotype essentially indistinguishable from the BAC treated antrally denervated rat (Huang *et al.,* 1993). NO has been shown to mediate the nonadrenergic, noncholinergic smooth muscle relaxation throughout the gastrointestinal tract (Boeckxstaens *et al.,* 1991) including relaxation of the gastric corpus (Desai *et al.,* 1991). In the NOS knockout mouse, the presumed loss of smooth muscle relaxation in the region of the antrum and pylorus had similar effects to those documented here.

A further possibility that may explain the high pressure resistance in the BAC treated antrum is that the BAC treatment has a direct effect on the muscle itself. It has

been apparent from early studies with BAC that following denervation of the small intestine there is significant thickening of both longitudinal and circular muscle layers of the intestine (Fox *et al.,* 1983; See *et al.,* 1988). In addition the muscle became spontaneously contractile with altered mechanical properties including the ability to generate three times the active stress of control smooth muscle in response to muscarinic agonists (Osinski and Bass, 1994). This was later shown to be a consequence of an inability to resequester Ca^{2+} in the sarcoplasmic reticulum leading to increased intracellular calcium (Osinski and Bass, 1995). Although it has been impossible to distinguish between the direct effect of BAC and the secondary effects of loss of normal innervation on smooth muscle function, Hadzijahic *et al.* (1993) have compared different methods of inducing myenteric denervation (Surgical vs BAC) and inflammation (intraluminal stasis and 5% acetic acid) to determine the underlying stimulus to the increased muscle thickness. Their results together with the observation of muscle thickening in the NOS knockout mice indicate that the changes in muscle thickness at least are a consequence of denervation and not a direct effect of BAC on smooth muscle.

Summary

Antral BAC produces selective lesions of defined populations of neurons in the stomach and leads to dramatic changes in gastric emptying. After antral BAC treatment there was retention of solids in rats fasted for 48 hr. Moreover, in antrally denervated rats with a gastric fistula, the emptying of saline, acid and peptone was substantially delayed. The cholecystokinin-dependent inhibition of gastric emptying

of peptone was preserved after antral BAC. Hence it appears that the innervation of the antrum is essential for normal emptying of both liquids and solids, but the inhibition of gastric emptying produced by cholecystokinin is not dependent on antral neurons.

Chapter Five

Antral denervation and G-cell Function

Introduction

The release of the antral hormone gastrin is controlled by the luminal contents of the stomach and by neurohumoral agents acting at the basolateral membrane of the G-cell. The main luminal stimulus is the presence of protein and protein-digestion products while the main luminal inhibitory factor is gastric acid (Dockray & Gregory, 1989; Walsh, 1994). The available evidence suggests that gastric acid releases somatostatin from antral D-cells which in turn inhibits gastrin release by a paracrine mechanism (Saffouri *et al.*, 1980; Chiba *et al.*, 1980; Walsh, 1994). The mechanisms by which luminal nutrients stimulate the G-cell are less certain. The luminal surface of the G-cell consists of tufts of microvilli that project into the lumen and this has led to the idea that luminal nutrients might act directly on the cell (Lichtenberger *et al.*, 1982; Delvalle & Yamada, 1990; Lichtenberger, 1982). However, it is also clear that luminal stimulation of the G-cell can be indirectly mediated by nervous pathways (Saffouri *et al.*, 1984; Schubert *et al.*, 1992).

It is well recognised that vagal efferent neurons stimulate gastrin secretion in the cephalic phase of digestion and in addition, there are local intrinsic nervous reflexes controlling the G-cell. There is evidence for cholinergic-muscarinic stimulation of the G-cell, particularly in the rat (Schubert & Makhlouf, 1993; Schubert *et al.*, 1992; Saffouri *et al.*, 1984), but in dog and man atropine typically has no effect on nerve-mediated gastrin release, or stimulates it - indicating the importance of non-cholinergic inputs to the G-cell (Dockray & Tracy, 1980; Feldman *et al.*, 1979;

Schiller *et al.*, 1980; Farooq & Walsh, 1975). The neuropeptide gastrin releasing peptide (GRP) which is well represented in antral mucosal nerve fibres (Dockray *et al.*, 1979), is thought to be the main non-cholinergic neurotransmitter stimulating the G-cell. In keeping with this, both antibodies and more recently selective antagonists to GRP have been shown to inhibit gastrin secretion in response to either luminal peptone, or electrical field stimulation of the antrum or vagal nerve stimulation (Schubert, *et al.*, 1985; Holst, *et al.*, 1990; Schubert, *et al.*, 1992). The experiments described in this chapter were performed in an attempt to define the relative importance of neural mechanisms compared to the direct stimulation by luminal factors on gastrin release. To this end I have studied the luminal factors that influence gastrin release in control and antrally denervated rats. The results indicate increased sensitivity to non-nutrient distension after antral denervation and suggest the existence of an antral inhibitory innervation in the rat that normally suppresses G-cell responses to non-nutrient distension.

Methods

Antrally denervated rats were prepared and after each experiment denervation confirmed by radioimmunoassay of appropriate neuropeptide markers in tissue extracts of antral muscle (see Methods). Gastric cannulas were placed at the time of antral denervation and all rats were allowed at least 11 days to recover after surgery. For the refeeding experiments, rats were fasted for 48 hrs on wire-bottomed cages and were then allowed to refeed for 30 minutes. The GRP antagonist (BW2258U89) {Moody et al Life Sci 56: 521-529} (2mg.kg^{-1} in 0.5ml of 0.14M saline) or saline, were injected subcutaneously 30mins before refeeding and then trunk blood was taken for assay of plasma gastrin. Samples of antral and corpus mucosa were taken for RNA extraction for analysis of gastrin, somatostatin, HDC and GAPDH mRNA abundance by Northern blot.

Gastric fistula rats:

As before, (chapter 4) the stomach was washed with warm 0.14M NaCl to remove solids at least one hour prior to experiments. Gastric acid output was determined by collecting gastric secretion over four sequential 15 min periods and titrating to pH 7.0 with 20mM NaOH using a pH autotitrator. Plasma gastrin responses were determined after gastric distension with the following test solutions: 2% (w/v) methyl cellulose (2%MC); 2%MC at pH6 (50mM ammonium acetate); 2% MC with 50mM HCl; 4.5% w/v peptone. In each case the stomach was distended by connecting the cannula to a reservoir of test solution and elevating the reservoir to 5cm H_2O pressure for 30mins.

Results

Basal plasma gastrin

Basal plasma gastrin concentrations were similar in control, fasted rats (7.6 ± 1 pM; Fig5.1) and control rats with a gastric fistula (11.8 ± 2 pM; Fig5.4). In BAC treated rats fasted for 48hrs plasma gastrin was significantly elevated (28.4 ± 7pM, $p<0.05$ vs control; Fig 5.1). In these animals there was also marked gastric retention compared to controls (see Chapter 4). It seemed likely that gastric retention in BAC-treated rats accounted for the increased plasma gastrin, since in fasted BAC-treated rats with a gastric fistula, plasma gastrin was similar to control (13.5 ± 3.2pM; Fig 5.4) when the gastric contents were removed by lavage via the cannula.

Refeeding experiments

When control rats were fed for 30min after fasting for 48hr, plasma gastrin increased approximately 3-fold. There was a comparable increase in plasma gastrin in BAC-treated rats but the absolute increase after refeeding was higher than that in control rats, due to the elevation of basal plasma gastrin concentration (Fig 5.1). In keeping with the idea that GRP mediates food-stimulated release of gastrin in the rat, I found that the GRP antagonist BW2258U89 (0.5 $mg.kg^{-1}$) inhibited the rise in plasma gastrin in control rats seen after refeeding for 30min following a 48 hr fast. In contrast, in BAC-treated rats, the post-prandial rise in gastrin was not dependent on GRP since there was no significant difference compared with BAC-treated rats that received vehicle in place of the GRP antagonist (Fig 5.2).

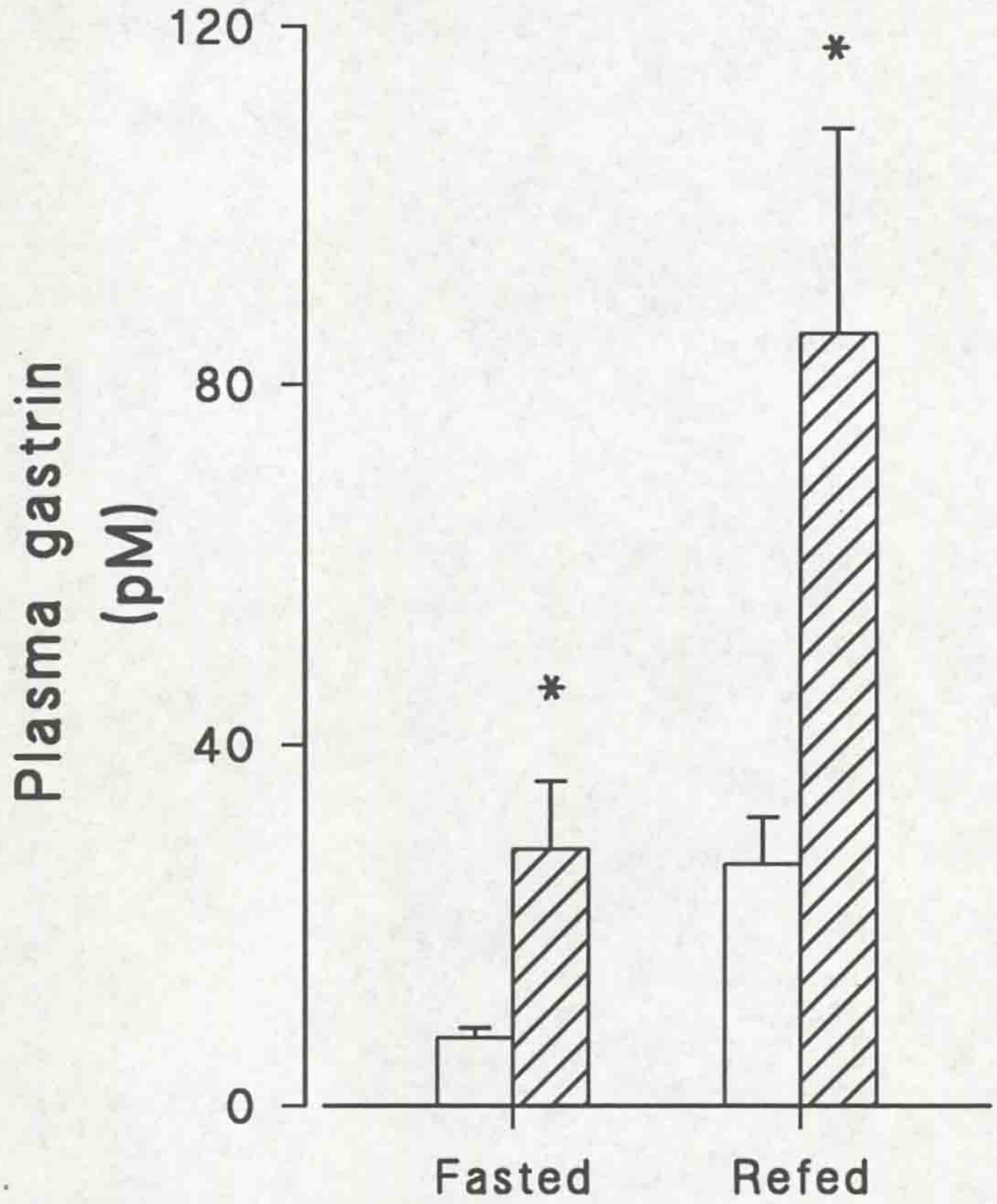


Figure 5.1 Plasma gastrin in fasted and refed rats 11 days after either sham operation (open bars) or antral denervation by 0.5% BAC (hatched bars). In fasted BAC-treated rats there was elevated fasting plasma gastrin. After refeeding, plasma gastrin increased approximately three-fold in both control and BAC-treated rats. (n=>6 for each group, * $p<0.05$ vs control response, Mann Whitney U test).

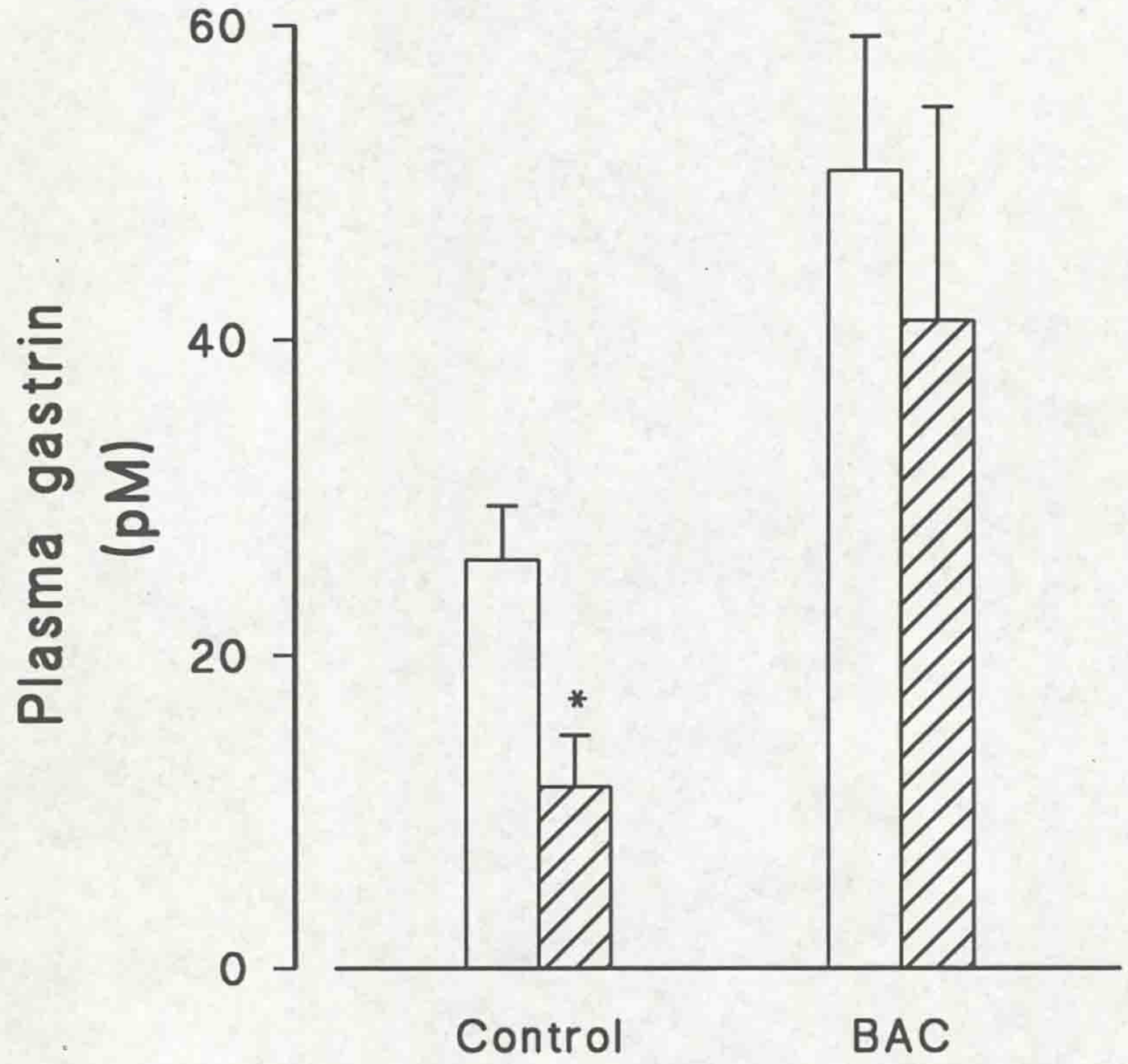


Figure 5.2 Plasma gastrin concentrations in control and antrally-denervated rats after administration of the specific GRP antagonist BW225U86 (hatched bars) or saline (open bars) 30mins prior to refeeding. In control rats, but not antrally denervated rats, the GRP antagonist significantly attenuated the post-prandial rise in plasma gastrin. (n=6 for all groups, * $p<0.05$ Mann Whitney U test).

Basal acid output:.

Under normal circumstances gastrin release is inhibited by luminal acid so that one potential explanation for the elevated plasma gastrin in BAC-treated rats was that BAC treatment of the antrum influenced acid secretion. In fact the range of basal acid output in BAC-treated rats overlapped with that in controls but the mean was approximately 3-fold lower (Fig 5.3). Complete antral denervation was required to reduce mean basal acid output as rats that received BAC only to the anterior surface of the stomach had a mean basal acid output comparable with controls.

Distension experiments

In control rats distension with the non-nutritive solution 2% MC had no effect on plasma gastrin. In contrast, distension of BAC-treated rats with 2% MC produced a significant rise in plasma gastrin (Fig 5.4). Although this appeared to substantiate the view that distension stimulates gastrin release in antrally denervated rats, there was also the possibility that alterations in pH may play a part in the response given the reduced acid output in BAC-treated rats. Therefore distension experiments were performed with buffered 2% MC (acidified and pH6) and with peptone as a positive control. In control rats distension with 4.5% w/v peptone increased plasma gastrin 3-fold but distension with the non-nutritive solutions 2 % methylcellulose (2%MC), or 2%MC pH 6 or 2%MC with 50 mM HCl did not increase plasma gastrin (Fig 5.5).

In BAC-treated rats, the effects of distension with peptone and 2%MC with 50mM HCl in methylcellulose were similar to control. However distension by either 2%MC or 2%MC pH 6.0 did stimulate gastrin release, the increase in plasma gastrin being comparable to that produced by peptone (Fig 5.5).

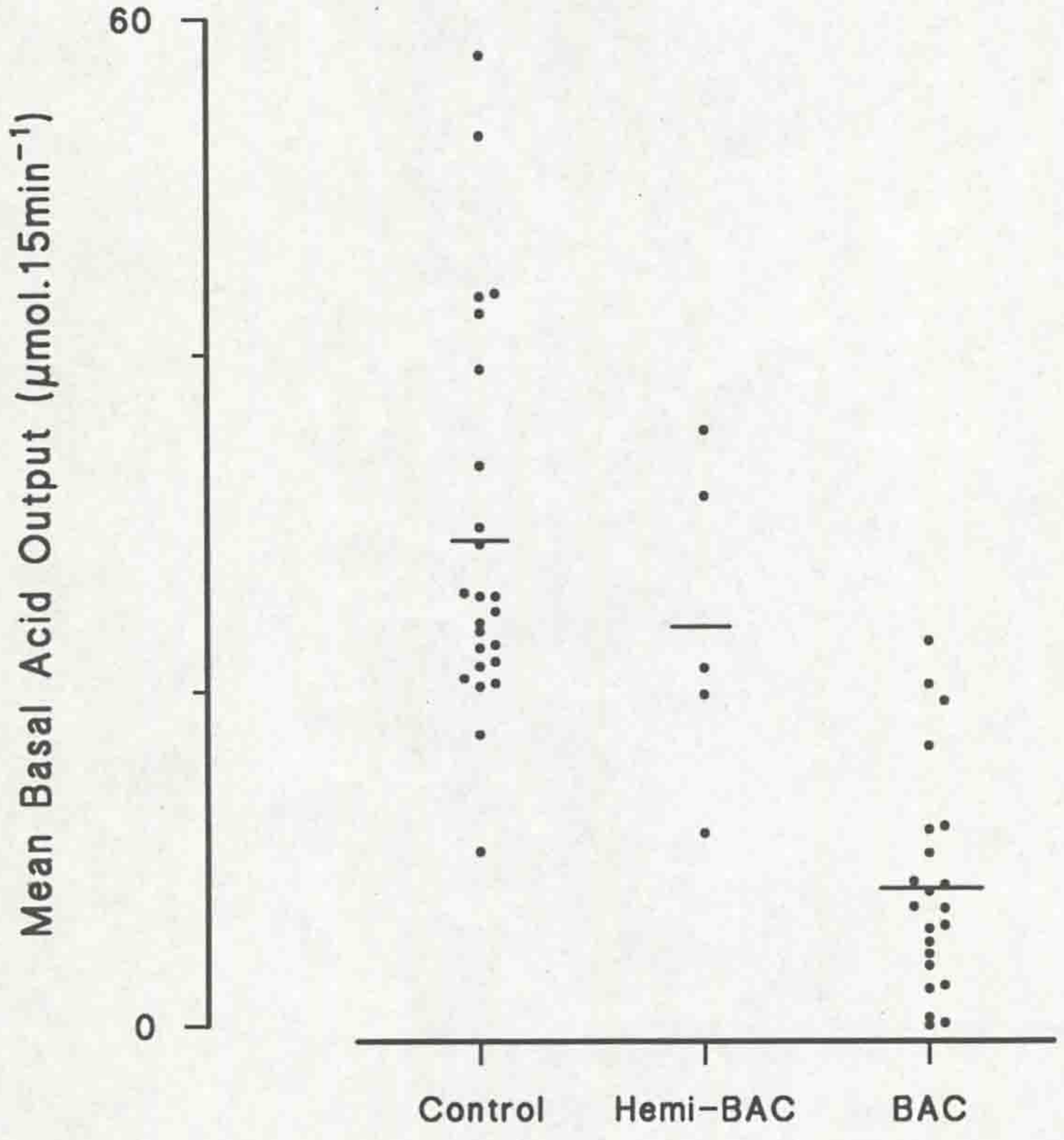


Figure 5.3 Mean basal acid output (mmol.15min^{-1}) in control and antrally denervated rats. Two groups of denervated rats were studied: either total antral denervation, or denervation of the anterior surface only (hemi-BAC). Each data point represents the mean acid output of four sequential 15min collections from one rat. Only in the rats with complete antral denervation was there a significant reduction in basal acid output. (* $p<0.05$ ANOVA).

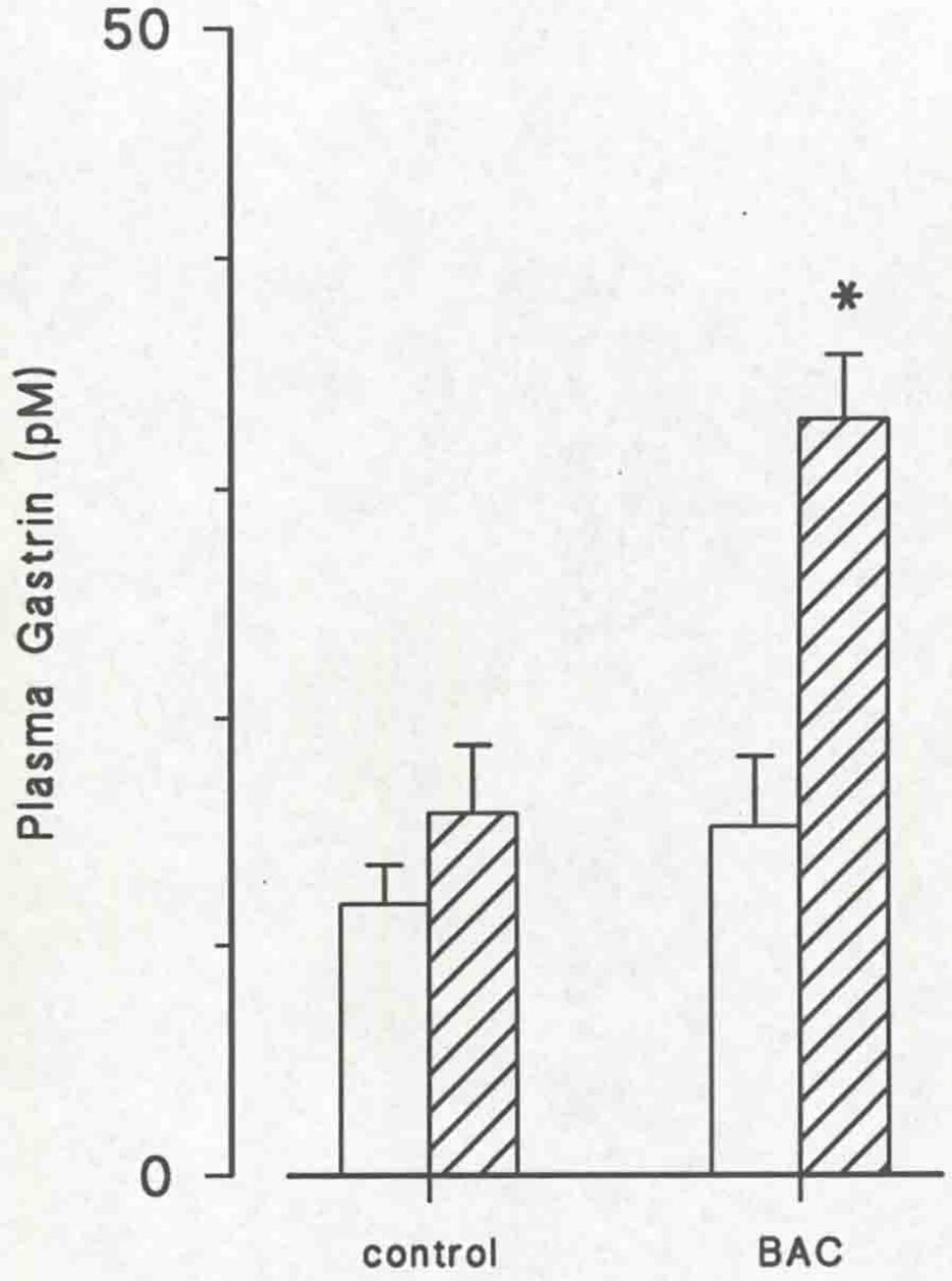


Figure 5.4 Plasma gastrin responses in control and BAC-treated rats after gastric lavage to remove residual gastric content. Rats were either left undistended (open bars) or were distended with the non-nutritive solution 2%MC for 30 minutes at a pressure of 5cm of H_2O (hatched bars). In BAC-treated rats but not controls, plasma gastrin was increased by distension with 2%MC. (n=6 or more for each group, * $p<0.05$ vs control response).

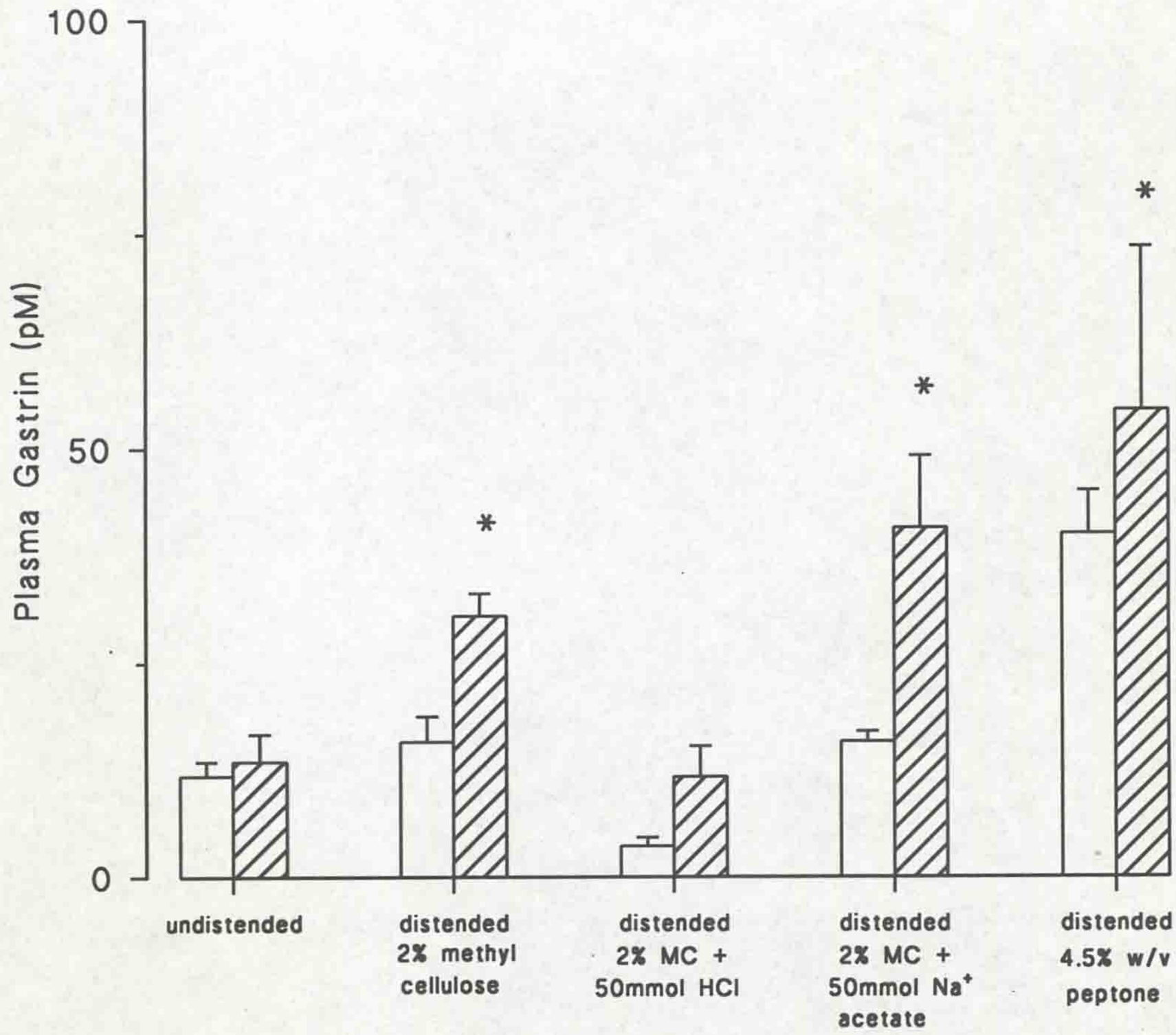


Figure 5.5 Plasma gastrin responses in control (open bars) and BAC-treated rats (hatched bars). Rats were either left undistended or distended for 30 mins with either 2%MC (buffered with 50mM HCL or to pH6) or 4.5% w/v peptone. Plasma gastrin after distension with 4.5% peptone was similar in control and antrally-denervated gastric fistula rats. In BAC-treated rats, but not controls, plasma gastrin was also increased by distension with 2%MC buffered to pH6.0. Neither group showed an increase in plasma gastrin after distension with 2%MC with 50mmol HCl. (n=6 or more for each group, * $p<0.05$ vs control response, Mann Whitney U test).

Gastrin, somatostatin HDC and GAPDH mRNA abundance

It is known that gastric endocrine cell responses frequently involve altered changes in the synthesis of active products, as seen by changes in mRNA abundance (Dockray *et al.* 1996). To determine whether antral denervation might alter the synthesis of regulatory molecules in gastric endocrine cells Northern blots of mRNA's encoding gastrin, somatostatin, HDC and GAPDH were analysed (Figures 5.6 & 5.7). There was no difference in the expression of GAPDH between control and BAC treated rats. In the antrum there was a significant reduction in antral mucosal somatostatin mRNA abundance in BAC-treated rats compared with controls and a similar decrease in gastrin mRNA abundance. In the corpus however, somatostatin mRNA abundance was unchanged in BAC-treated rats compared with controls, while HDC mRNA was variably increased. The latter is compatible with the sustained increase in plasma gastrin in these animals.

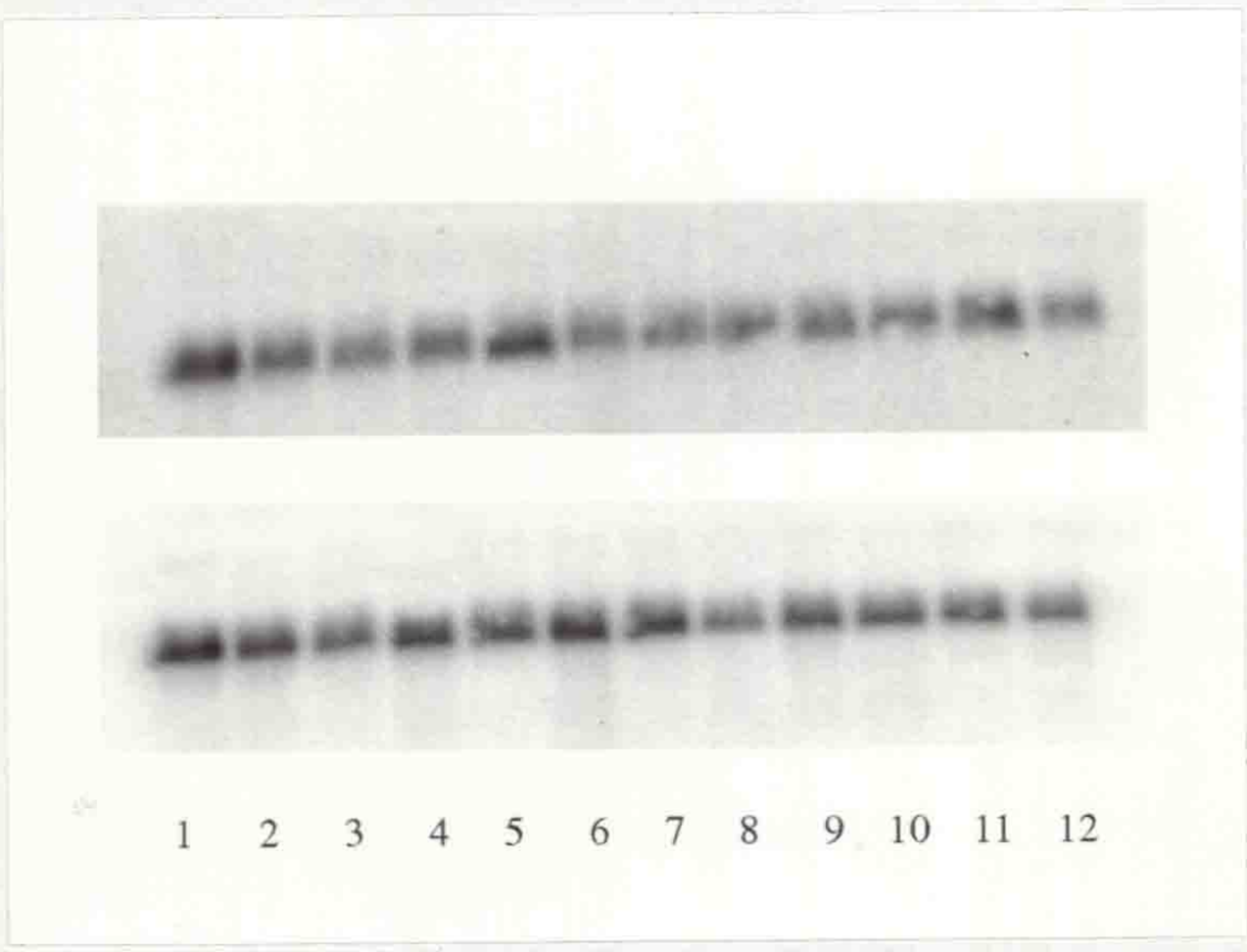


Figure 5.6 Representative Northern blots of total antral mRNA hybridised with specific cRNA probes for gastrin (upper panel) and somatostatin (lower panel). Lanes 1-6 are control and lanes 7-12 BAC-treated rats. BAC treatment significantly lowered gastrin and somatostatin mRNA abundance. These changes were considered specific as BAC treatment had no effect on GAPDH mRNA abundance (results not shown).

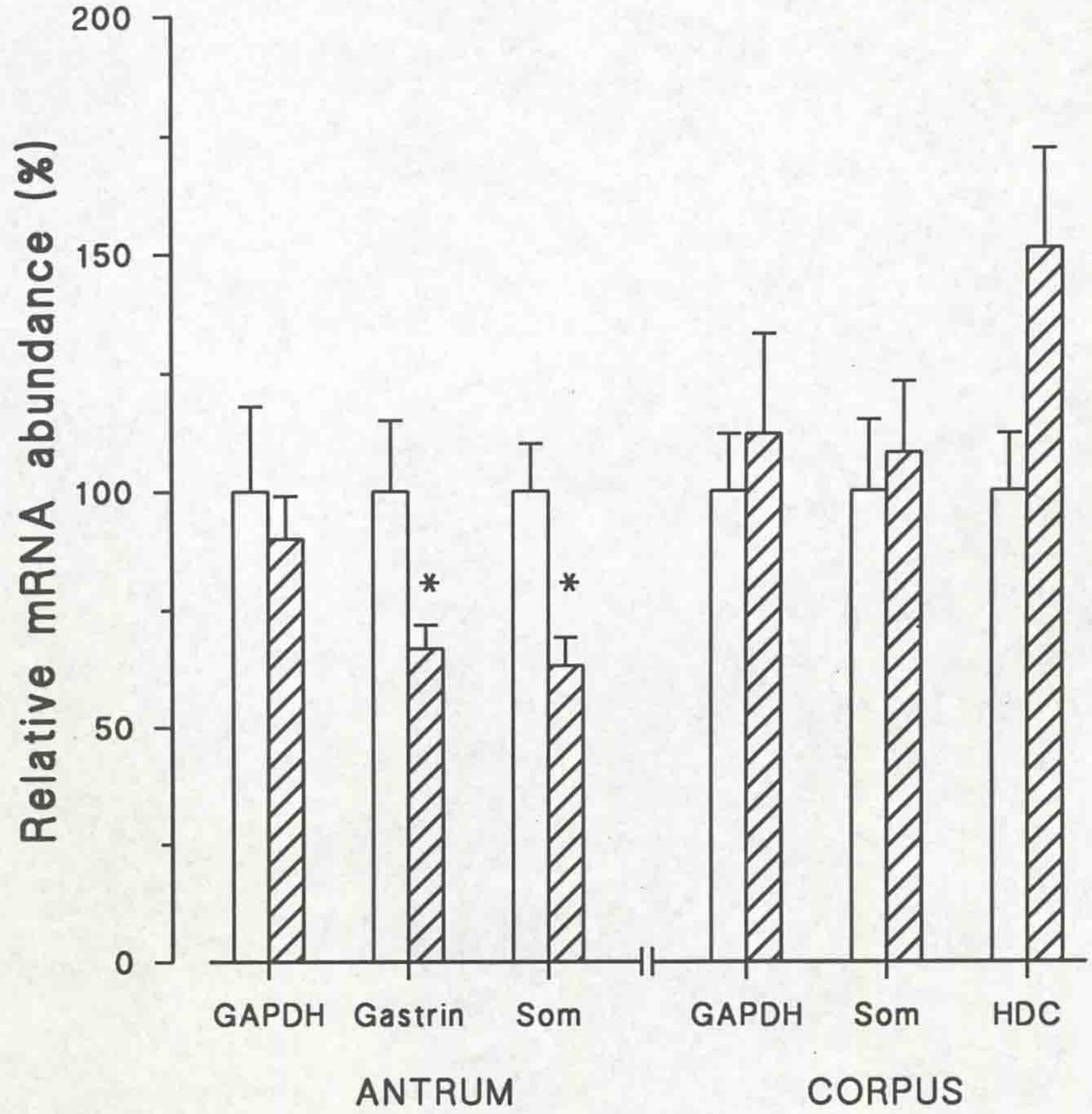


Fig 5.7 Relative mRNA abundance in the antrum of GAPDH, gastrin and somatostatin, and in the corpus of GAPDH, smatostatin and HDC in control (open bars) and BAC-treated rats (hatched bars). The relative abundance of each mRNA species has been normalised by taking as 100% the abundance in controls. Note the abundance of the non-regulated marker GAPDH was similar in control and BAC treated rats. In the antrum BAC decreased gastrin and somatostatin mRNA (n=6 in each group; * $p<0.05$, Students *t* test).

Discussion

In previous studies that have examined the neuronal regulation of G-cell function the extrinsic innervation has been manipulated by stimulation, lesioning or pharmacological blockade (Dockray & Gregory, 1989; Walsh, 1994); the role of intrinsic neurons has been largely determined by pharmacological approaches using neurotransmitter antagonists or antibodies (Schubert *et al.*, 1985; Schubert *et al.*, 1992). In the present experiments antral denervation by BAC was used to lesion both the intrinsic and extrinsic innervation of the rat antrum. The subsequent experimental data support the conclusions that the post-prandial gastrin response in control rats is largely mediated by the neuropeptide transmitter GRP; in antrally denervated rats, a post-prandial gastrin response is maintained but this is no longer inhibited by a GRP antagonist. Instead, it appears that non-nutritive distension becomes a strong stimulus for the denervated G-cell and this explains for the most part the fasting and meal stimulated hypergastrinaemia seen in the antrally denervated rat. The results are unexpected given that it is generally thought that G-cell responses to distension are mediated by nervous reflexes (Dockray & Gregory, 1989; Walsh, 1994; Schubert & Makhlouf, 1993).

Since gastrin release is inhibited by luminal acid, one potential explanation for the elevated plasma gastrin in antrally denervated rats might be reduced acid output. In fact there was a decrease in basal acid secretion after denervation. However, the capacity of the denervated G-cell to respond to distension was increased even when intragastric pH was buffered at pH 6.0 suggesting that G-cell responses secondary to

changes in acid output are unlikely to be important in the response to denervation. Furthermore, in undistended BAC-treated rats with a gastric fistula plasma gastrin was comparable to control rats suggesting that basal acid output was sufficient to maintain inhibition of gastrin release. In man, gastric distension is a good stimulus to acid secretion (Soares *et al.*, 1977), but in the conscious rat, it has been reported that non-nutritive distension of the stomach inhibits acid secretion by activating a sympathetic reflex (Dimaline *et al.*, 1986). Hence the chronic gastric retention after antral denervation may lead to inhibition of acid output by this mechanism.

In support of this argument, it is clear that after lesioning the antral innervation there was a pronounced gastric retention of solids, even after 48hr fasting. After complete emptying of the stomach in antrally denervated rats fitted with a gastric fistula, basal plasma gastrin was similar to control levels, suggesting that the hypergastrinaemia in denervated rats without a gastric cannula was attributable to the gastric retention.

Studies in the isolated perfused rat stomach have provided evidence that GRP mediates the release of gastrin in response to luminal peptone (Schubert *et al.*, 1992). The observation that the post-prandial increase in plasma gastrin concentration was blocked by a GRP antagonist suggests that a similar mechanism operates in conscious rats *in vivo*. However, the post-prandial increase in gastrin in antrally denervated rats was not blocked by a GRP antagonist suggesting that an additional mechanism comes into play after denervation. Interestingly, the gastrin response to gastric distension

with peptone in antrally denervated rats was no greater than that to non-nutritive distension. Two inferences may be drawn from this: First, that the stimulatory effect of nutrient is normally mediated by antral neurons, and second that antral neurons normally inhibit G-cell responses to non-nutrient distension. Studies in the isolated perfused rat stomach have shown that antral neurons mediate stimulation of gastrin release after distension at low pressures, and inhibition at high pressures (Schubert & Makhlouf, 1993). The latter may explain why methylcellulose at pH6 did not stimulate gastrin release in the intact stomach. The novel finding in the present experimental series is that there exists a mechanism for distension-evoked release of gastrin which is independent of the antral innervation.

It is generally thought that gastrointestinal responses to mechanical distension are mediated by nervous reflexes. Two possible mechanisms might account for the unexpected observation that the denervated G-cell responds to non-nutritive distension. First, the G-cell or a nearby cell in the antral mucosa might be mechanosensitive (which is suppressed by the antral innervation), or develops such sensitivity after denervation. In this context it has been recognised for many years that enterochromaffin cells (EC) cells are mechano-sensitive (Bulbring and Lin, 1958; Kirchgessner *et al.*, 1992); denervated G-cells might therefore take on EC-cell like properties, or alternatively antral EC cells might regulate G-cell function for example through a paracrine mechanism. Second, non-nutritive distension of the stomach could release an endocrine factor from the gastric corpus, which increases gastrin release

from the denervated, but not innervated, antrum. In the dog, Debas *et al.* (1975) showed that distension of the corpus increased gastrin release from an extrinsically denervated antral pouch suggesting the existence of an oxynto-pyloric endocrine reflex. The existence of a similar reflex in the rat might explain the present findings. If so, however, it would appear that such a reflex is not normally activated in the innervated antrum.

Hypergastrinaemia stimulates the enterchromaffin-like (ECL) cells of the gastric corpus which are an important source of histamine. The increased HDC mRNA abundance observed in BAC-treated rats is compatible with the hyper-gastrinaemia of antral denervation. Increased histamine synthesis and secretion might be expected to stimulate increased acid output, and the reduced acid secretion observed would therefore appear not to be secondary to inhibition at the level of the ECL cell. In the antrum we found marginally reduced gastrin and somatostatin mRNA abundance. Gastrin synthesis is regulated both at the level of gene transcription and at the level of mRNA translation (Bate *et al.*, 1996). The present data raise the possibility that after denervation there is independent control of release of synthesis at a post-transcriptional level. Furthermore, given that somatostatin and gastrin mRNA abundance appeared to be reduced following denervation without an overall depression in gene transcription (indicated by similar abundance of GAPDH in control and BAC treated rats) it seems possible that the innervation of the antral mucosa normally may regulate transcription of specific genes in target endocrine cells. Taken together with the possibility that following denervation G-cells may themselves

take on the EC-cell property of mechanosensitivity, it would appear that antral neurons play some role in the maintenance of the normal differentiated state of the G-cell.

Unfortunately the factors that regulate the differentiation of G-cells have proved difficult to study not least because of the problems of generating G-cell lines. However the mechanisms of differentiation in endocrine cells per se have been studied in pancreatic islet cells. Interestingly, gastrin is expressed in the foetal pancreas at the time of endocrine cell development (Brand and Fuller, 1988) and there is good evidence to suggest that gastrin acts in conjunction with TGF-α to promote normal pancreatic endocrine cell differentiation (Wang *et al.*, 1993). In adult rats, following partial pancreatectomy, pancreatic islet cell regeneration occurs and is associated with expression of the Reg gene (*reg*) (Terazono *et al.*, 1988) implying that *reg* expression may be involved in differentiation. The latter is of interest because the *reg* gene has been shown recently to be present in the gastric ECL cell (Ashahara *et al.*, 1996), a cell whose gene expression is well known to be regulated by gastrin. In addition *reg* expression is increased during the regeneration phase following acute gastric mucosal injury (Ashahra *et al.*, 1996).

It is plausible to suppose therefore that the remodelling of gastric mucosal function after denervation involves gastrin itself, acting in conjunction with other factors to regulate differentiation of endocrine cells, including the ECL cell. The

studies detailed in the next chapter begin to investigate the possibility that gastrin regulates *reg* expression.

Summary

In the intact rat, luminal nutrient releases gastrin via activation of neurons secreting GRP whilst the antral innervation normally inhibits gastrin release in response to non-nutrient distension. After antral denervation, post-prandial gastrin release is maintained but by a mechanism independent of neurons secreting GRP. Moreover, gastric distension with a non-nutrient solution becomes an adequate stimulus for gastrin release. Despite reductions in gastrin and somatostatin mRNA abundance following BAC treatment, hypergastrinaemia occurred and produced changes in ECL-cell gene expression although basal acid output was reduced. The altered G-cell function that occurred after denervation and the possibility that this effected changes in gastric endocrine cell differentiation remains to be determined.

Chapter Six

Control of *reg* expression in rat corpus mucosa and AR42J cells

Introduction

The work of several laboratories indicates that the *reg* gene is expressed during pancreatic or gastric mucosal regeneration (Terazono *et al.*, 1988; Miyaura *et al.*, 1991; Ashahara *et al.*, 1996). Since *reg* is expressed in pancreatic β cells and gastric ECL cells it is reasonable to suppose that it may be implicated in endocrine cell growth or differentiation. Gastrin is known to stimulate growth of ECL cells both in the human (Borch *et al.*, 1985; Bordi *et al.*, 1986; Roucayrol and Cattan, 1989; Solcia *et al.*, 1991) and the rat (Havu 1986; Hakanson and Sundler, 1990) and is essential for normal gastric (Koh *et al.*, 1997) and pancreatic (Wang *et al.*, 1993) endocrine cell development. In the latter case, gastrin must act in conjunction with growth factors for normal differentiation to occur. However, the possible interactions between gastrin, growth factors and *reg* expression have not been directly studied.

AR42J cells are an amphicrine cell line with the capability to differentiate into either acinar or endocrine cells depending on the tissue culture conditions (Christophe, 1994). These cells express both the gastrin/CCK-B receptor and the *reg* gene (Lambert *et al.*, 1991; Zenilman *et al.*, 1996), suggesting that they might be a useful system in which to investigate the relationships outlined above. The experiments performed in this chapter were undertaken first to determine whether gastrin might regulate *reg* gene expression in rat gastric corpus and second to study in the AR42J cell line some of the features of gastrin-induced *reg* expression. The results indicate that *reg* expression is regulated by gastrin acting via the CCK-B receptor. In addition,

although EGF potentiated gastrin-stimulated *reg* expression the reported data suggest that *reg* expression is associated with endocrine cell differentiation and not growth.

Methods

Animal Studies

Rats were either fasted for 48 hrs on wire-bottomed cages or fed ad libitum and given omeprazole (400μmol.kg^{-1}) by gavage for 5 days. Rats fed *ad libitum* were used as controls. At the end of treatment, trunk blood was taken for assay of plasma gastrin and samples of corpus mucosa were taken for RNA extraction and analysis of HDC and *reg* mRNA abundance by Northern blot.

Tissue Culture

Culture of AR42J cells and treatment with amidated and gly-extended gastrin, L740093 (1nM), EGF (10pM-1μM) and wortmannin (5 x 10^{-8}M) was performed by Dr. LA Bishop. Cells were cultured at a density of 1x10^{6} for 24 hrs in depleted media before appropriate treatment. All treatments were given for 24 hrs except for wortmannin (4 hrs). At the end of the culture period total cell mRNA was extracted by the lithium chloride precipitation method and reg mRNA abundance analysed by Northern blot (see Chapter 2).

Results

Reg expression in rat gastric corpus

In keeping with previously published observations (Dimaline *et al.*, 1993; Swarovsky *et al.*, 1994), treatment of Wistar rats with omeprazole (400μmol.kg^{-1}) for five days lead to a five to ten-fold increase in plasma gastrin and an associated three-fold increase in HDC mRNA in gastric corpus mucosa, compared to rats fed ad libitum. Conversely, fasting significantly reduced plasma gastrin and HDC mRNA abundance (Fig 6.1). In addition however, omeprazole-induced hypergastrinaemia also resulted in altered *reg* gene expression in the gastric mucosa, the changes in magnitude of *reg* mRNA abundance being almost identical to those of HDC (Fig 6.1).

Reg expression in AR42J cells

To examine in more detail how gastrin might regulate *reg* gene expression, AR42J cells were cultured in the presence of increasing concentrations of both amidated (G17-NH_2) and glycine-extended gastrin (G-gly). Amidated and glycine-extended gastrin both produced dose-dependent increases in *reg* expression with G-gly being approximately 1000-fold less potent than its amidated counterpart (Fig 6.2 & 6.3). To determine the relevant gastrin receptor mediating the *reg* response, AR42J cells were treated with either G17-NH_2 (10^{-8} M) or G-gly (10^{-6} M) in the presence of the specific CCK-B receptor antagonist L-740093. The increase in *reg* mRNA abundance stimulated by G17-NH_2 was completely inhibited by the CCK-B receptor antagonist (Fig 6.4). The response to G-gly, although significantly attenuated by L-740093, remained increased by approximately two-fold (Fig 6.5).

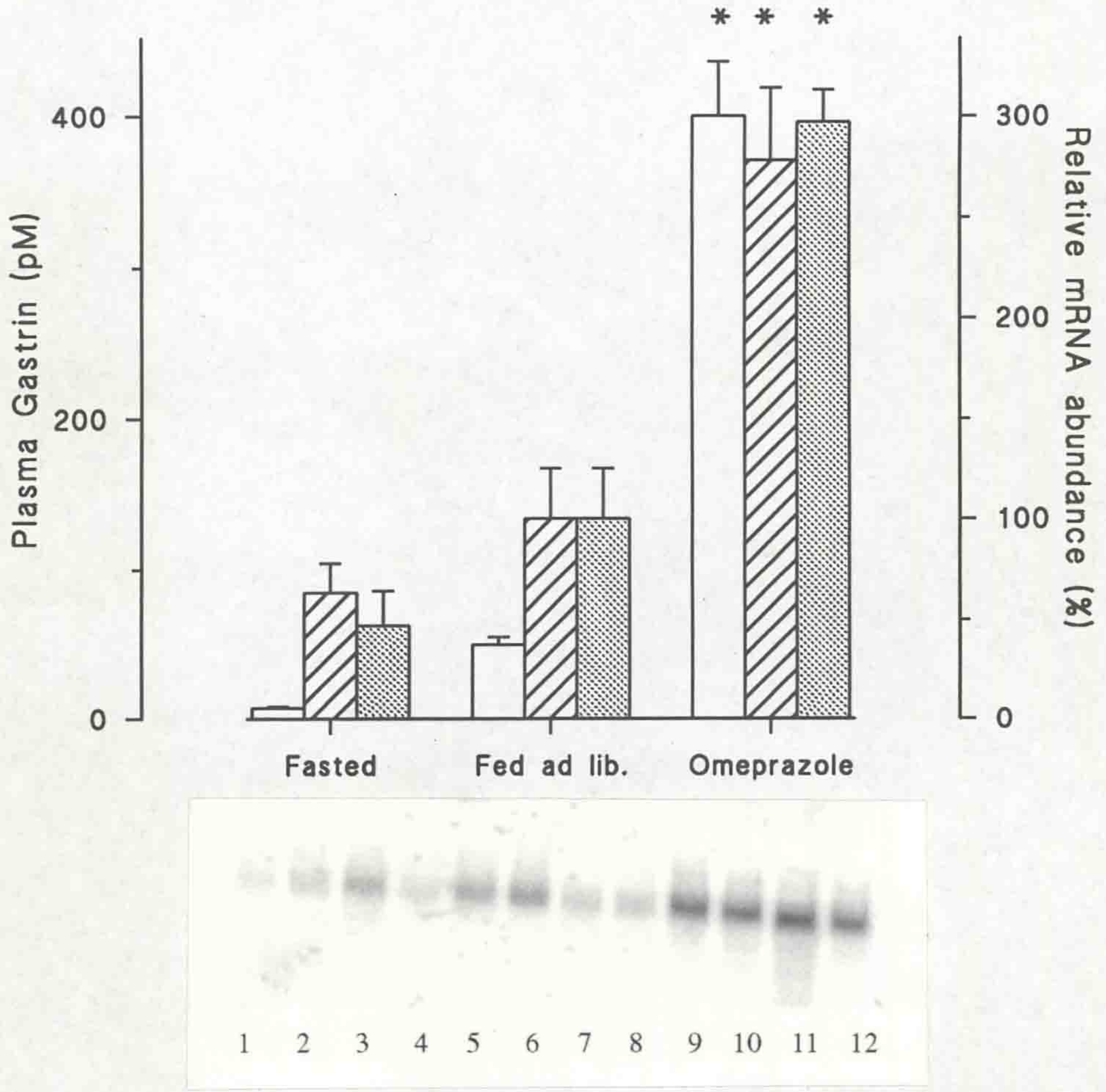


Figure 6.1 *upper panel:* Effect of fasting and omeprazole treatment on plasma gastrin (open bars), *reg* mRNA (hatched bars) and HDC mRNA abundance (stippled bars). Note *reg* mRNA abundance was decreased by fasting and increased by omeprazole treatment, the magnitude of the change paralleling that of HDC mRNA. (n=6 for each group, * $p<0.05$ vs control response, Mann Whitney U test). *lower panel:* Representative Northern blot of *reg* mRNA abundance in 48 hr fasted (lanes1-4), fed ad lib. (lanes 5-8) and omeprazole treated rats (lanes 9-12).

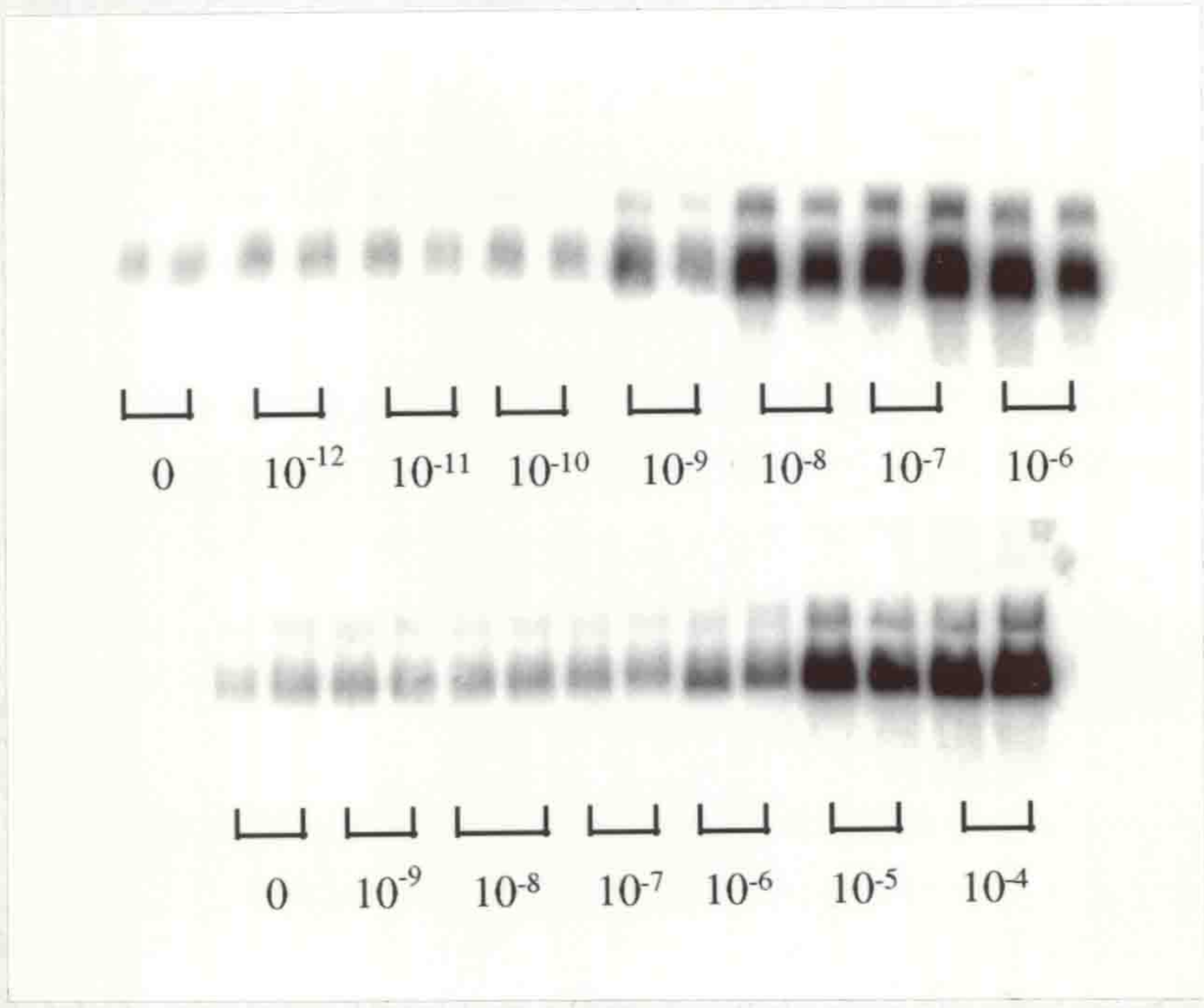


Figure 6.2 Representative Northern blots for *reg* mRNA abundance in total RNA extracted from duplicate cultures of AR42J cells treated with increasing molar concentrations of either G17-NH_2 (upper panel) or G-gly (lower panel). Note the dose range of the response is approximately 3 log units higher for G-gly.

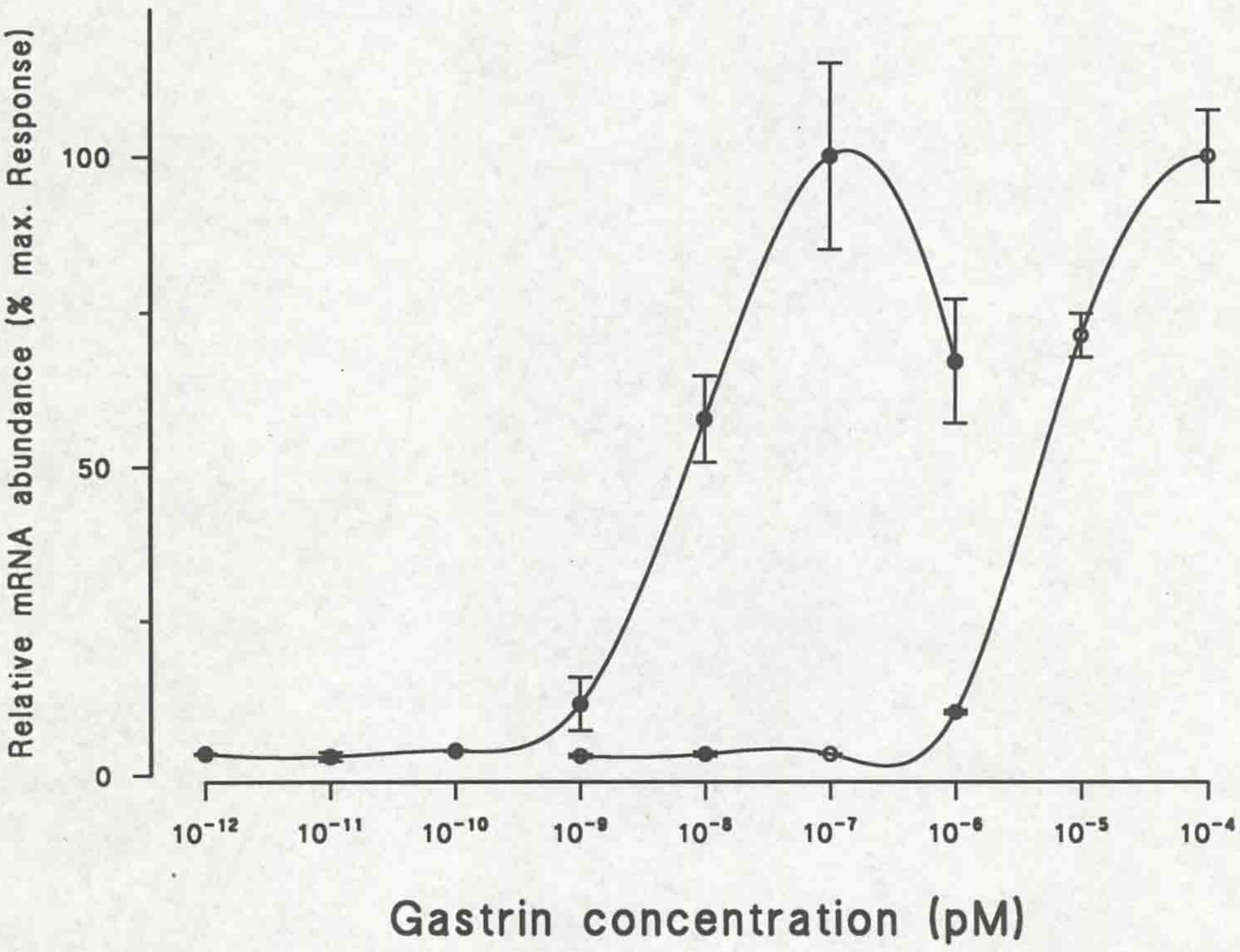


Figure 6.3 Dose response relationship between G17-NH_2 (closed circles) and G-gly (open circles) and *reg* mRNA abundance in AR42J cells. The A_{50} for G17-NH_2 was approximately 10^{-8}M and that for G-gly approximately $5x10^{-5}$M.

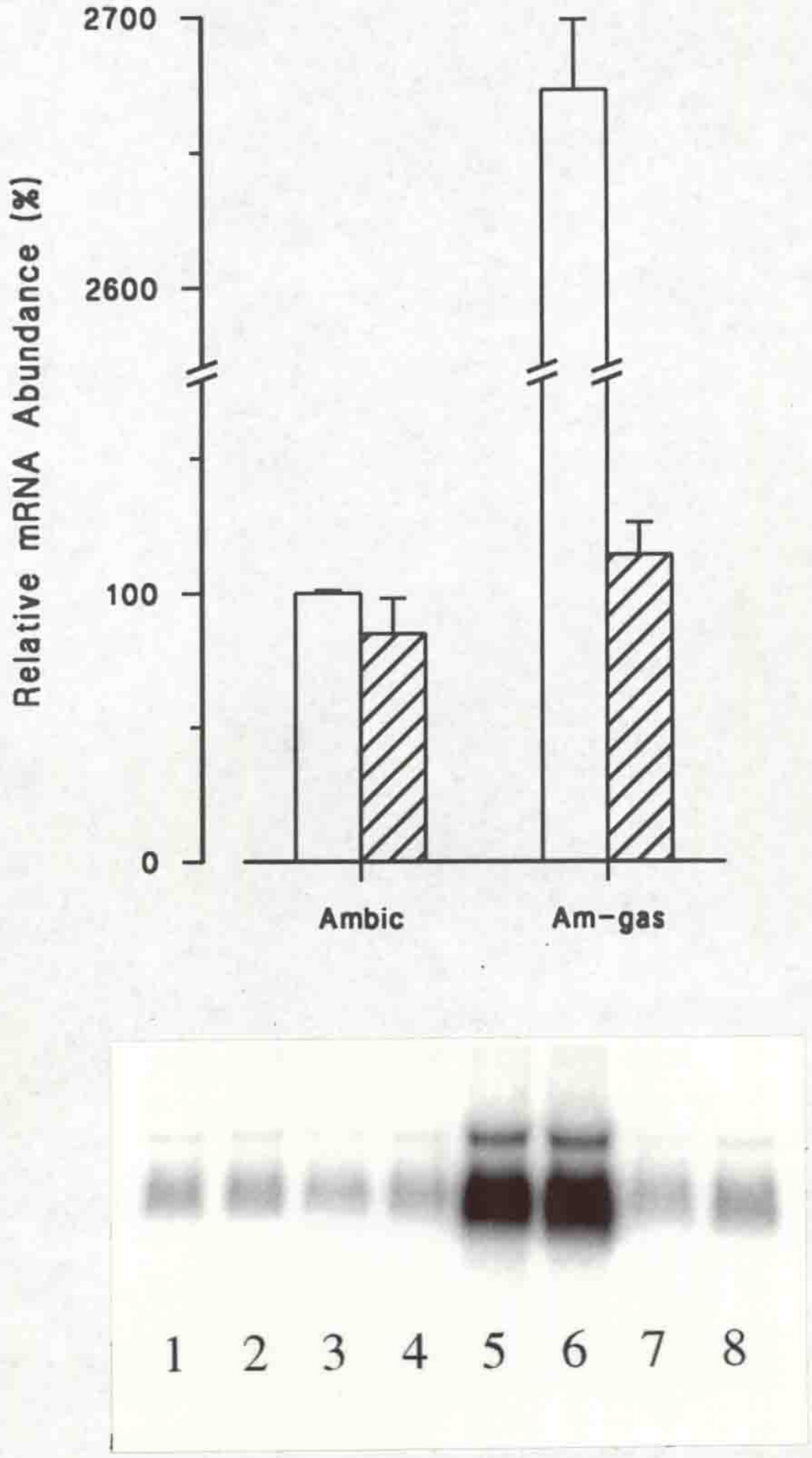
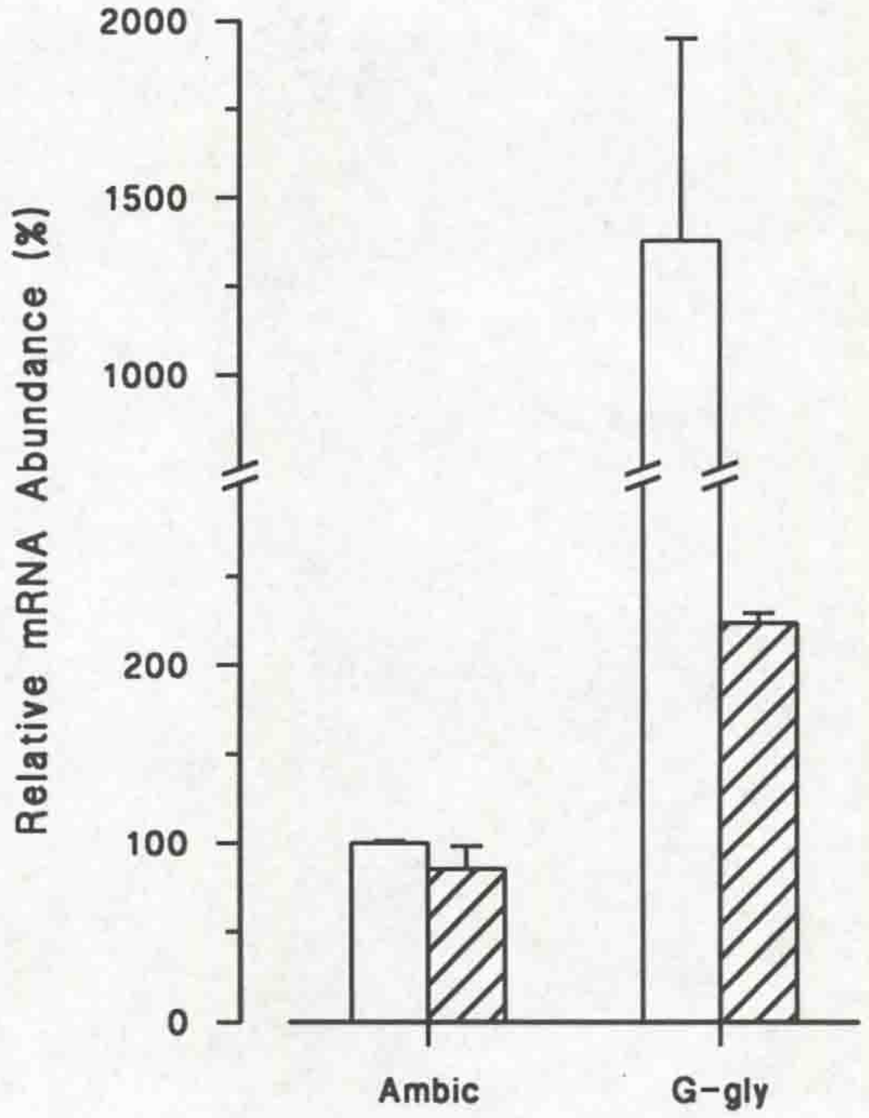


Figure 6.4 Effect of 10^{-9}M L-740093 (hatched bars) on the *reg* mRNA response to 10^{-8}M G17-NH_2 (upper panel) and a representative Northern blot (lower panel) of *reg* mRNA abundance in total RNA extracted from duplicate cultures of AR42J cells treated with vehicle (ammonium bicarbonate; lanes 1&2), L-740093 (lanes 3&4), G17-NH_2 (lanes 5&6) and G17-NH_2 and L-740093 (lanes 7&8). The twenty five-fold increase in mRNA abundance produced by G17-NH_2 was completely reversed by L-740093.



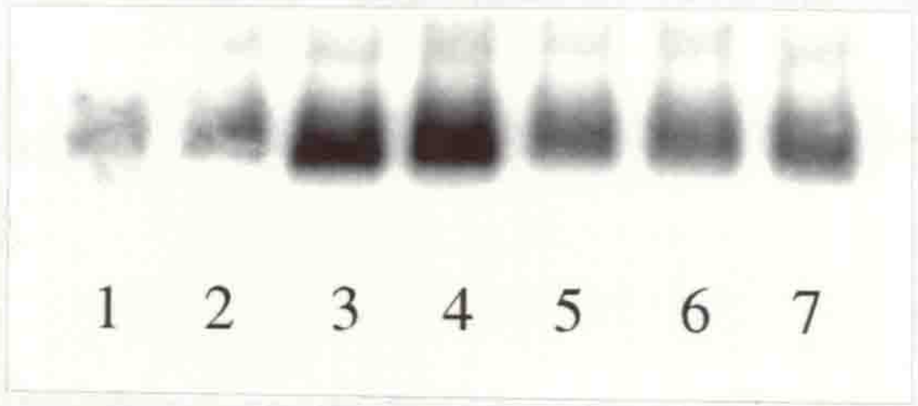


Figure 6.5 Effect of 10^{-9}M L-740093 (hatched bars) on the *reg* mRNA response to 10^{-6}M G-gly (upper panel) and a representative Northern blot (lower panel) of *reg* mRNA abundance in total RNA extracted from cultures of AR42J cells treated with ambic (lanes 1&2) G-gly (lanes 3&4) and G-gly and L-740093 (lanes 5-7). Although the response to G-gly was significantly attenuated by L-740093, *reg* mRNA abundance remained increased by approximately two-fold.

EGF and *Reg* expression in AR42J cells

The effects of EGF on *reg* expression is shown in Fig 6.6. At the highest dose of EGF (10^{-6}M) there is no significant upregulation of *reg* expression even though this concentration of EGF has been shown to induce proliferation of AR42J cells. However, EGF, at a concentration of 10^{-9}M potentiated the effects of amidated and glycine-extended gastrin on *reg* expression (Fig 6.7). Hence the ten to twenty-fold increase in *reg* expression induced by 10^{-8}M G17-NH_2 was further enhanced ten-fold by EGF (10^{-9}M).

Wortmannin and *Reg* expression in AR42J cells

Inhibition of the PI3 kinase in cultured human foetal pancreatic cells has been shown to induce differentiation towards an endocrine cell phenotype (Ptasznik *et al.*, 1997). Treatment of AR42J cells with the PI3 kinase inhibitor wortmannin (5×10^{-8} M) for four hours, increased *reg* expression in both unstimulated and G17-NH_2-stimulated AR42Jcells (Fig 6.8).

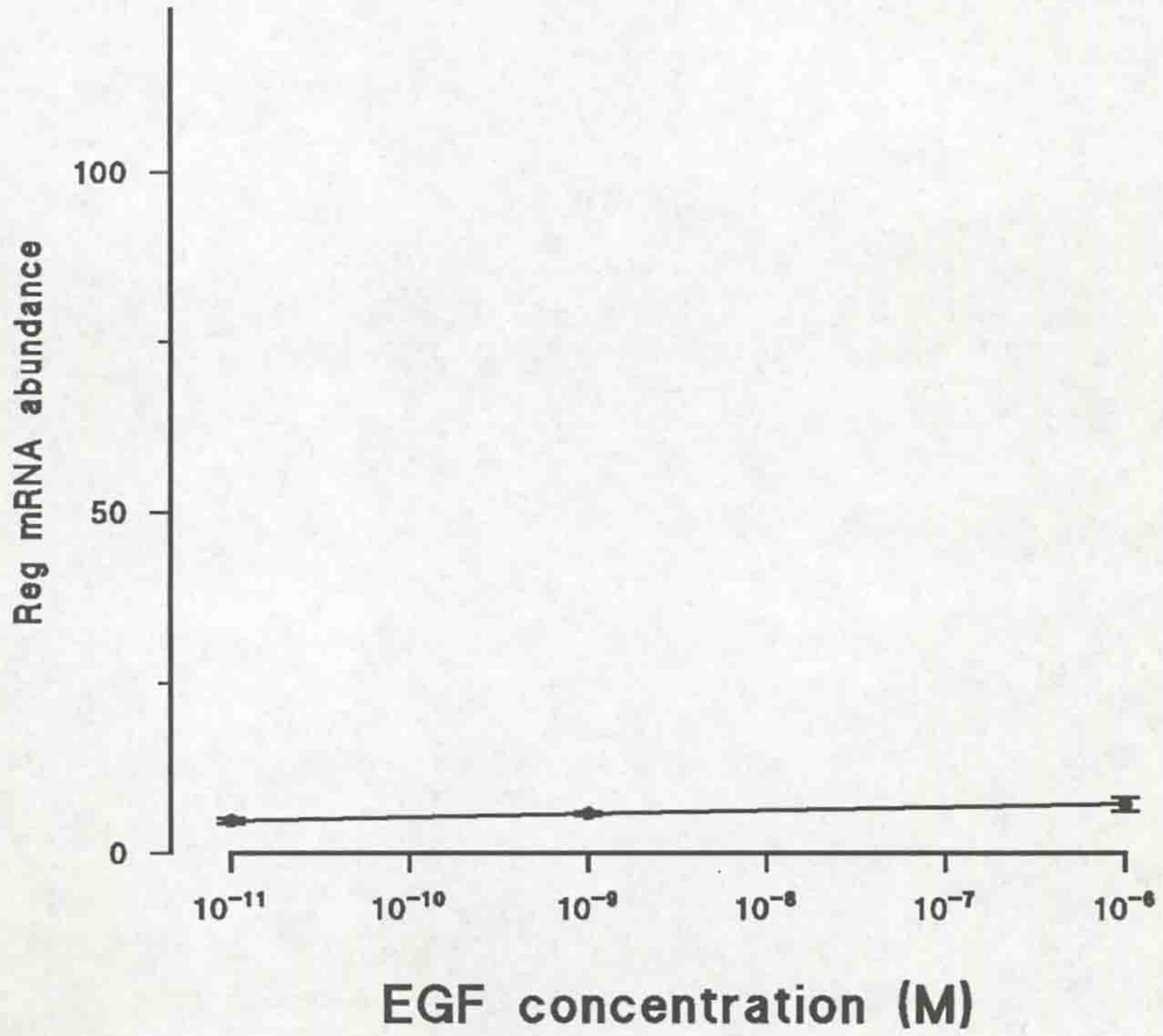


Figure 6.6 u*pper panel:* Relationship between increasing doses of EGF and *reg* mRNA abundance in AR42J cells. Results are expressed relative to mRNA expression in control cultures arbitrarily defined as 5% of a predicted maximum response. Even at 10^{-6}M EGF there is no increase in *reg* mRNA abundance. *lower panel:* Representative Northern blot of *reg* mRNA in duplicate cultures of AR42J cells exposed to increasing concentrations of EGF.

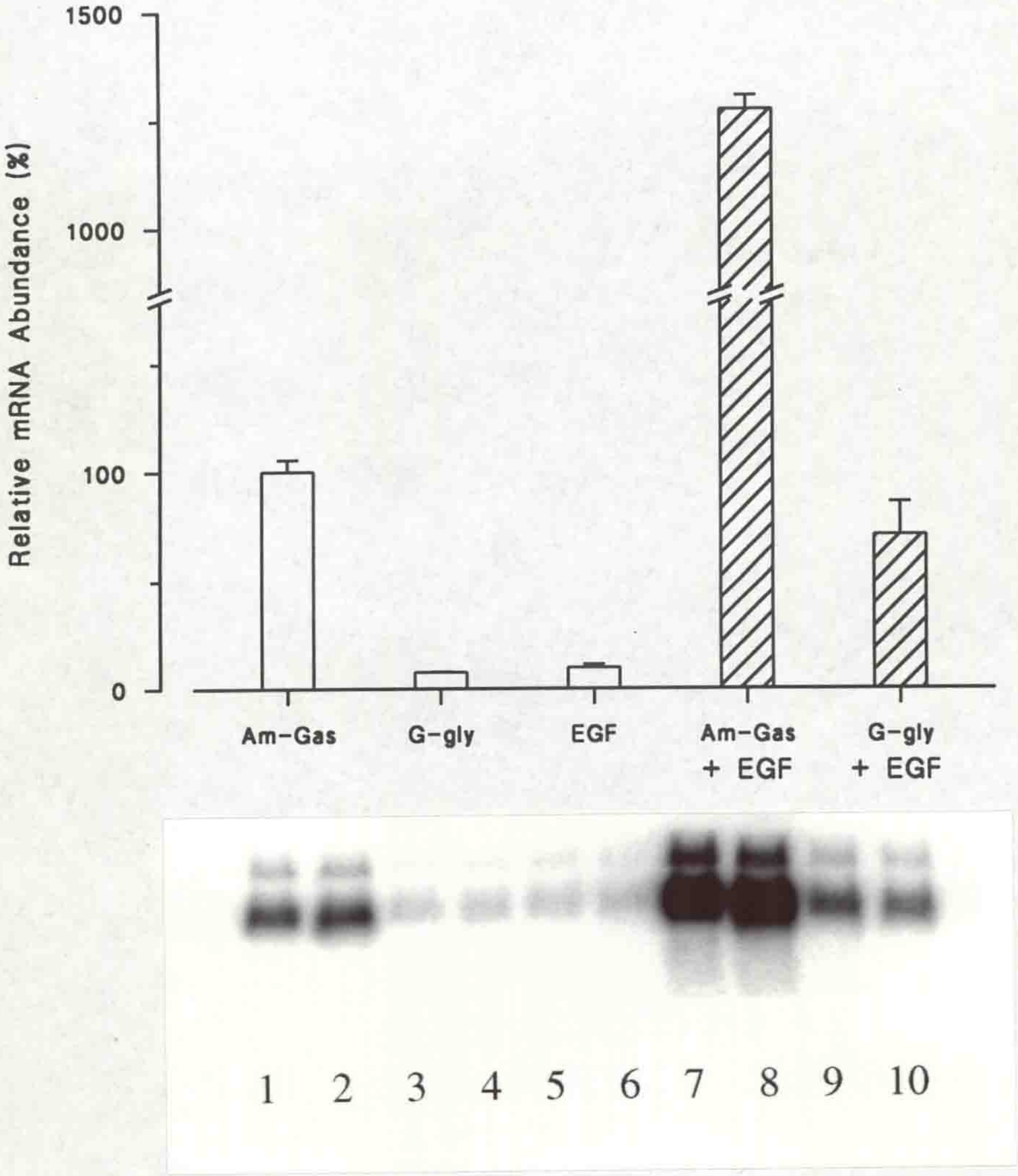
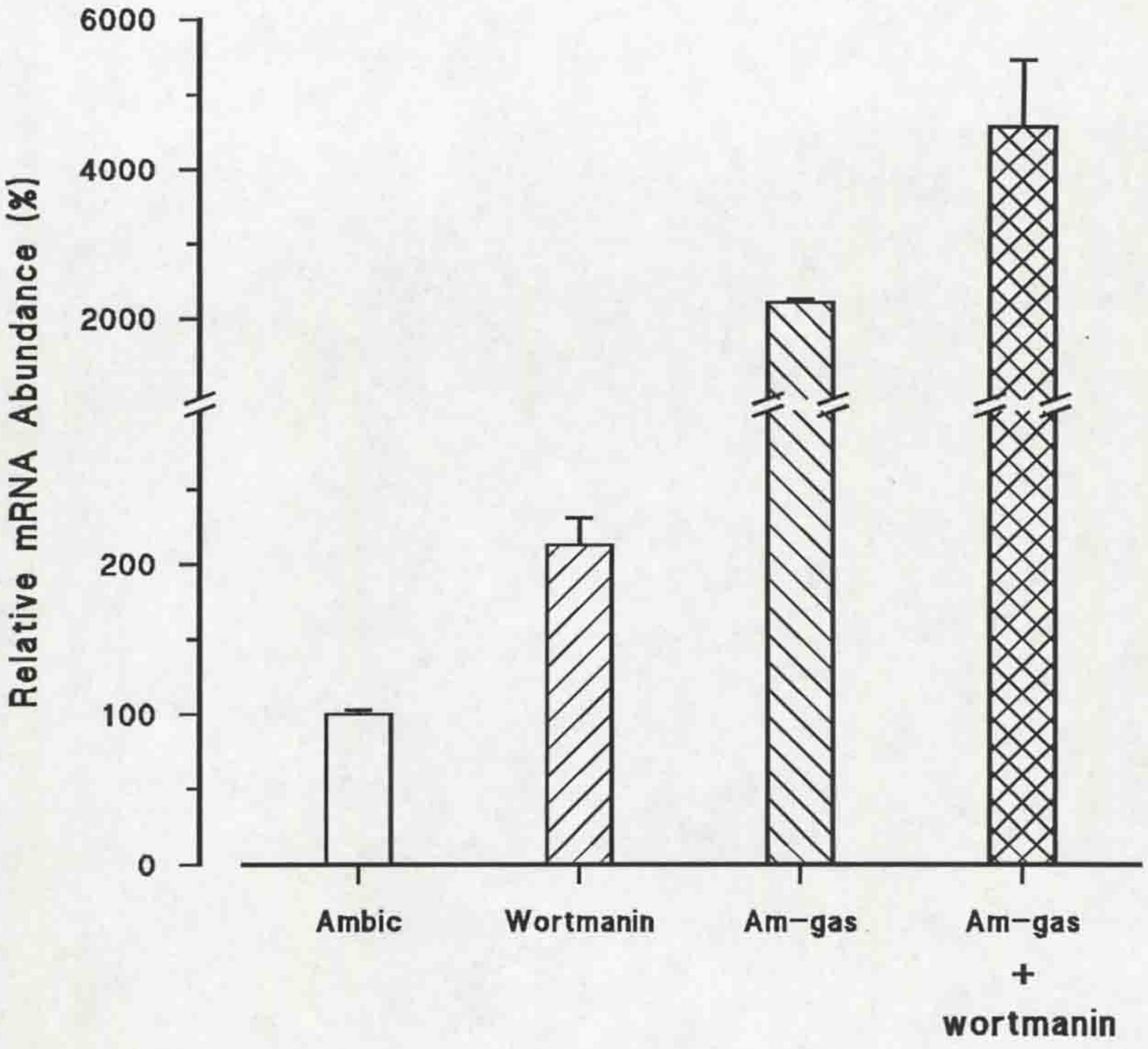


Fig 6.7 *upper panel:* relationship between G17-NH_2 (10^{-8}M) or G-gly (10^{-7}M), EGF (10^{-9}M) and *reg* mRNA abundance in AR42Jcells. Results have been normalised to the response to 10^{-8}M G17-NH_2 taken as 100%. The response of AR42Jcells to either G-gly or EGF alone was approximately 10% of the G17-NH_2 response. However, EGF increased the response of AR42J cells to G17-NH_2 by fifteen-fold and to G-gly by seven-fold. *lower panel:* Representative Northern blots for *reg* mRNA in duplicate cultures of AR42J cells treated with G17-NH_2 (lanes 1&2), G-gly (lanes 3&4), EGF (lanes 5&6), G17-NH_2 and EGF (lanes 7&8) and G-gly and EGF (lanes 9&10).



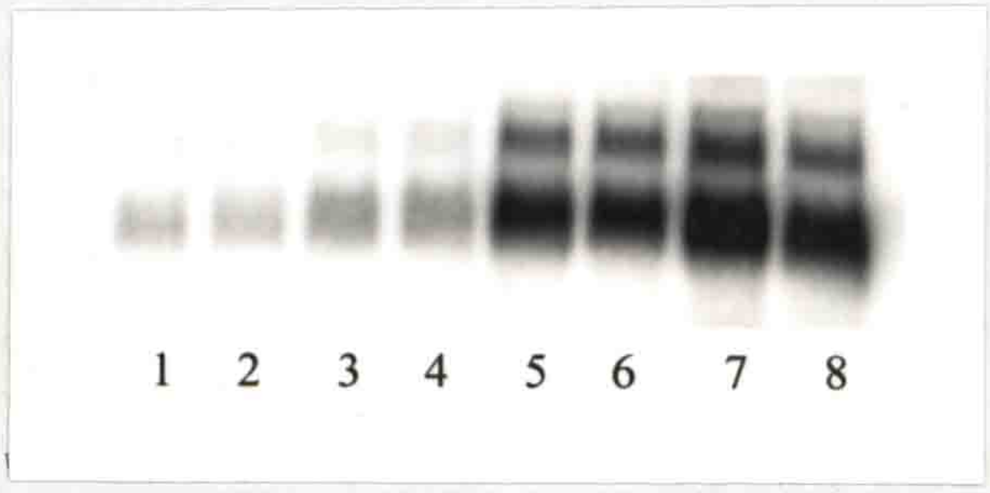


Fig 6.8 *upper panel:* relationship between G17-NH_2 (10^{-8}M), wortmannin (5 x 10^{-8}M) and *reg* mRNA abundance in AR42Jcells. The gastrin-stimulated response in *reg* mRNA is further increased two-fold by wortmannin. Note that wortmannin also stimulates *reg* mRNA expression in control cultures. *lower panel:* Representative Northern blots for *reg* mRNA in duplicate cultures of AR42J cells treated with Ambic (lanes 1&2), wortmannin (lanes 3&4), G17-NH_2 (lanes 5&6) and G17-NH_2 and wortmannin (lanes 7&8).

Discussion

The data presented in this chapter provide evidence that *reg* expression in rat corpus mucosa responds to changes in plasma gastrin and in AR42J cells is regulated by amidated gastrin acting through the gastrin/CCK-B receptor. In addition, whilst EGF alone had no effect on *reg* expression it markedly potentiated gastrin stimulated *reg* mRNA abundance. Furthermore, in culture conditions that have been shown to favour differentiation of AR42J cells into insulin-secreting endocrine cells, *reg* gene expression was increased.

Regulation of ECL cell gene expression by plasma gastrin has been demonstrated for a variety of genes including HDC (Dimaline *et al.*, 1991; Swarovsky *et al.*, 1994;).The observed threefold increase in HDC mRNA abundance following 5 day omeprazole treatment was in keeping with previously reported data. The presence of *reg* in rat ECL cells has been reported only recently (Ashahara *et al.*, 1996) and the results presented here demonstrate for the first time that *reg* gene expression in vivo can be regulated by plasma gastrin.

The present results contrast with those of Zenilman *et al.*, (1997) who showed that gastrin, CCK and glucagon (all 10nM) had no effect on reg mRNA abundance in AR42J cells. However, the tissue culture protocol in the experiments reported by Zenilman did not incorporate 24 hrs of culture in serum depleted media prior to the period of treatment. AR42J cells have been reported to synthesise and secrete gastrin

that may then act in an autocrine manner (Blackmore and Hirst, 1992) and this may explain the discrepancy between the above findings and the lack of effect of gastrin seen by Zenilman and colleagues. An additional explanation of the results of Zenilman *et al.*, is the influence of the cell density of AR42J cells on *reg* gene expression. In preliminary experiments it was found that *reg* gene expression was upregulated (approximately tenfold) as cell density reached confluence. Zenilman *et al.* conducted their experiments on confluent cells unlike the experiments reported here.

The gastrin stimulated *reg* expression in AR42J cells appears to act via the gastrin/CCK-B receptor known to be present on these cells (Lambert *et al.*, 1991). The reported dissociation constant for gastrin at this receptor in AR42J cells is 4nM (Zhou *et al.*, 1992). The A_{50} of the *reg* response to G17-NH_2 was ≈10nM and the complete inhibition of the response by the specific gastrin/CCK-B receptor antagonist L-740093 indicates that this response is mediated via the gastrin/CCK-B receptor. The *reg* response to G-gly also was inhibited by L-740093 but only by ≈80%. The dose of G-gly required to increase *reg* expression was much higher than that for G17-NH_2 and given that L-740093 is a competitive antagonist at this receptor it seems likely that the effects of G-gly on *reg* expression also are mediated via the gastrin/CCK-B receptor. The parallel rightward displacement of the G-gly dose-response curve compared to that of G17-NH_2 also suggested that G-gly was acting at the same receptor as G17-NH_2 but with lower affinity.

Perhaps by coincidence, the magnitude of change in *reg* mRNA abundance in the rat gastric mucosa was identical almost to that of HDC. Given that gastrin regulates HDC gene expression via the gastrin/CCK-B receptor (Hollande *et al.*, 1994), this might suggest a common intracellular pathway regulating HDC and *reg* gene expression. The rat and human HDC promoter has been shown to be regulated by a protein kinase C dependent pathway (Hocker *et al.*, 1996; Zhang *et al.*, 1996). For the human HDC gene a palindromic sequence (5'-CCCTTTAAATAAAGGG-3') that confers transcriptional responsiveness to gastrin has been identified and defined as a gastrin response element. A similar sequence is present within the rat HDC promoter region (5'-CCCTTAAATAAGAGGG-3'). In each case the putative response element lies within 25 base pairs of the transcriptional start site. The 5'-regulatory region of the rat *reg* gene has also been described in AR42J cells (Miyashita *et al.*,1994). The regulatory sequence of most significance indicates a region -256 to -237 upstream of the transcriptional start site that has significant sequence homology with the consensus sequence of the pancreatic exocrine enhancer (Boulet *et al.*, 1986). Although this would suggest a separate regulatory mechanism for *reg* gene transcription in AR42J cells, the response to gastrin was not studied. However, there does not appear to be a sequence similar to the putative gastrin response element of the rat HDC gene within the reported promoter region of the rat *reg* gene. Nevertheless the data clearly indicate that gastrin regulates *reg* expression in AR42J cells via a gastrin/CCK-B receptor mediated pathway.

Other effects of gastrin on AR42J cells that have been shown to depend on a gastrin/CCK-B receptor mediated pathway include stimulation of amylase secretion (Lambert *et al.*, 1991) and stimulation of growth (Pradel *et al.*, 1993; Seva *et al.*, 1994; Zenilman *et al.*, 1997). This raises the possibility that *reg* gene expression may be part of the growth response of AR42J cells to gastrin. This seems unlikely for two reasons: Firstly, the growth promoting effects of G17-NH_2 on AR42J cells is relatively weak compared to that of EGF (Watson *et al.*, 1993). Despite this, the data above demonstrate that 10^{-6}M EGF had no effect on *reg* gene expression although this dose would be expected to produce a large trophic response. This indicates that *reg* expression, although present in AR42J cells, is not required for cell growth. Secondly, treatment of AR42J cells with the PI3 kinase inhibitor wortmannin which has been shown to inhibit cell growth and promote endocrine cell differentiation (Ptasznick *et al.*, 1997) was found to increase *reg* gene expression. The effect of wortmannin on AR42J cell *reg* expression was not confined to unstimulated cells as it also potentiated the effects of gastrin on *reg* expression.

Several other lines of evidence indicate that *reg* gene expression is associated with differentiation towards an endocrine cell phenotype. Treatment of AR42J cells with dexamethasone has been shown to promote differentiation of an acinar cell phenotype and concurrently reduced *reg* expression (Zenilman *et al.*, 1997). Conversely, treatment with activin A, betacellulin or HGF stimulates differentiation of AR42J cells towards an endocrine cell phenotype (Mashima *et al.*, 1996a; Ptasznik *et al.*, 1997; Mashima *et al.*, 1996b) and HGF has been shown to increase *reg* expression

in human foetal pancreatic cells (Otonkoski *et al.*, 1994). Taken together, the experimental evidence suggests that *reg* expression in AR42J cells is associated with differentiation towards an endocrine cell phenotype. If so, then this process would be expected to be favoured by treatment with gastrin and potentiated by either EGF or wortmannin. The data are of interest as AR42J cells may provide a useful model to elucidate the intracellular pathways that mediate these effects.

The interaction between growth factors, gastrin and the differentiation of endocrine cells has been described in the mouse pancreas (Wang *et al.*, 1993). Here at least, both are required in conjunction for normal development of the endocrine pancreas. In the stomach, gastrin does not seem to be essential for normal ECL cell development as these cells can still be found in the gastric mucosa of gastrin knockout mice (Koh *et al.*, 1997). However, in this model ECL cell numbers were significantly reduced implying that gastrin regulates the number of mature ECL cells in the stomach perhaps by influencing the growth and differentiation of precursor cells. There are no data as yet on *reg* expression in such experimental situations but the provisional data presented thus far indicate that *reg* expression may be a marker for ECL cell function and more specifically may play a role in the maintenance of a normal ECL population within the gastric mucosa. The clinical implications of this are the subject of the next chapter.

Summary

Reg expression in rat gastric corpus was increased by 5 day omeprazole treatment. In AR42J cells *reg* expression was shown to be regulated by amidated gastrin acting through the CCK-B receptor. EGF alone had no effect on *reg* expression but markedly potentiated the gastrin stimulated response. In culture conditions that favour differentiation of AR42J cells into insulin-secreting endocrine cells, *reg* gene expression was increased. The observations of others clearly indicate that growth factors acting together with gastrin may regulate endocrine cell growth and differentiation in the gastrointestinal tract. AR42J cells may provide a useful model to elucidate the intracellular pathways that mediate these effects.

Chapter Seven

Reg gene expression in human gastric mucosa.

Introduction

In rodents hypergastrinemia secondary to long-term treatment with H_2-receptor antagonists or proton pump inhibitors (PPI) may lead to gastric carcinoid tumours (Hava, 1986; Poynter and Selway, 1991; Wangberg *et al.*, 1995). This has prompted extensive reviews of the natural history of these tumours in man (Solcia *et al.*, 1991; Thomas *et al.*, 1994; Rindi, 1995; Modlin *et al.*, 1995). It is now clear that hypergastrinaemia from whatever cause may result in hyperplasia of the gastric ECL cell. Significant ECL-cell hyperplasia has been demonstrated in one third of patients rendered hypergastrinaemic by *H pylori* infection and coincident treatment with a PPI (Eiselle *et al.*, 1997). The majority of patients with CAG display some degree of ECL cell hyperplasia, the achlorhydria of CAG leading to hypergastrinemia that is generally accepted to be a stimulus to ECL cell growth (Creutzfeld, 1988; Hakanson and Sundler, 1990; Bordi *et al.*, 1995). In fact, chronic atrophic gastritis (CAG) with or without pernicious anaemia is the commonest cause of enterochromaffin-like (ECL-) cell hyperplasia and in 5-10% of patients there is a progression to gastric carcinoid tumours (Borch *et al.*, 1985; Bordi *et al.*, 1991; Bordi *et al.*, 1995).

The recent demonstration of *reg* gene expression in rat ECL cells together with the evidence suggesting that the reg protein may be important for pancreatic endocrine cell growth and differentiation imply a possible role for *reg* in the control of

ECL cell growth and differentiation. Gastrin has been shown to be important for entero-pancreatic endocrine cell development (Wang *et al.*, 1993; Koh *et al.*, 1997), acting in concert with growth factors such as TGF-α. In keeping with these observations, the results presented in the previous chapter showed gastrin stimulation of *reg* expression, potentiation of this response by EGF and upregulation of *reg* mRNA in AR42J cells exposed to culture conditions that favour differentiation towards an endocrine cell phenotype. The experiments performed in this chapter attempt to determine whether *reg* expression in the human gastric corpus is affected by plasma gastrin concentration and whether *reg* may have a pathophysiological role in ECL cell tumour development in patients with chronic hypergastrinaemia.

Methods

Human studies

The details of the patients that provided samples for the experimental studies described in this chapter are shown in Table 7.1 Plasma gastrin was assayed by RIA using antibody L2. Endoscopic biopsies were taken from the gastric corpus and from any corpus nodules if present from hypergastrinaemic patients that required a gastroscopy. Duplicate biopsies were sent to Dr G Armstrong (Consultant Histopathologist, Hope Hospital) for Histology. Total mRNA was extracted from biopsies and *reg* mRNA analysed by Northern blot and RT/PCR, cloning and sequencing (see Chapter 2). For the Northern blots, *reg* mRNA is expressed relative to the non-regulated mRNA for GAPDH.

Octreotide Suppression Test

Two of the patients with carcinoid tumours (JH & KD) consented to an octreotide suppession test. The intended study was explained and informed consent obtained from both patients. Endoscopic biopsies were taken from ECL cell nodules and apparently normal corpus prior to and at the end of a 72hr infusion of octreotide ($25mg.hr^{-1}$). Plasma samples were taken before, during and after the octreotide infusion for assay of gastrin.

PATIENT	AGE	SEX	DIAGNOSIS	PLASMA GASTRIN (pM) G-NH2	ECL Cell Polyps	Polyp size
J. H.	44	F	P.A. Nodules	850	>50	>1cm
K.D.	42	M	P.A. Nodules	600	2	1cm + <5mm
K.W.	72	F	P.A. Nodules	1200	>20	<1cm
P.S.	47	M	Chronic Renal Failure Diabetes Mellitus	650	<10	<5mm
M.B.	56	F	Z.E./MEN1	280	<10	<5mm
P.G.	65	F	P.A.	450	<10	<5mm
P.M.	61	F	P.A.	400	<10	<5mm
W.S.	72	M	P.A.	480	<10	<5mm
J.C.	47	M	Z.E.	250	Nil	
T.O.	72	M	P.A.	570	Nil	
D.K.	46	M	P.A.	440	Nil	
H.W.	75	F	P.A.	180	Nil	
B.C.	62	M	P.A.	250	Nil	
S.B.	47	F	P.A.	1300	Nil	
E.H.	84	F	P.A.	850	Nil	
P.T.	56	F	Cystic fundic gland polyps	7	Nil	<5mm
M.L	70	F	Cystic fundic gland polyps	28	Nil	upto 1cm
M.L.	66	F	Cystic fundic gland polyps	20	Nil	<5mm
H.B.	86	F	Cystic fundic gland polyps	14	Nil	<5mm
J.T.	43	F	Cystic fundic gland polyps	20	Nil	<5mm
A.M.	63	F	Dyspepsia, Normal OGD	15	Nil	
E.H.	72	F	Iron Deficieny, Normal OGD	26	Nil	
E.Q.	50	F	Dyspepsia, Normal OGD	13	Nil	
T.B.	43	M	Dyspepsia, Normal OGD	25	Nil	
A.L.	63	M	Dyspepsia, Normal OGD	12	Nil	

Table 7.1 Details for all patients that provided tissue biopsies for either Northern blotting of *reg* mRNA abundance or RT/PCR to generate cDNA transcripts of *reg* mRNA for sequencing purposes. Hypergastrinaemic patients are shown above the control subjects that were selected on the basis of a normal oesophago-gastric-duodenoscopy (OGD) and normal plsama gastrin.

Permanent cell transfection

Two construct of human *reg* were selected for permanent transfection. These were the normal full length construct and the *reg* sequence that included a misense mutation at the initiator methionine residue (**A**TG→**G**TG). Stably transfected hamster insulinoma tumour (HIT) cell lines expressing wild type or mutant human *reg* were produced by electroporation of $5x10^6$ cells in 0.8ml at 200V and 1070μF in incomplete medium containing 10μg of linearised pcDNA *reg* construct. Cells were grown in complete RPMI in 10cm dishes for 48 hours prior to selection with zeocin ($750\mu g/ml^{-1}$) (Invitrogen). Six colonies expressing each construct were cloned and screened for *reg* expression by northern blot.

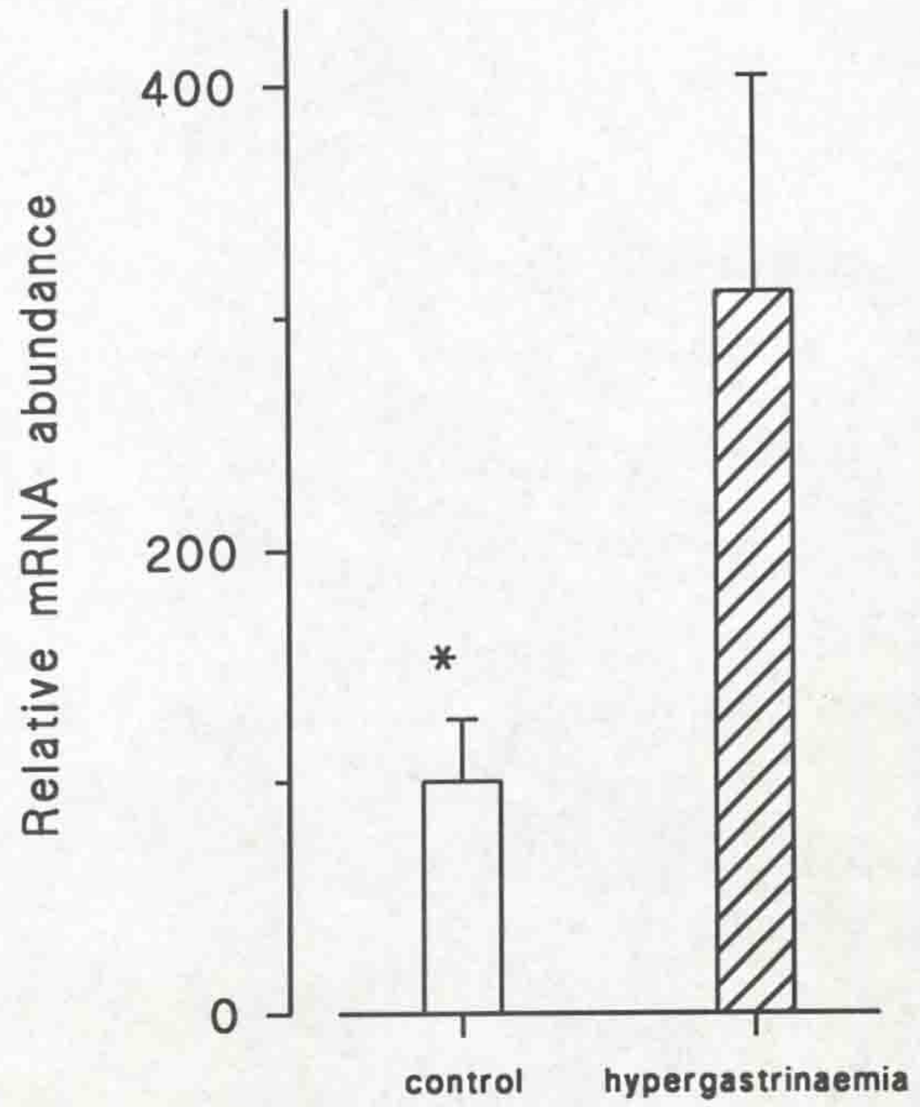
Results

Reg expression in human gastric corpus.

Reg mRNA abundance was determined in hypergastrinaemic patients and compared to that of control subjects (figure 7.1). In patients with sustained hypergastrinaemia (plasma gastrin <400pM) *reg* mRNA abundance was increased approximately threefold. In addition, suppression of plasma gastrin by a 72hr infusion of the somatostatin analogue octreotide, was associated with a significant downregulation of HDC (n=2) and *reg* mRNA (n=1) in tumour biopsies. The effect of octreotide on plasma gastrin and HDC mRNA abundance are shown for patient JH in Figure 7.2. The effect of octreotide on *reg* expression was determined in patient KD and is shown in Figure 7.3.

Reg expression in endocrine and non-endocrine cell polyps

Total RNA extracted from biopsies that were confirmed histologically to be non-endocrine in type (fundic cystic gland hyperplasia) showed a reduction in reg mRNA abundance compared to controls. Conversely, *reg* mRNA abundance was greatly increased in some ECL cell nodules compared to the patient's own corpus but not in all cases. In fact the abundance of reg mRNA in ECL cell tumour biopsies was very variable (Figure 7.4) and did not relate directly to plasma gastrin concentrations.



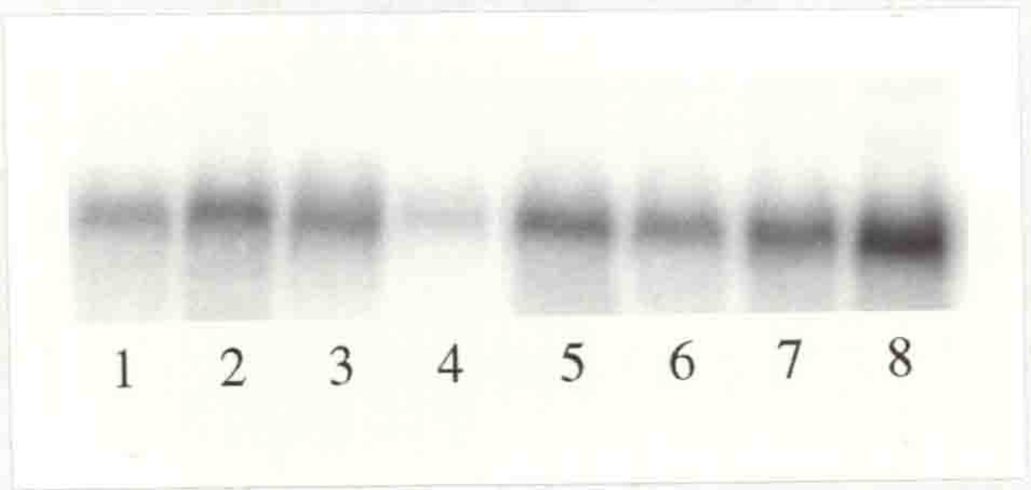
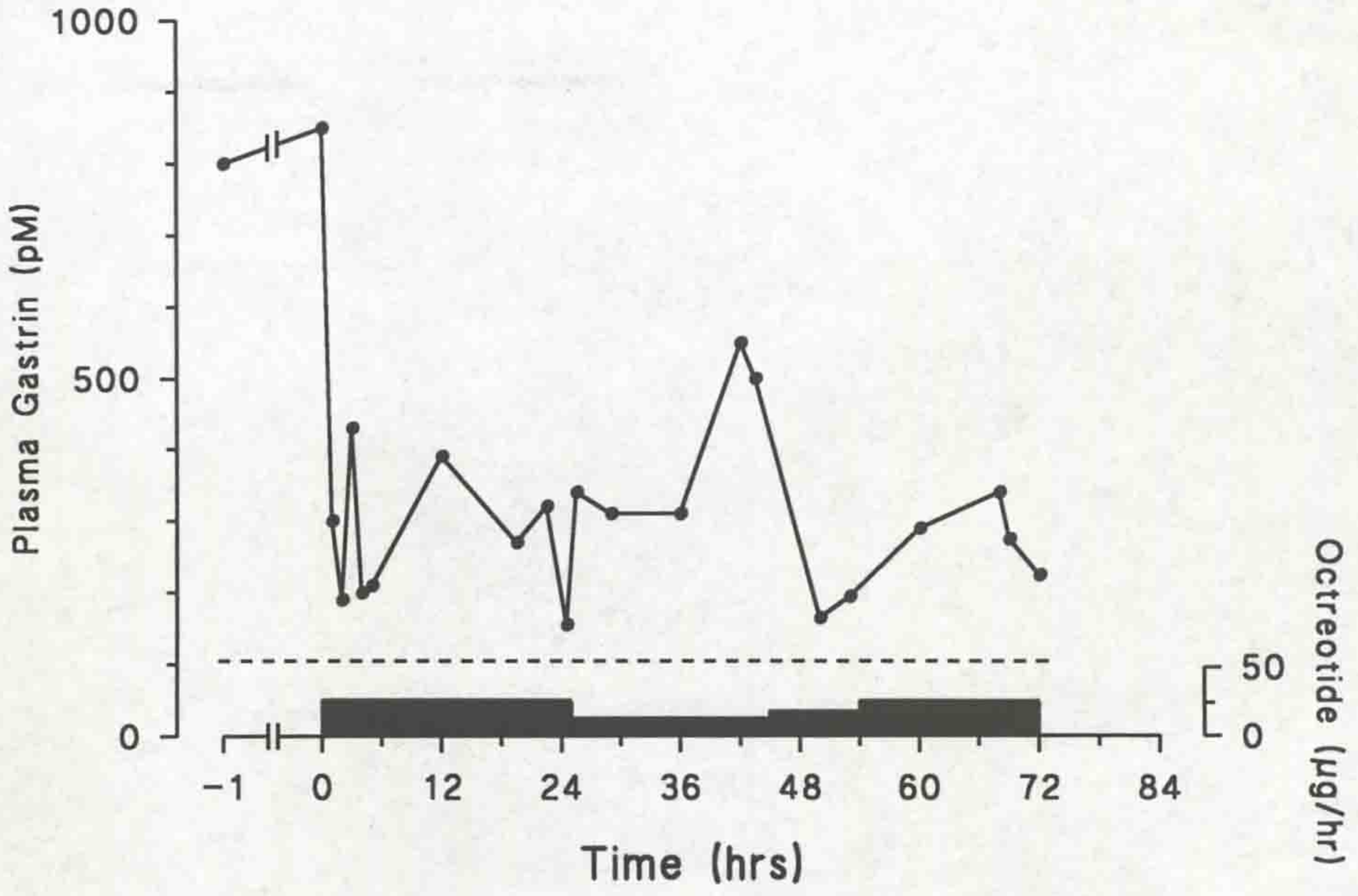


Fig 7.1 ***Upper panel Reg*** mRNA abundance in patients with hypergastrinaemia (plasma gastrin >400pM) compared to control subjects (plasma gastrin <100pM). There is a threefold increase in *reg* mRNA in patients with hypergastrinaemia (n =10 for each group; * $p<0.05$, compared with control, Mann Whitney U test). *Lower panel* Representative Northern blot of *reg* mRNA in controls (lane 1-4) and hypergastrinaemic patients (lanes 5-8) demonstrating the increased *reg* mRNA in hypergastrinaemic corpus.



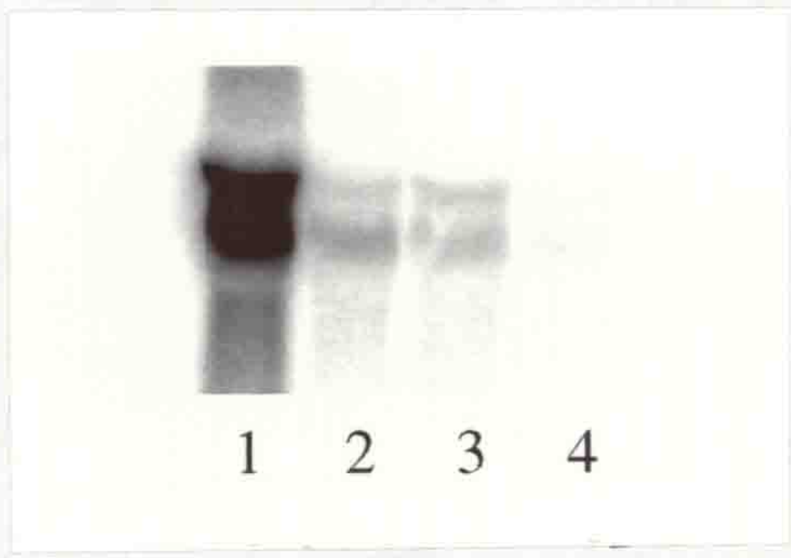


Fig 7.2 *Upper panel* Effect of octreotide infusion on plasma concentrations of amidated gastrin in patient JH. Plasma gastrin concentrations decreased during the infusion but remained above the upper limit of normal (indicated by dashed horizontal lines). The dose of octreotide is indicated by the black bar graph. *Lower panel* Northern blot for HDC mRNA in total RNA extracted from biopsies of carcinoid nodule (lane 1) and corpus (lane 2) *pre-* octreotide and from carcinoid nodule (lane 3) and corpus (lane 4) *post*-octreotide. HDC mRNA was reduced in both nodule and corpus following the octreotide infusion.

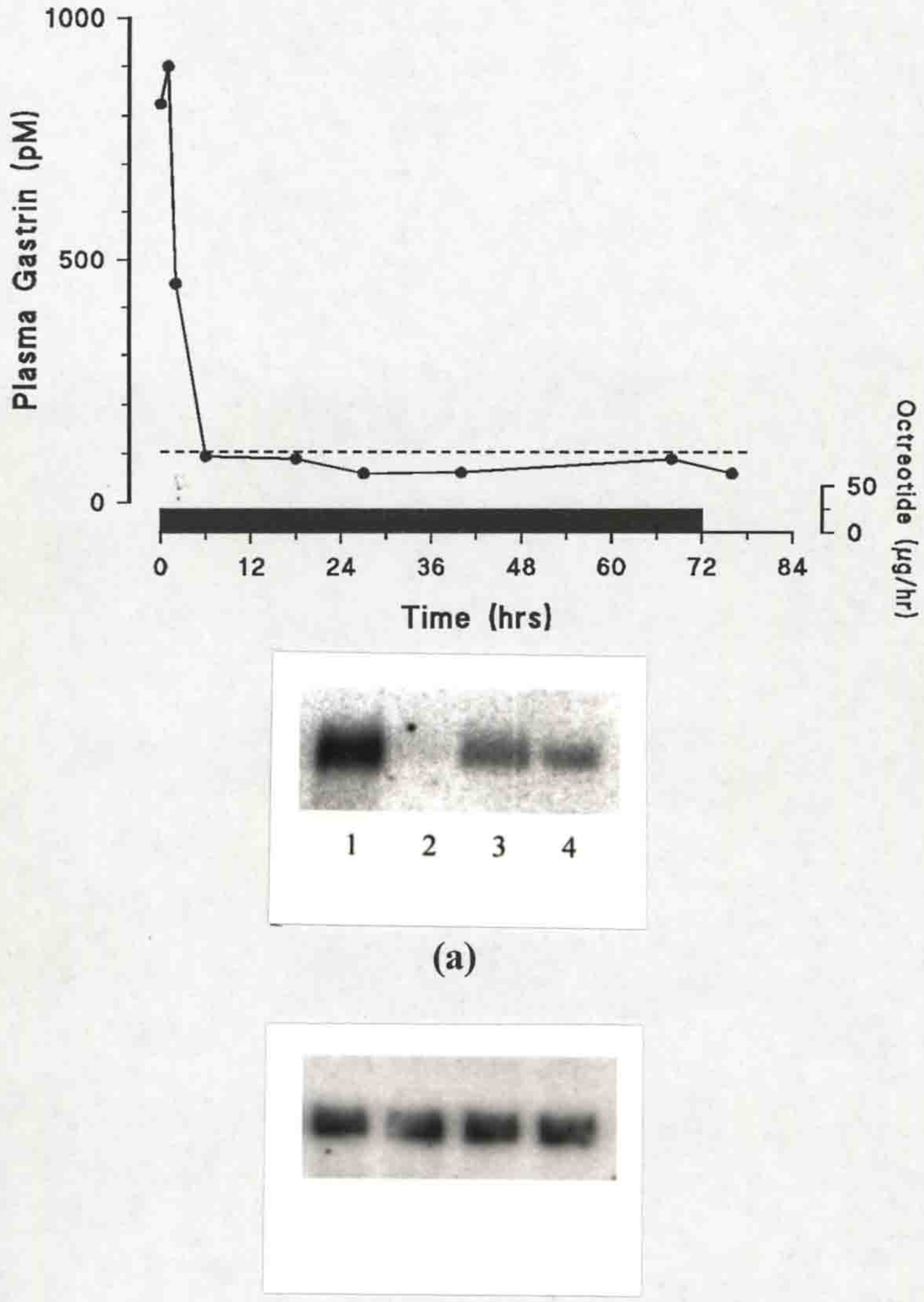
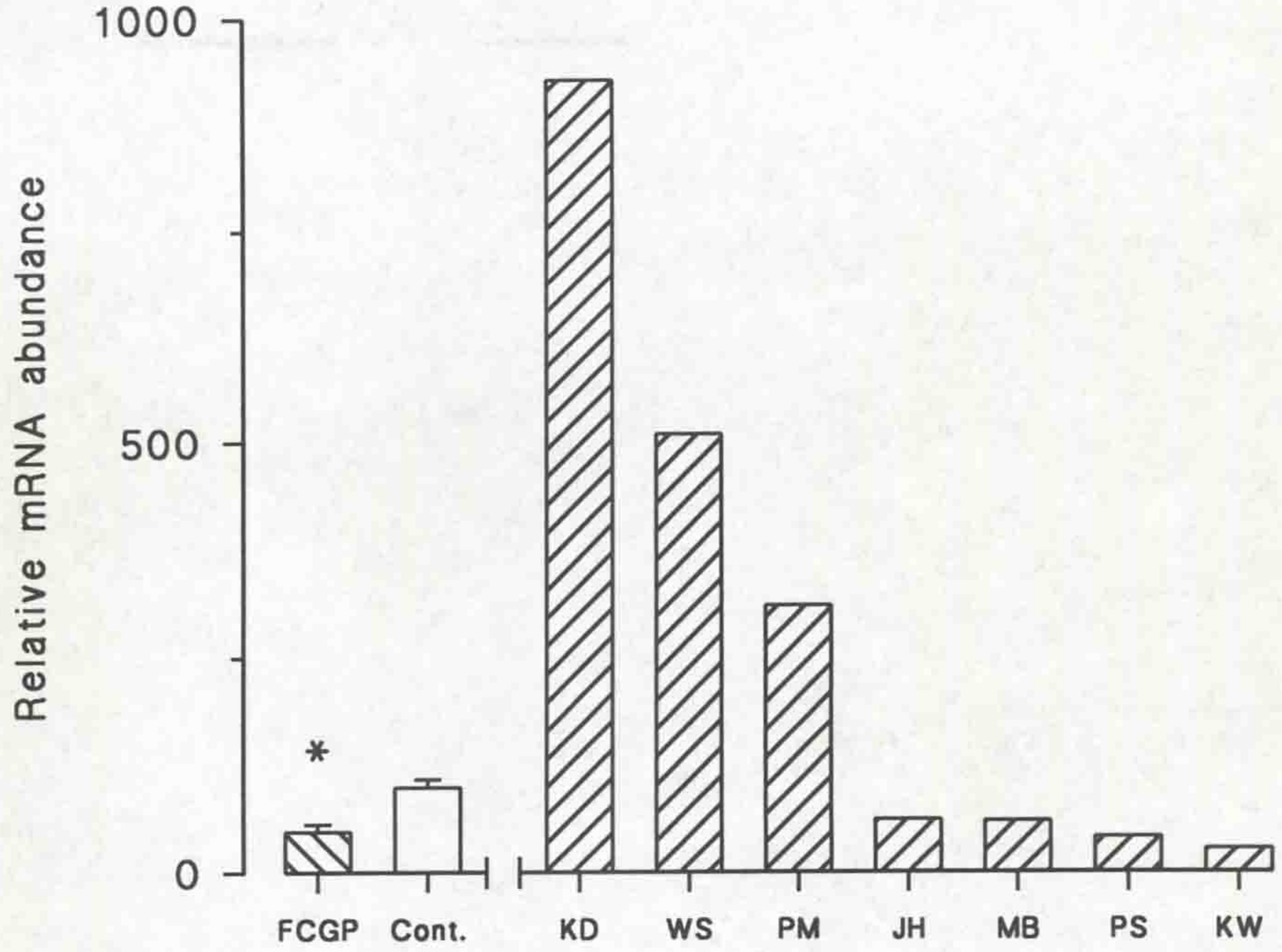


Fig 7.3 *Upper panel* Effect of octreotide infusion on plasma concentrations of amidated gastrin in patient KD. Plasma gastrin concentrations decreased during the infusion falling to within the normal range (indicated by dashed horizontal lines). The dose of octreotide is indicated by the black bar graph. *Lower panel a)* Northern blot for *reg* mRNA in total RNA extracted from biopsies of carcinoid nodule (lane 1) and corpus (lane 2) *pre-* octreotide and from carcinoid nodule (lane 3) and corpus (lane 4) *post*-octreotide. *Reg* mRNA was reduced in carcinoid nodule following the octreotide infusion. *b)* Northern blot of the same membrane for GAPDH mRNA.



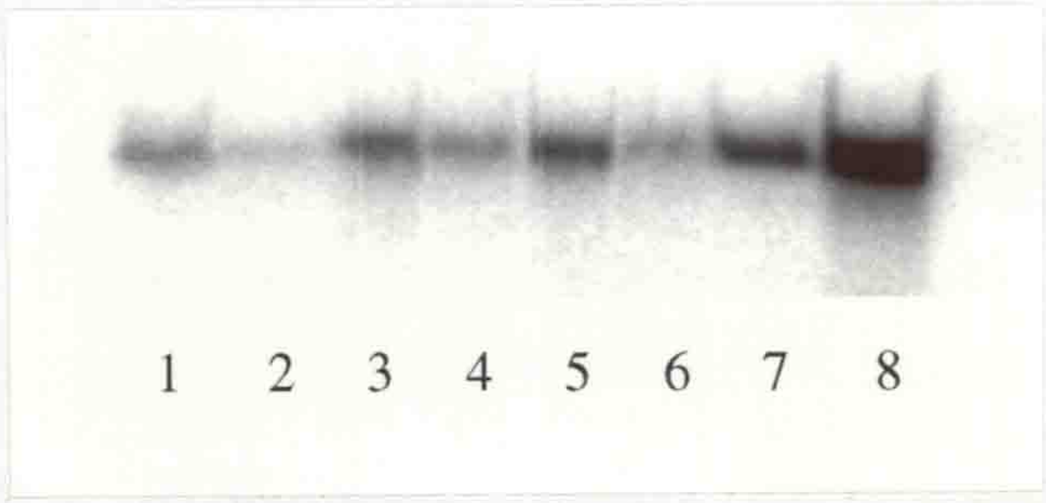


Fig 7.4 *Upper panel Reg* mRNA abundance in fundic cystic gland polyps (FCGP n=5) compared to controls (n=6). *Reg* mRNA was reduced by ≈50% in the non-endocrine polyps (* $p<0.05$ Mann Whitney U test). To the right of the axis break is shown the *reg* mRNA abundance in ECL cell tumours in individual patients (initials on X axis) relative to their own corpus *reg* mRNA abundance normalised to 100 arbritary units. Note the wide variation in tumour *reg* mRNA abundance *Lower panel* Representative Northern blot for *reg* mRNA in total RNA extracted from biopsies of normal corpus (lanes1,3 and 5) non-endocrine cell polyps (lanes 2,4 and 6) hypergastrinaemic corpus (lane 7) and ECL cell nodule (lanes 8).

Reg Sequence and ECL cell tumours

The cDNA sequence of clones derived form RT/PCR of *reg* mRNA was determined in six hypergastrinaemic patients without ECL cell tumours and five patients with tumours. At least two clones were sequenced and data on the full sequence length obtained. All patients without ECL cell tumours had a normal *reg* sequence. Three of the five patients with nodules had at least one mutation of their *reg* sequence in either corpus or nodule. The sequence information from patients with ECL cell nodules is presented in Table 7.2. JH had multiple presumably acquired mutations identified from the biopsies of her nodules. PS had a single mutation identified in both corpus and nodule suggesting a germ line mutation. Interestingly this mutation also occurred in JH and immediately appeared to suggest a pathophysiological mechanism. The mutation was a point mutation in the codon for the initiator methionine (**A**TG → **G**TG) and this would result in either the failure of initiation of protein translation or initiation of protein translation at a suitable upstream methionine residue. If protein translation commenced at the next upstream methionine this would result in loss of the first eight amino acids of the signal peptide with a failure of the protein translated to enter the secretory pathway (Fig 7.5).

PATIENT	AGE	SEX	DIAGNOSIS	PL. GASTRIN (pM)	cDNA sequence mutations	Amino Acid subtitutions
J. H.	44	F	P.A. Nodules	850	**Nodule**	
					1. **A to G** @ positon 1	**Met to Val**
					2. **G to A** @ position 473	**Gly to Asp**
					3. **T to G** @ position 25	**Met to Arg**
					G to A @ position 58	**Ser to Asn**
					4. **A to G** @ position 255	Normal
					5. Normal	Normal
					Corpus	
					1. Normal x 2	Normal
K.D.	42	M	P.A. Nodules	600	**Nodule**	
					1. Normal x 2	Normal
					Corpus	
					1. Normal x 2	Normal
K.W.	72	F	P.A. Nodules	1200	**Nodule**	
					1. **A to G** @ position 404	**His to Arg**
					2. Normal	
					Corpus	
					1. Normal x 2	
P.S.	47	M	Chronic Renal	650	**Nodule**	
			Failure		1. **A to G** @ position 1	**Met to Val**
					2. Normal	
					Corpus	
					1. **A to G** @ position 1 x 2	
						Met to Val
M.B.	56	F	Z.E./MEN1	280	**Nodule**	
					1. Normal	
					Corpus	
					1. Normal	

Table 7.2 Sequence data from cDNA transcripts of *reg* mRNA extracted from hypergastrinaemic patients with ECL cell tumours. Three of the five patients with nodules had at least one mutation in their *reg* gene identified from either nodule or corpus. All patients without nodules had normal *reg* sequence. PS had a single mutation identified from both nodule and corpus suggesting a germ line mutation. Patient JH had multiple, presumably acquired mutations identified from her tumour RNA. Note the same mutation in the initiator methionine residue occurred in both JH and PS.

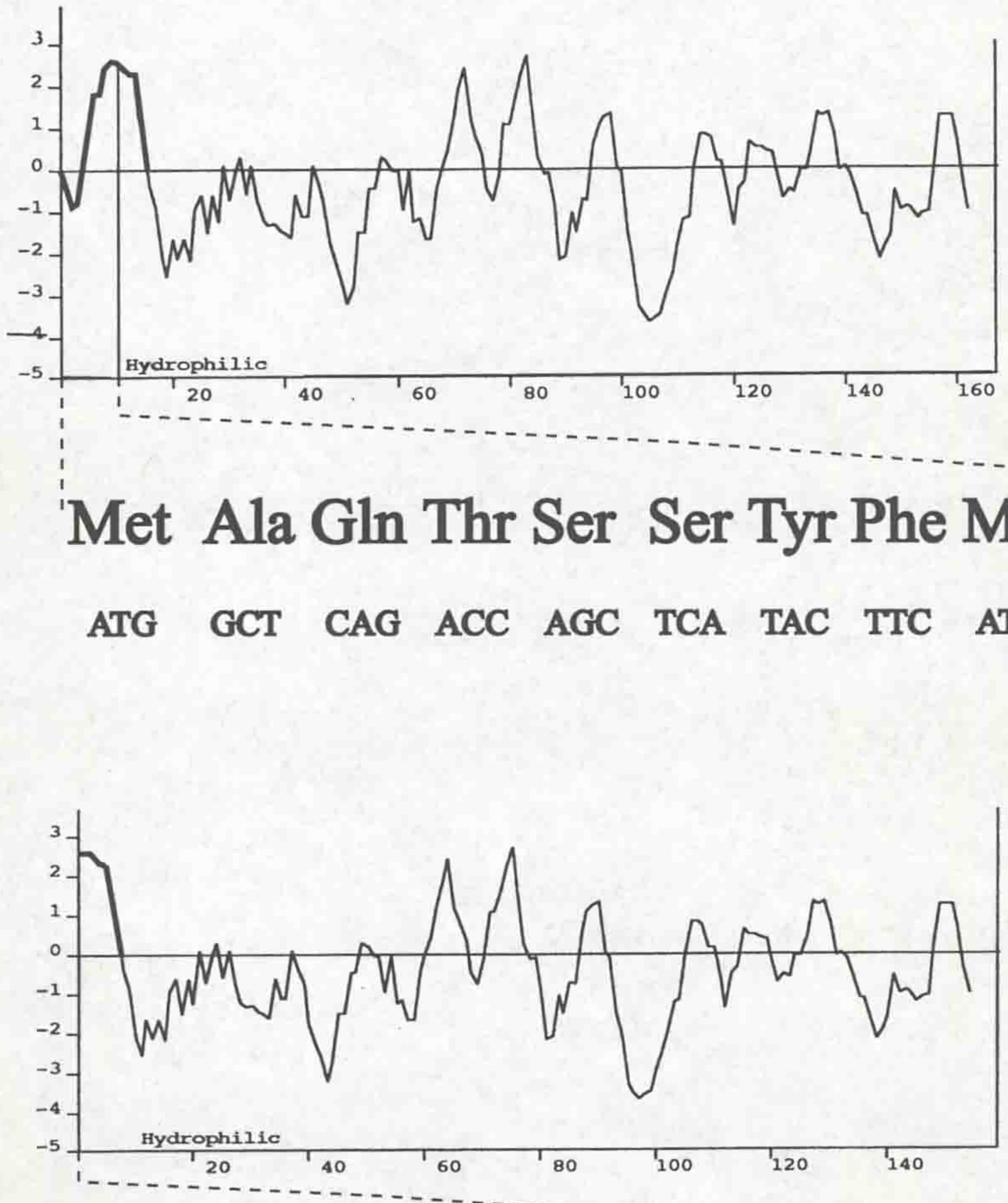


Fig 7.5 *Upper panel* Hypdrophobicity plot of normal Reg protein showing the hydrophobic tail of the signal peptide (bold line) and below this the first ten amino acids of the protein sequence. *Lower panel* Initiator methionine mutant *reg* showing the point mutation resulting in the initiator methionine residue being substituted by valine. Above this is shown the predicted hydrophobicity plot assuming protein translation occurs at the next upstream methionine. Note loss of the membrane spanning hydrophobic signal peptide.

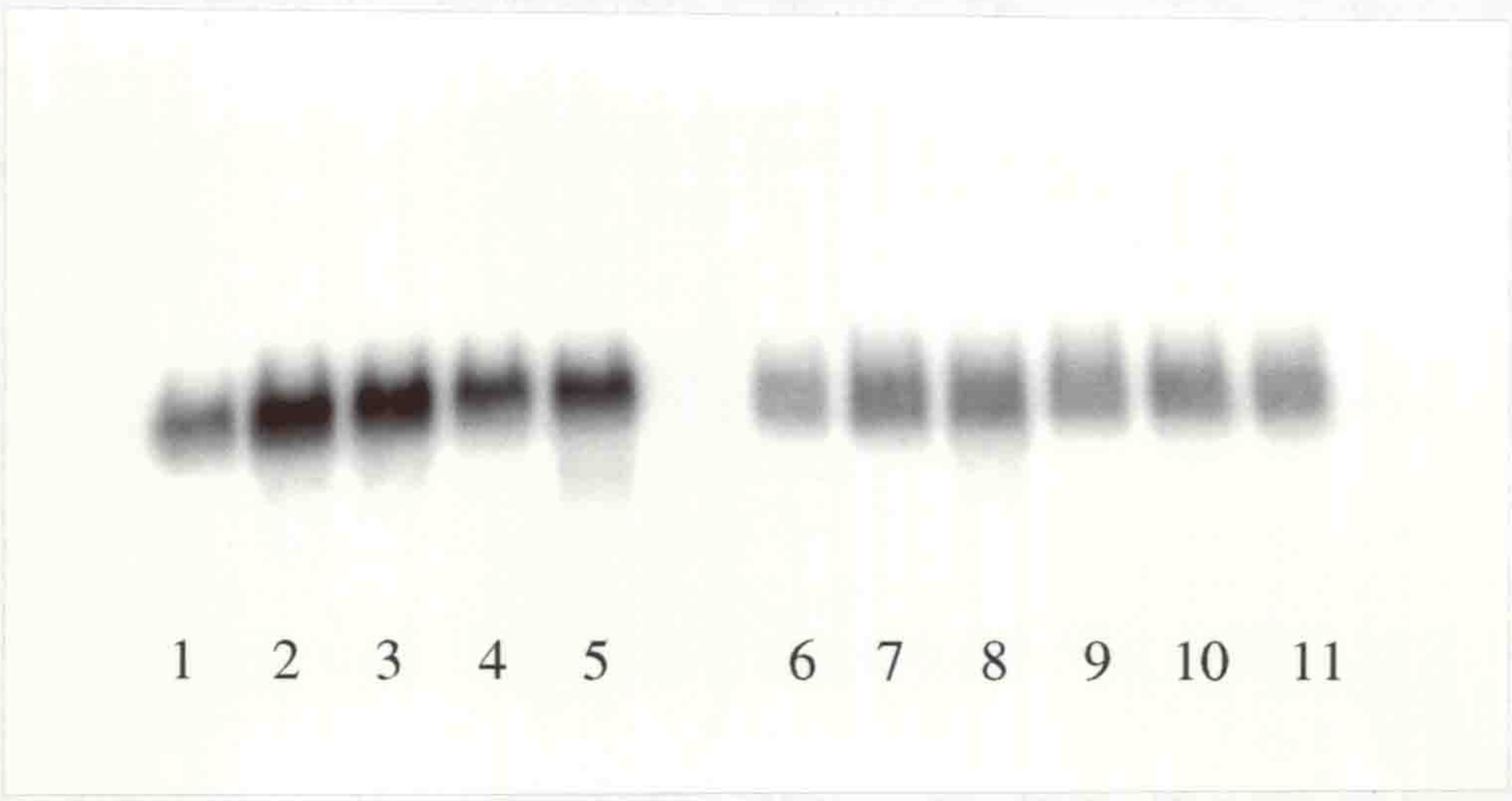


Fig 7.6 Northern blot of *reg* mRNA abundance in 5 clones of HIT cells permanently transfected with mutant *reg* (lanes 1-5) and 6 clones of HIT cells permanently transfected with wild type *reg* (lanes 6-11). Although there is variability, all clonal cell lines express *reg* mRNA.

Discussion

The data presented in this chapter demonstrate for the first time that *reg* expression in the human gastric corpus is increased in patients with hypergastrinaemia and may be suppressed in carcinoid tumour nodules by a 72hr infusion of octreotide. Perhaps of more significance was the demonstration of an association between mutations of *reg* and the development of carcinoid tumours in patients with hypergastrinaemia.

It is now generally accepted that gastrin has a trophic action on the ECL cell and high circulating levels of gastrin promotes ECL cell hyperplasia. Hypergastrinaemia induced by H_2-receptor antagonists leads to the progressive development of ECL cell hyperplasia and gastric carcinoid tumours in rodents (Hava 1986; Poynter and Selway, 1991; Wangberg *et al.*, 1995). Anti-secretory therapy in man may lead to significant ECL cell hyperplasia (Eiselle *et al.*, 1997) but no cases of carcinoid tumour have been reported. This may be because the hypergastrinaemia induced by such therapy is rarely high enough or sustained. Conversely, conditions associated with persistent and large elevations in plasma gastrin are associated with carcinoid tumour development.

Several genes expressed by ECL cells, including those for HDC, VMAT2 and chromogranin A, are upregulated by an elevated plasma gastrin (Dimaline *et al.*, 1993a; Dimaline and Struthers, 1996; Dimaline *et al.*, 1993b). The ability of gastrin to

stimulate new protein synthesis and growth of ECL cells can be blocked by specific gastrin/CCK-B receptor antagonists or by somatostatin and its analogues (Dimaline and Sandvik, 1991; Bordi *et al.*, 1993; Ferraro *et al.*, 1996). The data presented here confirm that HDC gene expression within the human gastric corpus mucosa was increased in patients with hypergastrinaemia and may be downregulated by a 72hr infusion of octreotide. *Reg* mRNA was downregulated in carcinoid nodule but not in normal corpus by octreotide(see below). In both cases, plasma gastrin fell during octreotide treatment although in the case of JH, plasma gastrin remained elevated above the upper limit of normal. Despite this there was profound depression in ECL cell function in the face of elevated plasma gastrin. This observation suggests that in addition to reducing the stimulus to ECL cell function mediated by gastrin, octreotide may itself exert direct inhibitory effects on ECL cell function. The ability of octreotide to influence gene expression within carcinoid tumour tissue suggests that this tissue is still under normal regulatory control.

In patient KD, *reg* expression changed in parallel with HDC. There was a marked increase in *reg* mRNA abundance in the patient's carcinoid nodule compared to his corpus mucosa and in his nodule at least, this was reduced by the octreotide infusion. Perhaps suprisingly, octreotide appeared to increase *reg* mRNA in gastric corpus. One possible explanation for this may be the variation in tissue sampling (see below). The elevated *reg* mRNA in gastric carcinoid tissue seen in KD was clearly not the situation for all patients with nodules as the abundance of *reg* mRNA in nodule

tissue was highly variable between patients. There may be several reasons for this. In rodents it has been suggested that as carcinoid tumours develop and progress, they develop autonomy and become less dependent on gastrin as a trophic stimulus. The variability in *reg* expression in patients perhaps may indicate patients at a different stage of their disease. Alternatively, the degree of inflammation in the surrounding corpus was not taken into account. The ability of growth factors to potentiate the gastrin stimulated *reg* mRNA response also may explain the observed variability in nodule *reg* mRNA abundance. Hence, perhaps as CAG progresses and the parietal cell mass falls, the degree of inflammation also subsides. Tissue sampling introduces another unknown variable although this maybe more relevant to corpus mRNA as macroscopic nodules represent reproducible sites to biopsy. Although biopsies were taken from corpus mucosa that appeared macroscopically normal through an endoscope, the presence of microscopic carcinoids within biopsies that were processed for corpus mRNA cannot be excluded. This probably explains not only the variability in *reg* expression between patients but also the somewhat surprising increase in corpus *reg* mRNA in KD after his octreotide infusion. Furthermore, part or all of the increase in corpus *reg* mRNA in patients with hypergastrinaemia may be explained by an increase in ECL cell number as a consequence of hyperplasia and also due to concurrent atrophy of other mucosal cell types in patients with CAG.

Carcinoid tumours occur most commonl y in patients with CAG, but are also associated with ZE and CRF. The incidence of carcinoid nodules in CAG is

approximately 5% and is similar to the incidence of carcinoid tumour occurring in the sporadic form of ZE syndrome. The incidence of carcinoid nodules in ZE associated with MEN1 syndrome rises to ≈30% (Lehy *et al.*, 1992). This suggests a genetic predisposition to carcinoid tumour development in this patient subgroup and is in keeping with the recently published observations of (Debelenko *et al.*,1997). They demonstrated that 75% of ZE patients with MEN1 syndrome have loss of heterozygosity at the MEN1 gene locus with deletion of the wild type allele. The MEN1 gene has recently been sequenced (Chandrasekharappa *et al.*, 1997) and is the tumour suppressor gene that carries the inherited risk for developing endocrine tumours that are the feature of MEN1 syndrome. Of interest also however, was the observation of a low incidence of loss of heterozygosity at the MEN1 gene locus (1 of 6 patients) in patients with CAG and carcinoid nodules (Debelenko *et al.*, 1997). This implies that another gene may be responsible for the risk of tumour development in these patients.

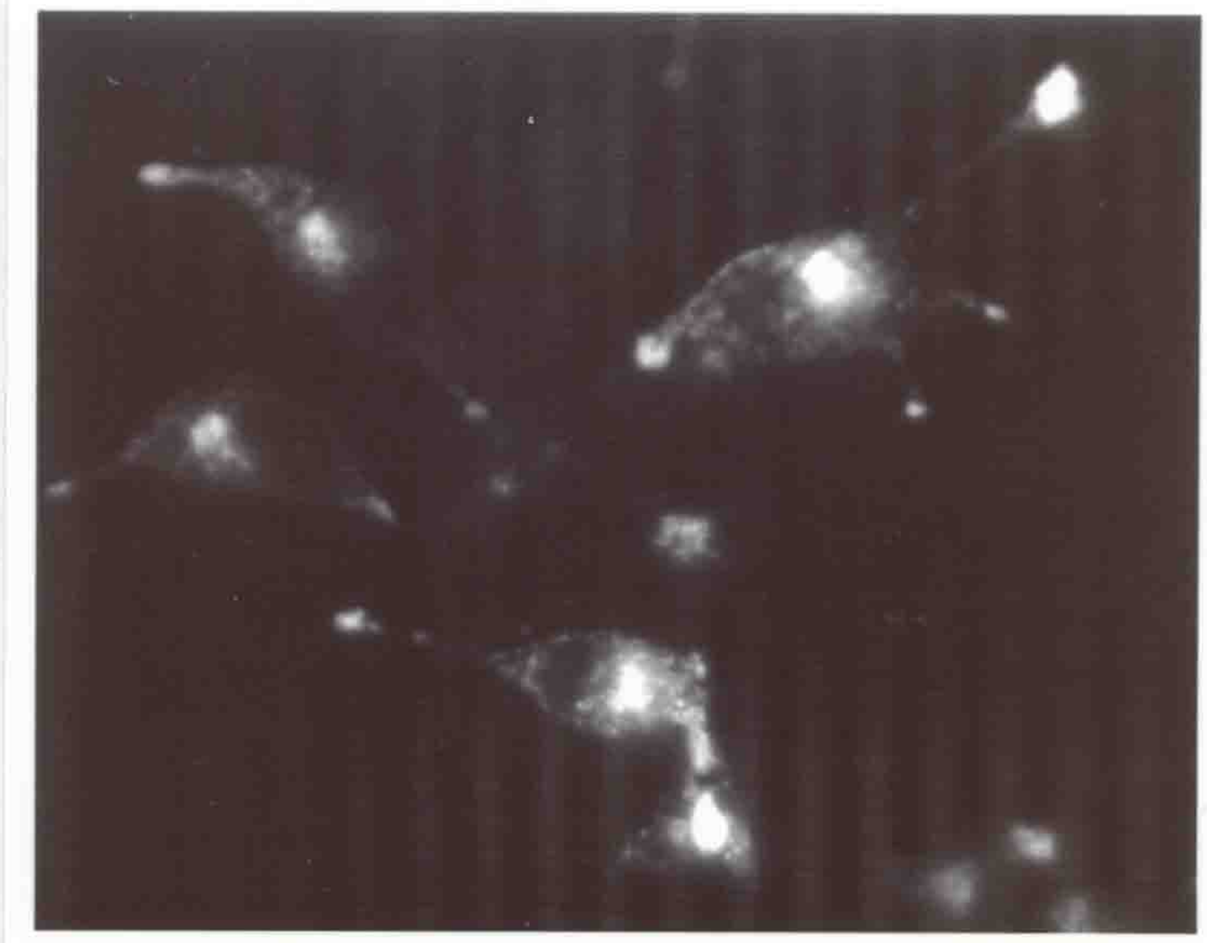
There is a growing body of evidence to suggest that the *reg* gene may play a role in the growth and differentiation of entero-pancreatic endocrine cells. It seemed reasonable to examine therefore whether mutations of *reg* may be associated with the development of carcinoid tumours in patients with hypergastrinaemia. The sequence data presented show that in 3 of 5 patients with hypergastrinaemia and carcinoid tumours there was at least one identifiable mutation in *reg* whereas in all hypergastrinaemic patients without carcinoid nodules the wild type *reg* coding

sequence was found. This identifies an association between mutations of *reg* and a risk of developing carcinoid tumours in the presence of hypergastrinaemia. However, a significant number of normal *reg* sequences were also obtained from both the corpus and the nodules of patients with tumours. Again this may reflect sampling error as each biopsy of tumour tissue will also have normal mucosal cells within it. To overcome this, microscopic dissection of fresh biopsy material to obtain clonal tumour cells will be required. It is also likely that the *reg* gene is not the only genetic mutation required or necessary to develop carcinoid tumours just as mutations of menin do not appear to be solely responsible for the development of carcinoid tumours in ZE associated with MEN1 (Debelenko *et al.*, 1997).

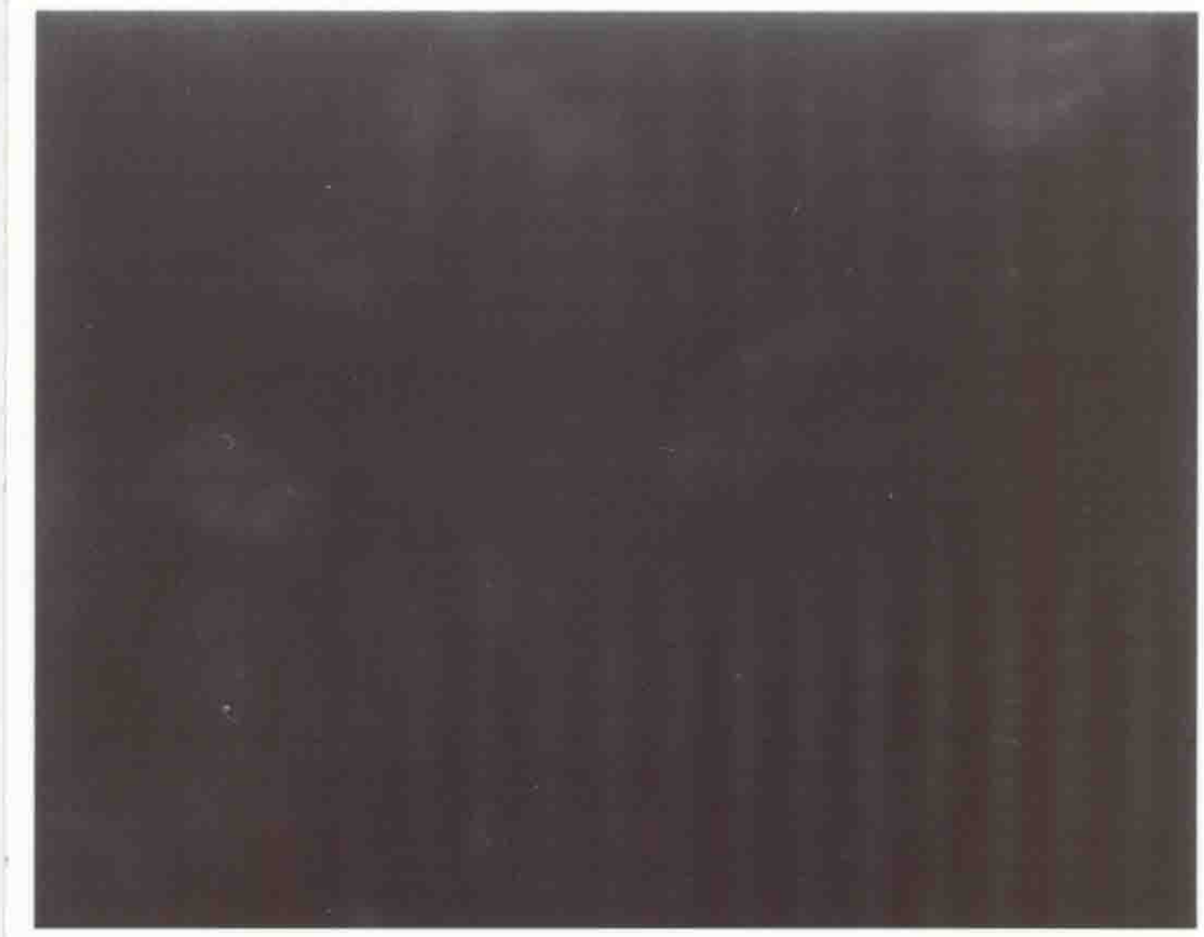
It seems likely that both acquired and germ line mutations of *reg* may occur in association with carcinoid tumours. Patient JH had multiple mutations of *reg* in several clones sequenced indicating multiple acquired mutations. Patient PS had only one mutation of his *reg* but it occurred in three of four sequences in both his corpus and nodule. This is suggestive of a germ line mutation. Interestingly the mutation identified in PS also occurred in patient JH and occurred at the methionine residue that initiates protein synthesis. The point mutation of **A** → **G** resulted in a loss of the initiator methionine, it being replaced by a valine residue. The possible results of this mutation include; i) a complete failure of protein translation, ii) protein translation occurs at the next upstream methionine that is "in frame", iii) protein translation occurs at the most appropriate methionine residue that may be "out of frame". In the case of the first and last instance either no protein or a

different protein will be produced with an expected loss of the normal function of *reg*. In the case of the second instance the protein produced will be missing eight amino acids of the N-terminal hydrophobic signal peptide. This should result in failure of the protein product to enter the secretory pathway as the signal peptide will be too short to span the hydrophobic membrane domain. The subsequent protein product may then either be degraded within the cytosol or act intracellularly. This could result theoretically in either a loss or gain in function.

According to the scanning model of mRNA translation proposed by Kozac (1978 and 1989), the sequence immediately adjacent to the initiator methionine codon determine the likelihood of protein translation occuring. The most important requirement is for a purine at position -3. In the abscence of a purine at position -3, a G at +4 is essential for efficient translation (Kozak, 1989 & 1991; Iida and Masuda, 1996). Examination of the transfected mutant *reg* sequence fails to identify any alternative ATG codon with a surrounding sequence that meets these criteria. This would suggest that translation is unlikely to occur at an alternative start site and if it does occur at the next upstream methionine residue then translation will be inefficient. To confirm that the initiator methionine mutant *reg* resulted in failure of the translated protein product to enter the secretory pathway, constructs of human *reg* were inserted into a mammalian expression vector (pcDNA3-ZEO^{+}). The constructs were then permanently transfected into the hamster insulinoma tumour cell line (HIT) by Dr L Bishop. Cells were cultured



(a)



(b)

Fig 7.7 Photomicographs of HIT cells permanently transfected with either wild type (a) or mutant (b) *reg* constructs and stained sequentially with monoclonal anti-human *reg* antibody (1:2000; gift from Dr J Wand, Boston USA) and FITC conjugated horse anti mouse antibody (1:50). Note, only in the cells expressing wild type *reg* is there clearly discernible staining within the golgi apparatus and secretory granules. (x 400 approx., Courtesy of Colin Blackmore)

under zeocin selection and surviving cell lines were confirmed as expressing the *reg* gene by Northern blot of total mRNA extracted from 10^6 cells (Fig 7.6). Clonal cell lines were then stained immuno-histochemically with a monoclonal anti-human reg antibody followed by FITC rabbit-anti-human secondary antibody. Intracellular distribution of reg protein was determined by immunoflourescence microscopy. (This work was performed by C. Blackmore, Dept of Physiology, Liverpool). The results of this work demonstrate that in the cell lines expressing wild type *reg* the protein enters the secretory pathway with clear staining of the Golgi apparatus and secretory granules (see photomicrograph 7.7a). In contrast, in cells expressing mutant *reg* there is no discernible pattern of staining (see photomicrograph 7.7b). Although this suggests that the protein either is not produced or is rapidly degraded, it remains possible that the shortened signal peptide remains attached to the N-terminal of the protein product and prevents the anti-human reg antibody from recognising the normal N-terminus of the reg protein. The specificity of the human reg antibody is being determined.

Summary

The *reg* gene is known to be present in the human stomach and there is an increasing body of evidence to suggest that it may play a role in the growth and differentiation of endocrine cells in the gut including the ECL cell. Gastrin is also known to influence enteropancreatic endocrine cell differentiation and has a trophic effect on the ECL cell. The data presented demonstrate that plasma gastrin influences *reg* gene expression in human gastric corpus mucosa. In addition, the development of

gastric carcinoid tumours in patients with hypergastrinaemia is associated with mutations of the *reg* gene. This raises the possibility of identifying patients at risk of carcinoid tumour development in the presence of sustained hypergastrinaemia.

Chapter Eight

Conclusions and Implications

The main objectives of the work contained within this thesis were to examine the role of antral neurons in the control of gastric emptying and the mechanisms that regulate gastrin release from G cells of the antrum, using a model of antral denervation in the rat. In addition, the significance of *reg* gene expression and carcinoid tumour development was examined by studying the relationships between gastrin and *reg* expression in the ECL-cells of the rat, in human gastric corpus and in a rat pancreatic cell line (AR42J).

Conclusions

1. Serosal application of BAC to the gastric antrum effectively lesions all myenteric and spinal afferent fibres of the antrum and the spinal afferent innervation of the gastric corpus. The intrinsic innervation of the proximal stomach is unaffected. Such chemically induced denervation provides a model for experimental studies of the intrinsic innervation of antral muscle and the antral mucosal endocrine cells of which the major cell of interest is the G cell.

2. In the antrally denervated rat there is retention of solid within the stomach that is apparent after a 48hr fast. In antrally denervated rats fitted with a gastric fistula, the emptying of liquid test meals such as saline, acid and peptone was delayed. However, the CCK-dependent inhibition of gastric emptying of peptone was preserved after antral denervation by BAC. It appears that innervation of the antral musculature is

essential for normal gastric emptying of liquids and solids but not CCK-mediated inhibition of gastric emptying.

3. In the intact rat, antral neurons normally inhibit gastrin release in response to non-nutrient distension of the gastric corpus and luminal nutrient produces gastrin release via activation of neurons that release GRP. Following antral denervation, post-prandial gastrin release was maintained but was no longer dependent on either antral neurons or release of GRP. Moreover, non-nutrient distension of the stomach becomes an adequate stimulus for gastrin release.

4. In AR42J cells *reg* mRNA expression was stimulated, in a dose-dependent manner, by amidated gastrin acting through the CCK-B receptor. *Reg* expression in rat and human gastric corpus was also dependent on plasma gastrin concentrations. Mutations of *reg* (a putative regulator of differentiation of gut endocrine cells) were associated with gastric carcinoid tumour development in patients with hyper-gastrinaemia. A major limitation of the studies thus far are that mRNA only has been measured and quantification of the protein product has not been performed.

Implications and future studies

Myenteric denervation by BAC was described first in the rat colon (Sato *et al.*, 1988), where its serosal application produced a phenotype similar to that of Hirschprung's disease. The effects of small intestinal denervation by BAC have also

been described both by serosal application (myenteric plexus ablation) and luminal application (submucosal plexus ablation). The particular advantages that have been described for BAC are that it can be applied to a strictly defined area of the gut and its application lesions only the myenteric intrinsic neurons and the extrinsic innervation of the region when applied to the serosal surface. Clearly, the effects of BAC on extrinsic afferent fibres may be more widespread depending on the anatomy of innervation. Nevertheless the lesion produced relies upon the ability of intestinal smooth muscle to recover whilst neuronal loss persists. There is a significant inflammatory element to the lesion during the first 7 days (See *et al.*, 1988). Thereafter, histologically inflammation subsides but muscle hypertrophy begins, although active stress generation by hypertrophied muscle does not exceed normal values until after the second week postoperatively (Herman and Bass, 1990).

Most of the studies performed in this thesis used animals towards the end of their second post-operative week. However, the possible confounding factors of continued inflammation and antro-pyloric muscle hypertrophy could conceivably contribute to some of the observations described. In addition, because of a lack of a neuropeptide marker for extrinsic vagal fibres, no attempt was made to determine the extent of vagal damage induced by BAC. Although antral BAC treatment almost certainly disrupts the antral vagal innervation, the preservation of CCK-mediated delay in gastric emptying suggests that the duodenal vagal afferent and gastric vagal efferent fibres were intact.

Inflammation of the gastric mucosa is known to result in increased levels of growth factors such as TGF-α (Polk *et al.*, 1992). Growth factors such as TGF-α and EGF have been shown to both stimulate gastrin release and inhibit acid secretion (Konturek *et al.*, 1984; Rhodes *et al.*, 1986). Furthermore, chronic gastric retention and delayed gastric emptying are well recognised complications of hypertrophic pyloric stenosis. In fact, chronic gastric retention is also associated with fasting and meal-stimulated hypergastrinaemia in the myenteric neuropathy that occurs as a result of *Trypanosomal* infection (Oliveira *et al.*, 1980; Troncon *et al.*, 1984).

Perhaps of more immediate relevance may be the mucosal inflammation produced by *H.pylori*. There have been reports that antral gastritis due to *H.pylori* may also induce loss of mucosal nerve fibres and hence lead to denervation (Stead *et al.*, 1996). Interestingly, and in keeping with the observations here, *H. pylori* infection produces basal and meal stimulated increases in plasma gastrin (Levi *et al.*, 1989; McKoll *et al.*, 1989; Tarnasky *et al.*, 1993) and reduces antral somatostatin mRNA levels (Kaneko *et al.*, 1990; Moss *et al.*, 1992) although gastrin mRNA apparently is unaffected. The exaggerated response to GRP in *H. pylori* infection (Graham *et al.*, 1991; McKoll and El-Omar, 1995) is in keeping with increased sensitivity seen following chronic denervation. The potential role of inflammation and growth factors in the BAC-treated rat was not examined.

Of more concern however, may be the potential relationship between trophic factors, elevated plasma gastrin and the growth of gastric mucosal cells given the association between *H. pylori* and the development of gastric tumours. Certainly, the

marked potentiation by EGF of the gastrin-stimulated expression of *reg* in AR42J cells is preliminary evidence for a similar potentiation occurring in gastric mucosal endocrine cells or their precursors. The wide variability of *reg* expression in hypergastrinaemic patients may indicate different degrees of mucosal inflammation, independent of the level of their plasma gastrin and was a variable that was not controlled for in these studies.

Finally, the role of gastrin and growth factors in the control of differentiation of gastric epithelial (endocrine and non-endocrine) cells is likely to become of increasing importance given the relationship between these factors, PPI therapy, *H. pylori* gastritis and gastric malignancy. The possible role of *reg* Iα as a tumour suppressor gene for endocrine cells of the stomach remains to be clarified but further studies of the *reg* gene family are likely to provide new insights into the cellular and molecular mechanisms that regulate the fundamental processes that lead to the differentiation and maintenance of cell phenotype. The role of *reg* Iα in the stomach and in AR42J cells provides a model system for testing the following hypotheses:

i. expression of the *reg* gene is regulated by gastrin,

ii. expression of the *reg* gene influences the progressive differentiation of precursor endocrine cells towards in the gastric mucosa,

iii. mutations of *reg* result in failure of differentiation and may cause endocrine cell tumour development in the gastric mucosa.

The following provides an experimental outline of the future studies that will test these hypotheses:

i) Does gastrin directly control *reg* expression?

a) Using the same techniques of tissue culture, mRNA extraction and Northern blot it would be possible to study gastrin receptor mediated events in AR42J cells using recognised gastrin receptor agonists, antagonists and inhibitors. The effects of somatostatin on *reg* expression could also be examined.

b) Gastrin response elements in the promoter region of human *reg* could be identified by transfection of AR42-J cells with different constructs of the promoter region coupled to a reporter luciferase gene. Exact localisation of the relevant sequences would then require scanning mutagenesis and DNA footprinting.

ii) Is an intact *reg* essential for normal differentiation of an endocrine cell?

a) The ability of *reg* to induce differentiation will be assessed. The ability of recombinant reg protein to induce differentation in responsive precursor cell lines will be studied and in addition, transfection of undifferentiated but responsive cells with wild type *reg* will also be performed. Expression of markers of differentiation will be assessed following stimulation with gastrin.

b) Immunohistochemical markers of differentiation (CGA, HDC) and proliferation (PCNA, MIB) will be correlated with *reg* expression in precursor stem cells and mature ECL cells in patients with and without hypergastrinaemia.

iii Does a *reg* mutation produce abnormal physiology?

a) The normal biosynthesis of the Reg protein will be assessed *in vitro* in gastric biopsies by pulse chase experiments incorporating ^{35}S methionine. Comparisons will be made between patients with and without mutations of *reg*.

b). The processing of wild type and mutant *reg* by Hamster Insulinoma Tumour (HIT) cells will be assessed by morphological and biosynthetic analysis. We have already permanently transfected wild type and mutant *reg* into HIT cells that are capable of processing and secreting transfected proteins.

c). Genomic DNA from hypergastrinaemic subjects with and without ECL cell dysfunction will be screened for mutant *reg*.

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